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Molecular Interaction between Fish Pathogens and Host Aquatic Animals

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We have studied the host-pathogen interactions between Atlantic salmon (*Salmo salar* L.) and *Aeromonas salmonicida*. Sequencing the genome of the bacterium allowed us to investigate virulence factors and other gene products with potential as vaccines. Using knock-out mutants of *A. salmonicida*, we identified key virulence factors. Proteomics studies of bacterial cells grown in a variety of media as well as in an *in vivo* implant system revealed differential protein production and have shed new light on bacterial proteins such as superoxide dismutase, pili and flagellar proteins, type three secretion systems, and their roles in *A. salmonicida* pathogenicity. We constructed a whole genome DNA microarray to use in comparative genomic hybridizations (M-CGH) and bacterial gene expression studies. Carbohydrate analysis has shown the variation in LPS between strains and reveals the importance of LPS in virulence. Salmon were challenged with *A. salmonicida* and tissues were taken to construct suppressive subtractive hybridization libraries to investigate differential host gene expression. We constructed an Atlantic salmon cDNA microarray to investigate the host response to *A. salmonicida*. Real-Time qPCR and NMR-based metabolomics have revealed important information about host responses to infection and to chronic stress. By linking genome sequencing, functional genomics, proteomics, carbohydrate analysis, metabolomics, and whole animal assays, we took integrated and innovative approach to pathogenesis research.

KEYWORDS host-pathogen interactions; genomics; proteomics; furunculosis; DNA microarrays; knock-out mutants

1. Introduction

The development of genomics, proteomics and metabolomics, have enabled the transition from sequential studies of single genes, proteins or metabolites to the simultaneous study of many components and their interactions at a variety of biological levels (from pathways to cells, tissues and whole organisms). The development of these fields has been supported by the concurrent development of new technologies and methods such as quantitative PCR or Real Time PCR, RNA interference (RNAi) assays, and *in situ* hybridization. These fields and their supporting technologies and methods are now widely used in the study of disease of higher vertebrates as well as in the development of new therapeutants and vaccines. The field of aquatic animal health has only recently begun to benefit from their application.

It is well known that farmed aquatic animals are susceptible to a wide variety of infectious diseases. Direct and indirect costs of preventing and controlling infectious diseases in aquaculture are estimated to exceed 10% of total production costs. This represents an appreciable loss of profits to the aquaculture industry. Coupled with this is an increasing global concern about the use of therapeutants (antibiotics and chemical treatments) in the production of food animals and issues related to animal welfare. Development of more effective vaccines for aquaculture is necessary to reduce losses due to disease, to enable further reductions in the use of therapeutants, and to improve animal welfare.

Vaccines against infectious diseases in fish have been effective and available for years, For example, multi-valent vaccines based on whole bacterins in formalin- or oil-based preparations have provided protection to salmon against *Aeromonas salmonicida*, *Listonella anguillarum*. Vaccines are also available against viral pathogens. However, it is notable that several problems are associated with commercially available vaccines for fish. Oil-based and formalin-based vaccines have been associated with adhesions and other pathologies including weight loss (Midtlyng and Lillehaug 1998), secondary infections (Bruno and Brown 1999), injury and inflammation (Mutoloki *et al*. 2004). Therefore, research into vaccines and vaccine delivery systems is ongoing.

Similarly, effective husbandry tools and practices have been used since the inception of aquaculture. Fallowing farm sites, separation of year classes, culling of infected broodstock, and reduction of anthropogenic stressors are all examples of successful husbandry techniques. However, these are largely based on trial and error and on empirical observations.

In order to devise effective aquatic animal health management tools in a timely and cost-effective fashion, it is essential that we identify meaningful biological targets, by knowing which antigens, host immune factors, and stress response genes to target. In order to do that, it is necessary to increase our understanding of the interactions between host aquatic animals and pathogens. Genomics, proteomics, and other biotechnological approaches help us to do that.

Aeromonas salmonicida is a non-motile, Gram-negative bacterium that is the etiological agent of furunculosis. Furunculosis has been recognized as a widespread important disease in wild and farmed fish since the 1890s (Emmerich and Weibel 1894). Due to its early recognition as an important pathogen it is arguably the best studied bacterial pathogen of fish. Several excellent reviews of earlier research on *A. salmonicida* and furunculosis are available (Bernoth 1997; Wiklund and Dalsgaard 1998; Hiney and Olivier 1999).

In this chapter we will demonstrate how a multi-disciplinary approach, combining genomics, proteomics and metabolomics with more traditional methods was used

within the National Research Council Genomics and Health Initiative Program "Genomics Approaches to Aquatic Animal Health Management," to understand the biology of the bacterial pathogen *Aeromonas salmonicida* and its interactions with one of its hosts, the Atlantic salmon (*Salmo salar* Linnaeus). We will also show how this approach was used to identify candidate protein and carbohydrate antigens for use in vaccines, in conjunction with whole animal assays.

2. Development of an *in-vivo* Growth System for *Aeromonas salmonicida*

It is well known that the conditions under which the bacterium are grown influence their gene expression and ultimately their phenotype (Dalsgaard *et al*. 1998). This was originally demonstrated for *A. salmonicida* grown within chambers in the host by Garduño *et al*. (1993). These authors demonstrated that cells grown under *in vivo* growth conditions had increased resistance to bacteriolysis, phagocytosis and oxidative killing. We developed a method for the *in vivo* growth of large volumes of *A. salmonicida* (Dacanay *et al*. 2003). Briefly this method involves the use of growth chambers made of autoclaved 12 to 14-kDa molecular-mass-cutoff dialysis tubes that are filled with *A. salmonicida* suspensions and surgically implanted into the abdominal cavity of Atlantic salmon for a period of 24 hours, then harvested. Bacteria grown within these implants have been used in studies of gene expression, for proteomics, carbohydrate analysis and for studying the host response to infection as described below.

3. Development of Genomics Resources and Tools for *Aeromonas salmonicida* subsp. *salmonicida* (A449)

The acquisition of full genomic sequences for pathogens has facilitated rapid advances in infectious disease research. Genomic sequencing has facilitated the development of new methods of pathogen detection, our understanding of genetic relationships between species and investigations into the nature of host-pathogen interactions. Through the use of bioinformatics methods, which allow for genomic comparisons between species, putative virulence factors, as well as genes that encode proteins that may be suitable for use in vaccines can be identified. Genomic sequencing also facilitates the use of other technologies such as the development of knock-out mutants, and other genomics (e.g., microarray), and proteomic studies, all of which serve to increase our understanding of host-pathogen interactions and aid in the rational design of effective vaccines

At the initiation of our research program there was little genomic information available for *A. salmonicida* or closely related species. In order to understand the infection process and possible interactions between *A. salmonicida* and its hosts, as well as to aid in the rational design of new vaccines the whole genome of wild-type strain A449 was sequenced and assembled (GenBank Accession #s NC_009348-009350). The A449 genome consists of a circular chromosome and five plasmids that encode more than 4700 genes (Boyd *et al*. 2003; Reith *et al*. unpublished). During this program a full genomic sequence became available for *Aeromonas hydrophila* (Seshadri *et al*. 2006). Comparison of the *Aeromonas salmonicida* genome with genomes of related organisms such as *A. hydrophila* enabled the identification of large numbers of genes encoding potential virulence factors (Boyd *et al*. 2003; Nash *et al*. 2006; Reith *et al*. unpublished). Virulence genes were targeted for future study and for use as vaccine candidates. Table 1 summarizes some of the results of the projects described above.

Genome arrays which contain large numbers of genes or whole genomes have

Table 1. Summary of *Aeromonas salmonicida* genes identified via genomic sequencing and knock-out mutants. We also elucidated the role of the expressed proteins in virulence

System	Gene(s)	Role	*In vivo*		*Ex vivo*	Reference
			Injection	Immersion		
Type I pili	*fimABCD*	Type I pilus subunit, tip and usher proteins	No change in virulence			
	No change in virulence					
Reduced covert infection?	Reduced adherence	Unpublished				
Type IV pili	*tapA*	Type IV pilus subunit	No change in virulence	Reduced virulence	No change	Boyd *et al.* 2008
	flp1	Type IV pilus subunit	No change in virulence	No change in virulence		
Type III Secretion (TTSS)	*ascC*	TTSS secretome protein	Avirulent	Avirulent	Altered macrophage cytokine profiles	Dacanay *et al.* 2006
	aexT	Secreted effector	Reduced virulence	No change in virulence	—	
	aopH	Secreted effector	Reduced virulence	No change in virulence	—	
	aopO	Secreted effector	No change in virulence	No change in virulence	—	
Iron sequestration	*fstB*	Anguibactin-like siderophore receptor	Avirulent	Avirulent	—	Unpublished
	fstC	Amonabactin-like siderophore receptor	No change in virulence	No change in virulence	—	
	hupA	Haemoprotein receptor		Reduced virulence	—	
Oxidative stress	*sodA*	Superoxide anion detoxification	No change in virulence	Reduced virulence	Increased enzyme levels in virulent strains	Dacanay *et al.* 2003 (unpublished)
	sodB	Superoxide anion detoxification	No change in virulence	Reduced virulence	Increased enzyme levels in virulent strains	Dacanay *et al.* 2003 (unpublished)
	katA	Hydrogen peroxide detoxification	No change in virulence	—	—	Unpublished

been used to study genomic variability among strains of a variety of bacterial pathogens through a process known as microarray-based comparative genomic hybridization (M-CGH) (Ong *et al*. 2004; Taboada *et al*. 2004). From the genomic sequence Nash *et al*. (2006) developed a DNA microarray of 2024 genes for *A. salmonicida* subsp. *salmonicida* and used M-CGH to compare a variety of *A. salmonicida* subspecies, strains and isolates obtained from different hosts and geographical locations. Using this technique these authors were able to show variable carriage of the virulence-associated genes and generally increased variation in gene content across sub-species and species boundaries. Variable genes included those encoding outer membrane proteins, pili, and flagella. Their data showed little correlation between geographic region and degree of variation for all isolates tested. The greatest variation was observed among genes associated with plasmids and transposons. However, many known virulence genes were common to all strains tested, including superoxide dismutase, and those associated with iron-restriction and sequestration.

Wang *et al*. (2007) examined the LPS structures of the same strains and isolates that were used by Nash *et al*. (2006) in their M-CGH studies. The carbohydrate analysis showed three distinct structural types. The separation of the isolates into the three LPS types corresponded to the clustering of the gene variation seen in the M-CGH studies (Nash *et al*. 2006). Wang *et al*. (2007) speculated that an LPS-based classification system of *A. salmonicida* sub-species consisting of two serologically distinct types: type A and non-type A, could be developed.

Identification of common proteins, carbohydrates, and other antigens is an important first step in identifying genes important in virulence, as well as vaccine candidates that are conserved across all strains known to cause disease. In addition the importance of some of these genes as virulence factors was confirmed with knock-out mutants as described below.

4. Transcriptional, Proteomics and Biochemical Responses of *A. salmonicida* Grown under Selected Conditions: Understanding Virulence Mechanisms of *A. salmonicida* Using Mutants and Live Challenges

In order to investigate effects of host responses and other growth conditions on *A. salmonicida* we conducted studies of gene expression and protein expression, as well as carbohydrate analysis. These studies examined how the phenotype of *A. salmonicida* changed in response to the host factors (*in vivo* growth condition) by comparing *in vivo*-grown bacteria to bacteria grown under variable *in vitro* conditions such as high temperatures, and within iron-restricted media. Such data can aid understanding the host-pathogen interaction as well as in the selection and development of antigens for use in vaccines. These data also highlight that interpretation of data from studies of expression single genes or gene sets, proteins or carbohydrates must be taken within the context of the strain, subspecies, or isolate used.

For example, the *A. salmonicida* genomic DNA microarray was used to examine changes in transcription levels and proteomics was used to examine changes in the translation levels for *A. salmonicida* in response to iron-restricted *in vitro* and *in vivo* growth conditions (Brown *et al*. unpublished). Based upon this work it was possible to identify genes that are differentially expressed when the organism is under these two conditions. Not surprisingly many of the genes that were identified as differentially expressed were involved in the utilization of iron or heme. However, there were several genes which were upregulated only *in vivo*, including genes homologous to an ABC-type galactoside transporter and components of the lateral flagellar system. Comparison of

the expression data to proteomic data obtained from the same samples showed that for many of the genes and proteins there was a strong positive correlation between gene expression level and protein expression, thus confirming the conclusions on the roles of the proteins and genes in virulence and pathogenicity. These results demonstrate how microarray analysis of *A. salmonicida* under varied growth conditions can be used to determine how different host factors influence gene expression, as well as emphasize the importance that interpretation of data from studies of expression single genes or gene sets, proteins or carbohydrates must be taken within the context of the growth conditions.

Within our research program two dimensional gel electrophoresis (2DE)-based proteomics methods were used to identify outer membrane proteins (OMP) of *A. salmonicida*, as well as to describe changes in the OMP profile resulting from *in vitro* culture in low iron media and *in vivo* culture (Ebanks *et al*. 2004, 2005). Ebanks *et al*. (2005) were able to identify 76 proteins from a carbonate-enriched *A. salmonicida* membrane preparation which corresponded to approximately 60% of all of the protein spots that could be visualized by 2DE (pI 4–7). Within these were a number of proteins such as endolase, which due to their lack of a classical export sorting signals were not predicted to be OMPs, i.e., based on motif sequence. Ebanks *et al*. (2005) determined that they were in fact, OMPs, based on protein analysis. Being able to identify these proteins within the OMPs of *A. salmonicida* demonstrates the value of combining genomic and proteomic approaches. In another study in our research program, outer membrane protein profiles of *A. salmonicida* were compared between *in vitro*-grown bacteria and bacteria grown in low iron media and *in vivo* (Ebanks *et al*. 2004). With respect to the OMPs, growth under both iron-restricted conditions and *in vivo* caused a marked up-regulation of two iron regulation systems (ferric siderophore and heme acquisition systems). Based on their results these authors felt that with respect to the OMPs that growth in low iron media was a good model system for *in vivo* growth. Microarray analysis of mRNA expressed in bacteria grown under the same conditions confirmed the results obtained in the proteomics experiments described above (Brown *et al*. unpublished). As these proteins were also seen to be expressed in avirulent strains of *A. salmonicida* under these conditions it is suggested that they may be necessary but not sufficient for bacterial virulence (Ebanks *et al*. 2004). Proteomics and RT-qPCR were also used to study the type III secretion system of *A. salmonicida* grown under selected conditions (Ebanks *et al*. 2006). In that study a variety of strains of *A. salmonicida* were grown *in vitro* at 16, 17 and 28°C, as well as *in vivo*. These authors demonstrated the increased expression, as well as assembly of, a functionally competent type III secretion system in cells grown at 28°C and in the presence of 0.19 to 0.39 M NaCl. Cells that were grown *in vivo*, in low iron media, at low pH or at high cell densities did not show increased expression of the type III secretion system.

Proteins are not the only virulence factors tested within the program. Wang *et al*. (2004) compared the structures of the capsular polysaccharide and lipopolysaccharide O-antigen of *A. salmonicida* grown on TBS and for 72 hours *in vivo*. This study demonstrated the presence of capsular polysaccharide and novel LPS O-chain polysaccharide in cells grown *in vivo* when compared to the *in vitro* grown bacterial inocula. These authors suggested that the use of *in vivo*-cultured cells for isolation and structural analysis of *A. salmonicida* polysaccharides leads to the development of more meaningful biological data.

The conclusions of Ebanks *et al*. (2004), based on proteomics data, were supported by the knock-out mutant results of Dacanay

et al. (2006). Those authors examined the contribution of the type III secretion system (TTSS) to virulence of *A. salmonicida*. The TTSS consists of an injection system (membrane proteins and a needle like structure) and a number of effector proteins that are transmitted into host cells, leading to modulation of components of both the innate and adaptive immune response that are advantages to the bacterium. In their study deletion mutant strains in the genes of the outer bacterial transmembrane pore (ΔascC) and three effector genes (ΔaexT, ΔaopH, ΔaopO) were generated and the virulence of these strains tested by challenge of Atlantic salmon. These authors demonstrated that the TTSS was essential for virulence of *A. salmonicida* as the ΔascC mutant strain was avirulent when challenged by both immersion and intraperitoneal (i.p.) injection. None of the three effector mutants showed significantly decrease virulence when compared to wild type in i.p. challenges. Interestingly, significantly lower morbidity of two of the mutant strains (ΔaexT and ΔaopH) when compared to wild type in immersion trials suggested a possible role of these effectors in host colonization. Furthermore fish that were i.p. challenged with the ΔascC mutant were not protected upon re-challenge with the wild type strain. The authors concluded that the type III secretion system is absolutely essential for virulence of the bacterium, and that removal of individual effectors has little effect on virulence but has a significant effect on colonization.

As described above for the type III secretion systems, using information provided by the genomic sequence a number of mutant strains of *A. salmonicida* have been constructed and tested for virulence by live animal challenge (Table 1). For example, knock-out mutants for genes (*sodA*, *sodB* and *katA*) involved in protection against oxidative stress have been created and used in challenge trials with Atlantic salmon (J. M. Boyd and A. Dacanay, unpublished data). When challenged by injection there was no significant change in virulence when compared to wild type, however the *sodA* and *sodB* mutants when used to challenge fish by immersion had reduced virulence (Table 1).

More recently, Boyd *et al*. (2008) examined the contribution of Type IV pili to the virulence of *A. salmonicida*. Pili are structures that allow bacteria to attach to surfaces such as host tissues and for this reason are important virulence factors for many pathogenic bacteria. In this work the authors constructed three mutant strains that were deficient in: a gene involved in encoding Tap pili (*tapA*), a gene involved in encoding FLP pili (*flpA*) and a double (*flpA-tapA*) mutant. Immersion challenge with the *tapA* and *flpA-tapA* mutants resulted in reduced cumulative mortality when compared to wild type. However, when challenged i.p. there was no significant difference between these mutants and the wild type. In contrast the Flp pili appear to contribute little to virulence as immersion and i.p. challenge with the *flapA* mutant resulted in mortalities that were not significantly different when compared to the wild-type (Boyd *et al*. 2008). The authors concluded that pili were essential factors of the initial adhesion and colonization processes.

Results within our research program showed that when determining the role of putative virulence factors of pathogens it is equally important to test various methods of presentation of the bacterial antigens, as seen by the studies cited above.

Knock-out mutants were also used to investigate pathways involved in host responses. For example, Fast *et al*. (2008, and unpublished data) using enriched cultures of head kidney macrophages exposed to the all of the type III secretion mutants, showed the same pattern of expression of Interleukin (IL)-8 and Tumour Necrosis Factor (TNF) when compared to cells exposed to wild type (parent) strain of *A. salmonicida*. With respect to expression of IL-10 and IL-12 the ΔascC mutant strain (avirulent when

challenged by both immersion and i.p. injection) did not stimulate their expression. Exposure to the mutant Aop3Δ (lacking 3 of the type III secretory system effectors) only stimulated IL-12 expression. Taken together these data suggest that the type III secretion system is not only essential for the virulence of the bacterium, but components of the system are also important for stimulation of host immune responses. IL-12 is an important cytokine released by infected macrophages, which stimulate T-helper type I cells driving towards cell-mediated immunity. As AscCΔ does not stimulate this cytokine, it may explain why infection with this knock-out does not provide protection from future A449 infection as immunological memory has not been triggered by the initial exposure. Furthermore, AscCΔ does not survive/replicate within macrophages as well as A449 (80% reduced).

Production of IL-10 by the wild type strain may assist in prolonging infection and down regulation of pro-inflammatory production and secretion, as IL-10 is inhibitory towards T_H-1 responses and generally towards inflammation, in mammals. Protection by Aop3Δ therefore, would be expected due to stimulation of IL-12, but possibly in the absence of significant virulence (no IL-10), as seen in the wild type strain.

5. Transcriptional Responses of Atlantic Salmon to Infection with *A. salmonicida* and Chronic Stress

At the beginning of this research program there were relatively few genomic sequences available for Atlantic salmon. In order to characterize genes involved in the immune response of Atlantic salmon to infection with *A. salmonicida* reciprocal suppression subtractive hybridization (SSH) six cDNA libraries were constructed for liver, head kidney and spleen tissues following i.p. challenge (Tsoi *et al*. 2004). Control (uninfected, injected i.p. with saline only) cDNA was subtracted against infected tissue, as well as the reciprocal subtractions, i.e., infected tissue was subtracted against that of the control fish. Tissues were kept separate, i.e., distinct libraries were each created for liver, head kidney, and spleen. This resulted in the identification of 1778 expressed sequence tags (ESTs) which were used to create a custom cDNA microarray that has been used to describe the transcriptional responses of Atlantic salmon following cohabitation challenge and Atlantic salmon macropahges exposed *in vitro* to *in vivo* and *in vitro* cultured *A. salmonicida* (Douglas *et al*. 2003; Ewart *et al*. 2005, 2007). Ewart *et al*. (2005) identified differentially expressed genes in head kidney, spleen and liver samples obtained from fish after 7 and 13 days of co-habitation challenge. Using this microarray and RT-qPCR validation they were able to identify in addition to known immune-related genes some unusual genes that were highly upregulated. These genes were homologous to pufferfish plasma high-affinity saxitoxin-binding protein and snake peptide neurotoxin (also know as differentially regulated trout protein). They were also able to identify a large number of unknown genes that were also differentially regulated. This same microarray was used to examine differences in the transcriptional profile of primary isolates of Atlantic salmon macrophages infected with *in vivo* and *in vitro* cultured *A. salmonicida* (Ewart *et al*. 2007).

As mentioned previously there are significant and biologically meaningful differences in the phenotype of *A. salmonicida* culture under these two conditions (Ebanks *et al*. 2004; Dacanay *et al*. 2006; Brown *et al*. unpublished). Examination of gene transcription patterns at 0.5, 1 and 2 hours post-infection revealed commonalities, as well as significant differences in gene expression patterns between macrophages exposed to *in vivo* and *in vitro* grown cells. Differences in expression of genes such as JunB and TNF-α that were evident 30 minutes after

infection led these authors to suggest that these differences may be due to differential production by the bacteria of diffusible products (Ewart *et al*. 2007). As in the earlier study a number of unknown genes were also identified to be differentially regulated making them interesting candidates for further study. Overall the use of microarrays to examine the transcriptional response of *A. salmonicida* has led to the development of more questions than answers.

Fast *et al*. (unpublished data) isolated macrophages from Atlantic salmon and incubated them with the wild-type parent strain and with two type III secretion system knockout mutants, Aop3Δ (deficient in three of the type III secretion system effector genes) and AscCΔ (deficient in the gene encoding the outer-membrane pore of the type III secretion apparatus). The authors also incubated Salmon Head Kidney (SHK) cell culture with the same wild-type and mutant strains. Gene expression within salmon cells was measured using Real-Time qPCR. Their results showed *A. salmonicida* infection stimulates inflammatory gene expression and that expression of IL-8 and TNF are not affected by mutations within the type III secretion system. However, they did observe that the outer-pore membrane secretion apparatus seems to be essential for expression of IL-12 and IL-10, and the three effector genes are also essential for IL-10.

While investigating the mechanisms of host-pathogen interactions it is extremely important to determine the effect of the physiological state of the host on those interactions. The fish species involved in these investigations are important to aquaculture, and are subjected to anthropogenic and natural stressors, such as handling, grading, vaccinations, and fluctuations in temperature and water chemistry. These and other factors, contribute to the physiological state of the animal, and can directly or indirectly affect their ability to respond to pathogens.

Fast *et al*. (2008) showed that repeated handling stress resulted in increased constitutive expression of IL-β in head kidney macrophages from Atlantic salmon, followed by decreased stimulation of leukocytes by extracellular antigens (LPS) and decreased leukocyte survival. This is important information and highlights the need for further studies. Aquaculture fish species can be subjected to acute and chronic stressors and it is essential to understand the effect of those stressors on the animals' ability to mount protective immune responses against pathogens. If the efficacy of vaccines is compromised because of the hosts' immune state, this will add to the cost of production to the farmer and will also lead to increased losses due to infection. If this can be avoided by avoiding stressors at the time of vaccination (for example), this would prove to be a low-cost approach to aquatic animal health management.

6. Metabolomics Responses of Atlantic Salmon to Infection with *A. salmonicida* and Vaccination

Metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind"—specifically, the study of their small-molecule metabolite profiles (Davis 2005). The metabolome represents the collection of all metabolites in a biological organism, which are the end products of its gene expression. Solanky *et al*. (2005) used NMR-based metabolomics to show that metabolites within the plasma of Atlantic could clearly separate into distinct groups: those fish that had been vaccinated with killed *Aeromonas salmonicida* bacterin, those that had been challenged, and those that had survived the challenge. Dacanay *et al*. (2006) showed that this technique can be used to investigate host response to specific virulence factors in pathogens, using plasma from fish challenged with selected knock-out mutants, and with the wild-type parent strain. Using

NMR-based metabolomics, the authors compared the plasma from vaccinated salmon immersion-challenged with the wild-type parent strain with plasma from fish challenged with the avirulent mutant strain AscCΔ. Their results showed that the plasma metabolite profiles correlated with the protective immune response, i.e., where a protective immune response was observed in whole animal challenge, the plasma profiles clustered separately. When there was no significant protection, the plasma profiles were indistinguishable (Dacanay *et al*. 2006). This study clearly demonstrates that metabolomics technologies have potential to provide non-invasive assays to monitor host immune responses.

7. Summary and Future Directions

In using these and other techniques, we have been able to identify virulence proteins and carbohydrates from *Aeromonas salmonicida*, and have been able to develop more effective vaccine candidates against the pathogen. Moreover, in examining host immune responses at the molecular level, we have developed antigen delivery strategies that target the immunomodulatory mechanisms of the host.

Our investigations into the molecular mechanisms of salmonid stress-related genes and disease susceptibility will allow us to develop more effective tools for husbandry. These and other studies will also aid in targeting specific disease-resistance markers for family selection and comprehensive breeding programs.

However, a word of caution is warranted. The tools and technologies as described above are very powerful, yet remain only tools. Genome sequencing, cDNA microarrays, EST libraries, etc., generate huge data sets, usually with hundreds, if not thousands of unknown genes and proteins. Currently, gene function is often tentatively assigned on the basis of BLAST annotation and homology to known genes (e.g., GO annotation). If useful tools and knowledge is to be derived from the use of these genomics technologies, then it must be validated by complementary studies of the biological function of the genes and proteins. Knockout mutants, transformative cell lines, and recombinant protein technologies are all useful in this sense. Ultimately, however, whole animal trials validate any assumptions made via the genomics and proteomics approaches described above. We have taken this systems approach in our research program.

Also required is an understanding of the physiological responses of the host, within the context of nutrition, stress, environmental effects. Equally important is knowledge of the biology of the pathogen: its genetic variability, its gene expression within the context of its environment.

As can be seen in describing the early and then later work of our team and others, these genomics and proteomics technologies can also be used to push earlier studies much further. Thus the techniques will allow us to achieve a greater understanding of the mechanisms of systems and processes that had been previously described at an higher organismal level.

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