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10 Pathogenesis and the gastrointestinal tract of growing fish

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A diverse range of bacteria and viruses is associated with diseases of fish. The skin, lateral line, gills and gastrointestinal tract or a combination of these organs are suggested to be infection routes. The purpose of this review is to present current knowledge on adhesion, colonization and translocation of pathogenic agents in the gastrointestinal tract of growing fish.

1. INTRODUCTION

As fish live in an aqueous environment, their external surfaces will be regularly exposed to potential pathogens, and water taken into the gastrointestinal (GI) tract during feeding can deliver them to the mucosal surfaces of this tract. Even in the non-feeding early stages of development of marine fish larvae, drinking of water is required for osmotic regulation (Tytler and Blaxter, 1988) and this provides an early entry into the GI tract for bacteria. As in all animals, the GI tract is the route of nutrient uptake and any perturbation by microbial action can be harmful. This is particularly so in the early stages of fish larval development. In contrast to mammals, where numerous bacterial and viral pathogens produce severe diarrhoeal disease there are no directly equivalent pathogens known for fish. However, a number of bacteria cause pathology in the gut of fish and this can be a route of systemic infection in many instances, comparable to that of invasive enteropathogens of mammals.

The bacterial pathogens of major importance in aquaculture are, with few exceptions, Gram-negative microorganisms. *Aeromonas salmonicida*, *A. hydrophila*, *Vibrio anguillarum*, *V. salmonicida*, *V. viscosus* (*Moritella viscosa* or *M. marina*) and *V. ordalii* belong to the Vibrionaceae; *Yersinia ruckeri*, *Edwardsiella ictaluri* and *E. tarda* are members of the Enterobacteriaceae, and *Piscirickettsia salmonis* is a member of Pisciriickettsiaceae. Of the Gram-positive bacterial pathogens, *Renibacterium salmoninarum* belongs to the Corynebacteriaceae, *Carnobacterium piscicola* to the lactic acid bacteria, and others, e.g. *Streptococcus iniae*, *S. difficile* and *Lactococcus garviae*, are Gram-positive cocci. Microbial pathogenicity has been defined as *the biochemical mechanisms whereby microorganisms cause disease* (Smith, 1990). Not all pathogens have an equal probability of causing infection and disease. In this review, the term infection will be used to describe successful persistence or multiplication of a pathogen on or within the host, while disease will be described as an infection which causes significant overt damage to the host.

Intensive fish production has increased the risk of infectious diseases all over the world (Press and Lillehaug, 1995; Karunasagar and Karunasagar, 1999), but to prevent microbial entry fish have various protective mechanisms, such as production of mucus by goblet cells, the apical acidic microenvironment of the intestinal epithelium, cell turnover, peristalsis, gastric acidity, lysozyme and antibacterial activity of epidermal mucus. At the same time, pathogenic microorganisms have evolved mechanisms to target the skin, gills or GI tract as points of entry. The three major routes of infection are through: a) skin (Kawai et al., 1981; Muroga and De La Cruz, 1987; Kanno et al., 1990; Magarinos et al., 1995; Svendsen and Bøgvold, 1997; Spanggard et al., 2001), b) gills (Baudin Laurencin and Germon, 1987; Hjeltnes et al., 1987; Svendsen et al., 1999), and c) the GI tract (Sakai, 1979; Rose et al., 1989; Chair et al., 1994; Olsson, 1995; Grisez et al., 1996; Olsson et al., 1996; Romalde et al., 1996; Jöborn et al., 1997; Robertson et al., 2000; Lødemel et al., 2001).

Pathogenicity can be divided into four different phases: 1) the initial phase where the pathogen enters the host's environment, including the GI tract, 2) the exponential phase where the pathogen adheres to and colonizes mucosal surfaces, replicates to sufficient numbers and/or translocates into host enterocytes, 3) the stationary phase where the pathogen replicates within the host and circumvents the host defence system; in this phase the host is moribund and this can quickly be followed by 4) the death phase.

In order to adhere successfully, colonize and produce disease, the pathogen must overcome the host defence system. It is well known that stress from environmental factors, such as oxygen tension, water temperature and water salinity, are important in increasing the susceptibility of fish to microbial pathogens. The water milieu can also facilitate transmission of these pathogens.

The purpose of this review is to present information on 1) adhesion of bacteria to mucosal surfaces, 2) protection against bacterial adhesion, 3) bacterial translocation, 4) invasion of host cells, 5) effect of diet in disease resistance and 6) data obtained from endothermic animals which may have relevance to pathogenesis of fish.

2. ADHESION OF BACTERIA TO MUCOSAL SURFACES

2.1. General factors

A number of environmental factors determine whether bacteria can adhere to and colonize the digestive tract of endothermic animals and these have been extensively reviewed by Savage (1983). Among these are: 1) gastric acidity (Gilliland, 1979); 2) bile salts (Floch et al., 1972); 3) peristalsis; 4) digestive enzymes (Marmur, 1961); 5) immune response; and 6) indigenous microorganisms and the antibacterial compounds which they produce. In order to replicate to a sufficient number to allow transmission to a new susceptible host, a microbial pathogen must enter a host, find a unique niche, circumvent competing microbes and host defence barriers, and obtain nutrients from the host.

Adhesion of bacteria to surfaces such as epithelial cells involves four different types of interaction, depending on the distance separating the bacteria from the surface. Attraction is initially by van der Waal's forces operating at distances greater than 50 nm, but at closer distances electrostatic interactions become more significant. As epithelial and bacterial cells are usually negatively charged, electrostatic repulsion normally prevents closer association. In regions of lower ionic strength closer interaction may occur allowing hydrophobic interaction and specific receptor–ligand binding within *circa* 1 μm separation. This leads to strong binding between bacteria and host cell surfaces (Fletcher, 1996).

2.2. Adhesins

Several bacterial surface components can be involved in specific binding to epithelial cell ligands. The best characterized bacterial adhesins are the fimbriae (or pili) which are widely distributed on Gram-negative bacteria (Smyth et al., 1996), but are also found on some Gram-positive bacteria (Klemm et al., 1998). Although fimbriae are the most widely used adhesins in Gram-negative bacteria, flagella, capsules, protein fibrils, outer membrane proteins (Gram-negative bacteria), surface proteins (Gram-positive bacteria) and crystalline protein surface arrays can all be used as adhesins (Henderson et al., 1999).

A range of fimbriae can be expressed by any one bacterial species; for example, 14 different types of fimbriae are known in *Escherichia coli*, more than one of which can be expressed at the same time (Hacker, 1992; Klemm et al., 1998; Nataro and Kaper, 1998). Type 1 fimbriae of *E. coli* are perhaps the best studied example and a single cell may express over 500 fimbriae. Of approximately 7 nm in diameter and 1 μm in length, type 1 fimbriae are composed of about 1000 copies of the major structural protein FimA, in a helical cylinder (Brinton, 1965) capped by the FimH protein which recognizes mannose-containing receptors on the target eukaryotic cell. Other minor proteins, FimF and FimG are involved in binding FimH to the FimA helix and other genes in the *Fim* complex are required for assembly of the fimbriae and translocation through the bacterial membranes (Krogfeldt et al., 1990; Klemm et al., 1998).

Despite their widespread occurrence in Gram-negative bacteria and their importance in the pathogenesis of numerous infections, fimbriae have not yet been proved to be important in bacterial infections of fish. Saeed (1983) observed that *E. ictaluri* was heavily piliated and suggested that this could be important in infection, although this awaits further examination.

The crystalline protein array (S layer or A layer) of *A. salmonicida* renders the surface of this bacterium extremely hydrophobic to the extent that bacteria in broth cultures autoagglutinate and sediment rapidly when allowed to stand unshaken. Loss of the S layer can occur spontaneously, or can be induced by culture at elevated temperature or by Tn5 mutagenesis; in all cases the loss of the S layer is accompanied by loss of virulence (Ishiguro et al., 1981; Belland and Trust, 1985; Trust, 1986) and loss of adherence to macrophages (Trust et al., 1983).

Flagella are important adhesins for bacteria such as *V. cholerae* (Guentzel and Berry, 1975; Richardson, 1991) and *Campylobacter jejuni* (Wassenaar et al., 1991; Nachamkin et al., 1993). Although flagella have been shown to be important for the virulence of *V. anguillarum*, this was not at the level of adhesion, as motility-deficient mutants which had much reduced virulence had similar adhesion levels to chinook salmon embryo (CHSE) cells as the wild-type organism (Ormonde et al., 2000). However, chemotactic motility and active motility are important for virulence in waterborne infections of fish (O'Toole et al., 1996, 1999; Ormonde et al., 2000).

Infectivity studies revealed that disruption of the flagellum and subsequent loss of motility correlated with an approximate 500-fold decrease in virulence when fish were inoculated by immersion in bacteria-containing water. Once the pathogens have reached the mucosal surface, several options exist: depending on their intrinsic colonizing or invasive capacities, the nature of the toxin(s) they produce and their ability to resist host defences.

2.3. Electron microscopy studies of adhesion of fish-pathogenic bacteria to tissues of the GI tract

In a recent study, Knudsen et al. (1999) tested pathogenic and non-pathogenic bacteria isolated from fish for their adhesion to cryosections from different mucosal surfaces of Atlantic salmon by immunohistochemistry. The majority of the bacteria tested – *V. anguillarum* serotype O1, *V. salmonicida*, *V. viscosus*, *Flexibacter maritimus*, “gut vibrios” and intestinal isolates of *V. salmonicida* – all adhered to mucus from the pyloric caeca, foregut and hindgut. In contrast to these results, *V. anguillarum* serotype O2 (O2a and O2b), did not adhere to mucus.

The past decade has seen an explosion of information on our understanding of bacterial adhesion at both the molecular and genetic level of endothermic animals, and electron microscopy has contributed significantly to this knowledge (Knutton, 1995). Although several papers have described pathogenesis in fish, few investigations have used transmission electron microscopy (TEM) and/or scanning electron microscopy (SEM) to evaluate the effect of bacterial infection on morphology in the

GI tract of fish. The advantage of using SEM is that large areas of the mucosal and cell surfaces can be examined rapidly for adherent bacteria. Adhesion can then be assessed qualitatively or quantitatively. For quantitative analysis a defined number of fields is selected at random, photographed, and bacterial adherence assessed to give an adhesion index consisting of numbers of bacteria per unit area (Yamamoto et al., 1991) or percentage of area colonized by bacteria (Knutton et al., 1991). The resolution of SEM is rarely sufficient to obtain detailed information about the mechanisms of adhesion, although it has proved useful to determine bacterial adhesion/colonization of gut enterocytes of fish (Magarinos et al., 1996; Ringø et al., 2001, 2002). Magarinos et al. (1996) demonstrated that *Photobacterium damsela* (*Pasteurella piscicida*) strains adhered strongly to the intestines from sea bream, sea bass and turbot in numbers ranging from 10^4 to 10^5 bacteria per gram of intestine depending on the bacterial isolate and the fish species employed. These results are clearly supported by scanning electron microscopy studies. Sometimes, bacteria colonizing the GI tract had their luminal ends protruding above the levels of the microvilli (figs. 1 and 2). Micrographs displayed clear differences in levels of bacterial association over a small area, as some enterocytes were heavily colonized while others had no associated bacteria. Ringø et al. (2001) showed that some enterocytes were heavily colonized by bacteria when charr were fed dietary soybean oil, whereas a different situation was observed when fish were fed dietary linseed oil (Ringø et al., 2002). In the latter situation, most bacteria associated with enterocytes were located at the apical brush border (fig. 3).



Fig. 1. Scanning electron micrograph of the apical aspects of enterocytes in the midgut of Arctic charr (*Salvelinus alpinus* L.) fed dietary soybean oil. The borders between adjacent cells are clearly visible, as microvilli which cover the cell apex. The luminal ends of bacteria located in the intestines between microvilli are also visible (arrows). $\times 7500$. After Ringø et al. (2001).

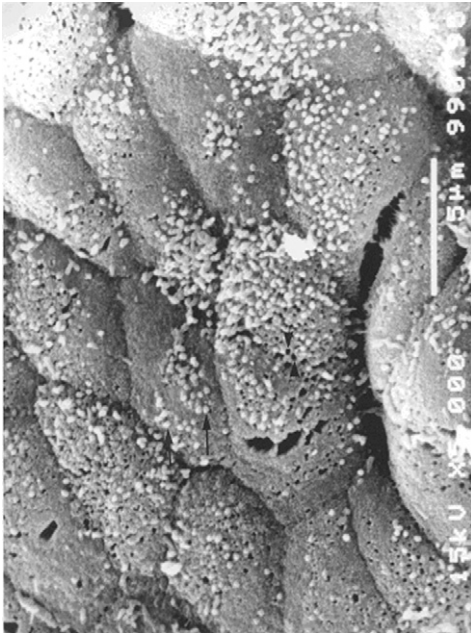


Fig. 2. Scanning electron micrograph showing cell apices in the hindgut of Arctic charr (*Salvelinus alpinus* L.) fed dietary soybean oil. Cell borders can be seen and all cells have associated bacteria (arrows), although numbers vary from cell to cell. Note the small spaces (arrowheads) between microvilli. These may represent the transit paths of more deeply embedded bacteria, or they may be created by bacterial loss. The latter may be an artefact of tissue preparation or a consequence of local bacterial cell division. $\times 5000$. After Ringø et al. (2001).

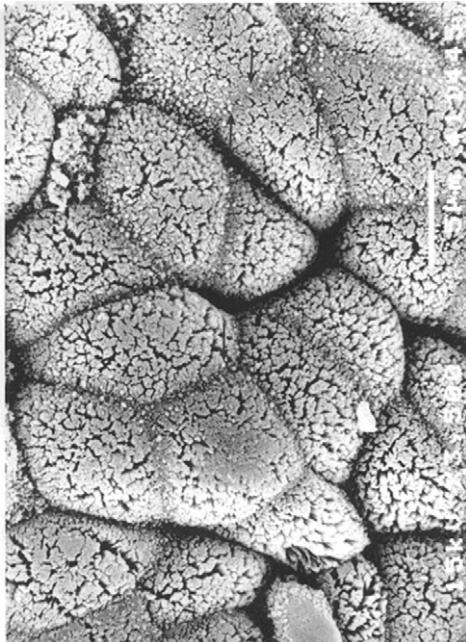


Fig. 3. Scanning electron micrograph showing bacteria associated with enterocytes in the hindgut of Arctic charr (*Salvelinus alpinus* L.) fed dietary linseed oil. Associated bacteria (arrows) are located at the apical brush border. $\times 7500$. After Ringø et al. (2002).

Fish pathogenic bacteria, such as *V. salmonicida* and *V. anguillarum*, have been shown *in vivo* to adhere to the intestinal epithelium of fish larvae and promote severe destruction of microvilli (Olafsen and Hansen, unpublished data). In contrast, Lødemel et al. (2001) did not show any destruction of microvilli in the pyloric caeca, midgut or hindgut regions of adult Arctic charr (*Salvelinus alpinus* L.) infected by *A. salmonicida* subsp. *salmonicida*. SEM investigations of human intestinal mucosa infected with enteropathogenic *E. coli* (EPEC) showed that EPEC adhere intimately in microcolonies and cause gross alterations of the brush border surface of infected enterocytes (Knutton et al., 1987, 1989; Knutton, 1995). These characteristic “attaching-and-effacing” lesions are formed on epithelial cells in a three-stage process. After initial adhesion, mediated by bundle-forming (type IV) fimbriae, a type III secretory system is activated in *E. coli* allowing secretion of a receptor (translocated intimin receptor) into the epithelial cell membrane which acts as a receptor for the *E. coli* outer membrane protein intimin. This leads to reorganization of the cellular actin cytoskeleton and formation of the characteristic elevated pedestal to which *E. coli* is bound.

2.4. Host cell ligands

A wide range of potential receptors is present on the eukaryotic cell membrane involved in the normal cellular functions of transport, signal transduction and cell–cell communication, and bacteria can bind to many of these molecules. In addition, proteins of the extracellular matrix, such as fibronectin, fibrinogen and collagen, are receptor molecules for which specific adhesins have been characterized in bacteria such as *Staphylococcus aureus* (Smeltzer et al., 1997). In glycoproteins, the sugar residues commonly act as receptor ligands for fimbriae; binding of *E. coli* to eukaryotic cells via type 1 or type 5 fimbriae is inhibited by mannose leading to the conclusion that mannose-containing glycoproteins are cellular targets for binding by this organism (Krogfeldt et al., 1990). Other sugars, e.g. fucose and galactose have been similarly identified as receptor targets for other types of fimbriae (Ofek and Doyle, 1994). Similar work by Wang and Leung (2000) has shown that strains of *Vibrio anguillarum* differ in the types of receptors used. Two invasive strains of the organism, G/Virus/5(3) and 811218-5W adhered strongly to three different fish tissue culture cell lines. Adherence of strain G/Virus/5(3), and of nine other vibrios, was inhibited by galactose-containing sugars, but adherence by strain 811218-5W was not affected by a range of sugars tested. As no fimbriae could be detected in either strain it was concluded that non-fimbrial adhesins were involved in both cases (Wang and Leung, 2000).

The ability of *Photo. damselae* subsp. *piscicida* to adhere to fish tissue culture cell lines was inhibited by galactose and mannose but not fucose, indicating a possible glycoprotein target for adhesins of this organism (Magarinos et al., 1996). However, prior treatment of bacteria with proteinase K did not affect their capacity

to bind to tissue culture cells and the *Photo. damselae* subsp. *piscicida* adhesin remains unidentified.

2.5. Consequences of bacteria/ligand interactions

As noted above, many cell surface molecules are receptors involved in transmembrane signalling. For bacteria, interaction with eukaryotic cells can lead to altered cell growth patterns, induction of adhesins, e.g. for enteropathogenic *E. coli* (Donnenberg and Kaper, 1992), or secretion of proteins required for invasion, e.g. for *Yersinia* (Cornelis and Wolf-Watz, 1997). For the eukaryotic cell, uptake of bacteria may result in cytokine release (Wilson et al., 1998), morphology alteration (enteropathogenic *E. coli*) (Nataro and Kaper, 1998) or intercellular adhesion molecule synthesis may be stimulated (enteroinvasive *E. coli*).

3. PROTECTION AGAINST BACTERIAL ADHESION

3.1. Mucus

The internal surface of the host is the first defence barrier to infection. Intestinal mucins secreted by specialized epithelial goblet cells located in the intestinal enterocytes form a viscous, hydrated blanket on the surface of the intestinal mucosa that protects the delicate columnar epithelium. This is thought to be a vital component of the intestinal mucosal barrier in prevention of colonization by pathogens in both fish and endothermic animals (Florey, 1962; Forstner, 1978; Westerdahl et al., 1991; Maxson et al., 1994; Henderson et al., 1999; Mims et al., 2000). Gastrointestinal mucus is thought to have three major functions: 1) protection of the underlying mucosa from chemical and physical damage, 2) lubrication of the mucosal surface, and 3) to provide a barrier against entero-adherence of pathogenic organisms to the underlying mucosal epithelium. Intestinal mucus is composed almost entirely of water (90–95%) and the electrolyte composition is similar to plasma, accounting for about 1% of the mucus weight. The remaining 4–10% is composed of high molecular weight glycoproteins (mucins), consisting of a protein core with numerous carbohydrate (fucose and galactose) side chains. Hydrolysis of intestinal mucus material of rainbow trout liberated increased amounts of N-acetylgalactosamine and N-acetylglucosamine (O'Toole et al., 1999), indicating that these carbohydrates may be present as mucin-bound moieties in fish intestinal mucus as is the case for mucus from other animal species (Roussel et al., 1988). The majority of intestinal mucus-associated lipids in rainbow trout partitioned to the organic phase during extraction with chloroform/methanol and this contained saturated and unsaturated free fatty acids, phospholipids, bile acid, cholesterol, and monoglycerides and diglycerides (O'Toole et al., 1999).

The mucous blanket is constantly renewed by the secretion of high molecular weight glycoproteins from individual goblet cells throughout the epithelium.

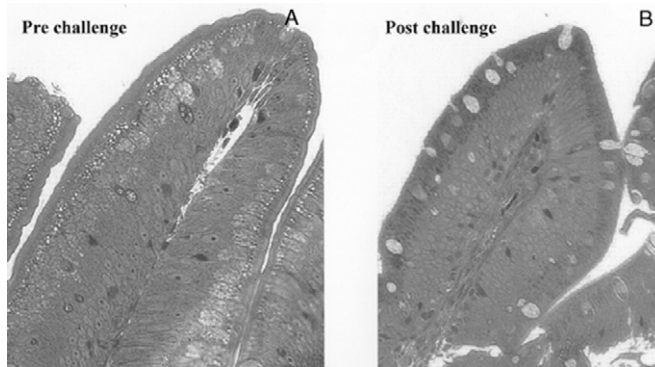


Fig. 4. Light microscopic view of villi in the midgut from Arctic charr (*Salvelinus alpinus* L.) fed soybean oil (A) post and (B) prior to challenge with *Aeromonas salmonicida* subsp. *salmonicida*. Note the substantial more conspicuous goblet cells (arrows) along the villi of infected fish. After Lødemel et al. (2001).

Goblet cells differentiate in the lower portion of the crypts of both small and large intestine and gradually migrate on to the villi or mucosal surface.

In an early study on histopathological changes caused by *V. anguillarum*, Ransom et al. (1984) found large amounts of goblet (mucus producing) cells in the anterior part of the GI tract of infected chum salmon. The first reaction of Arctic charr (*Salvelinus alpinus* L.) infected by pathogenic bacteria (*A. salmonicida*) is to slough off the infected mucus by increasing goblet cell production (fig. 4A) compared to uninfected fish (fig. 4B). A similar reaction to that found in infected fish is also observed in rabbits and rats infected by pathogenic bacteria (Enss et al., 1966; Mantle et al., 1989, 1991), and this may be considered a normal host response to particular intestinal infections (Mims et al., 2000).

Gastrointestinal mucus is rich in nutrients that organisms, including pathogens, may utilize for growth (Blomberg et al., 1995; Wadolowski et al., 1988). Many endothermic studies have implicated growth in mucus as a critical factor for intestinal colonization by pathogens and several outer membrane proteins are necessary for establishment of an infection focus (Freter et al., 1983; Myhal et al., 1982; Krivan et al., 1992; Burghoff et al., 1993). Olsson et al. (1992) suggested that the GI tract is a site of colonization of *V. anguillarum* as the pathogen could utilize diluted turbot (*Scophthalmus maximus* L.) intestinal mucus as its sole nutrient source. More recently, Garcia et al. (1997) examined the ability of *V. anguillarum* to grow in salmon intestinal mucus, which they concluded is an excellent growth medium for this species. This is an important aspect of the pathogenesis of this organism.

3.2. Ultrastructural changes in enterocytes caused by dietary manipulation

Recently, Olsen et al. (1999, 2000) showed that extensive accumulation of lipid droplets occurred in Arctic charr enterocytes when the fish were fed a diet containing linseed oil and this caused significant damage to the epithelium with focal loss

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Fig. 5. Linseed-oil-fed Arctic charr (*Salvelinus alpinus* L.). These enterocytes are obviously damaged by the fat vacuoles (droplets). Note especially cellular membrane ruptures (arrows). Bar = 7 μ m. After Olsen et al. (1999).

of enterocytes and consequent loss of the epithelial barrier. Such damage (fig. 5) is likely to be pathological and therefore detrimental to fish health. Rupture in the membranous system may also represent a major microbial infection route for potentially pathogenic bacteria provided they are present in sufficient numbers in the gut.

The prebiotic potential of dietary fibres is well known in endothermic animals (Gibson, 1998), and may also have interesting applications in aquaculture. However, a recent study clearly demonstrated that feeding Arctic charr a diet supplemented with 15% inulin led to the occurrence of a large number of spherical lamellar bodies in the enterocytes of the pyloric caeca and the hindgut. These structures were not observed when fish were fed 15% dextrin (Olsen et al., 2001). Feeding inulin had a destructive effect on microvillus organization which may increase translocation of pathogenic bacteria if they are present in relatively high concentrations in the GI tract.

3.3. Autochthonous bacteria and antagonistic activity

Savage (1983) defined bacteria isolated from the digestive tract as being either indigenous (autochthonous) or transient (allochthonous) depending on whether or not they are able to colonize epithelial surface of the digestive tract of the host animal. Recently, Ringø and Birkbeck (1999) presented a list of criteria for testing autochthony of bacteria from the GI tract of fish. These were that they should i) be found in healthy animals, ii) colonize early stages and persist throughout life, iii) be found in both free-living and hatchery-cultured fish, iv) grow anaerobically, and v) be found associated with epithelial mucosa in the digestive tract. The presence of an autochthonous microflora fitting the above criteria was demonstrated recently by Ringø et al. (2002) in that bacteria in the gut were found closely associated with the intestinal epithelium and between the microvilli. On the basis of this observation, one might hypothesize that the autochthonous microflora of fish which is associated closely with the intestinal epithelium forms a barrier serving as the first defence to limit direct attachment or interaction of pathogenic bacteria with the mucosa as reported for endothermic animals (van der Waaij et al., 1972; Snoeyenbos, 1979;

Tancrède, 1992). In fish, situations such as stress, antibiotic administration, or even small dietary changes affect the GI tract microflora. Stability of this microflora is important in natural resistance to infections produced by bacterial pathogens in the intestinal tract. The existence of antibacterial substances produced by bacteria isolated from the digestive tract of fish has been demonstrated in several studies (Schröder et al., 1980; Dopazo et al., 1988; Strøm, 1988; Westerdahl et al., 1991; Olsson et al., 1992; Bergh, 1995; Sugita et al., 1996, 1997, 1998; Jöborn et al., 1997; Gram et al., 1999; Ringø, 1999; Ringø et al., 2000). However, a recent study by Gram et al. (2001) demonstrated that *in vitro* activity in well diffusion assays and broth cultures cannot be used to predict a possible *in vivo* effect even if a reduction of *in vivo* mortality was observed in another system (Gram et al., 1999). These studies underline the importance of developing and testing cultures for each specific combination of different pathogens, different fish species and environment that might occur.

4. BACTERIAL INVASION AND TRANSLOCATION MECHANISMS

The indigenous intestinal flora is prevented from gaining access to other sites in the body by a single epithelial cell layer on the mucosa. In endothermic animals the M cells of the intestinal epithelium are specialized structures that may allow natural entry of bacterial pathogens (Jones et al., 1995; Neutra et al., 1996; Vazques-Torres and Fang, 2000). Information about the interactions between intracellular pathogenic bacteria and M cells in fish is not available, however, and is a topic of further studies.

The mechanisms by which bacteria can translocate from the gut to appear in other organs are an important phenomenon in the pathogenesis of “opportunistic” infections by indigenous intestinal bacteria (Finlay and Falkow, 1997). Once inside a host cell, pathogens have a limited number of ways to ensure their survival whether remaining within a host vacuole or escaping into the cytoplasm.

In endothermic animals the primary defence mechanisms preventing indigenous bacteria from translocating from the gastrointestinal tract are: a) a stable GI tract microflora preventing bacterial overgrowth of certain indigenous bacteria or colonization by more pathogenic exogenous bacteria, b) the host immune defences and c) an intact mucosal barrier. More than one of these defence mechanisms can be involved, depending upon the animal model or clinical situation. An example of this is *Lactobacillus casei*, which can prevent *E. coli* infection in a neonatal rabbit model and inhibits translocation of *E. coli* in an enterocyte cell culture model (Mattar et al., 2001). However, in fish these defence mechanisms are not well understood.

The pathogenesis of *V. cholerae* infections in mammals is primarily a non-invasive toxin-mediated gut infection but such infections have not been found in fish. Translocation of intact *Vibrio* antigens and bacterial cells by endocytosis has been reported in the gastrointestinal tract of fish larvae (Hansen and Olafsen, 1990, 1999; Hansen et al., 1992; Olafsen and Hansen, 1992; Grisez et al., 1996). However, when discussing endocytosis, the development of the digestive tract is an important

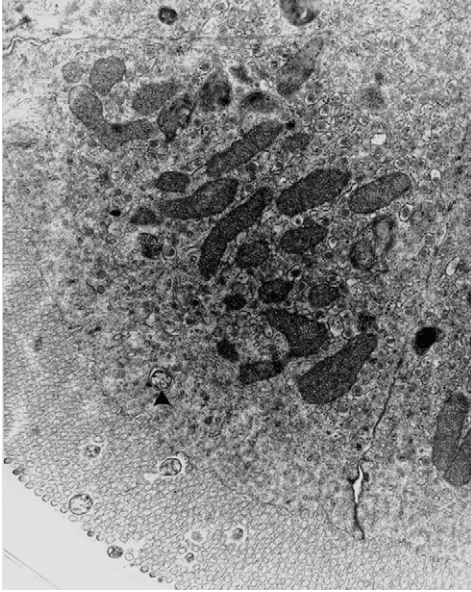


Fig. 6. Transmission electron micrograph of the apical regions of enterocytes in the pyloric caecum of adult Arctic charr (*Salvelinus alpinus* L.). Bacterial profiles are seen scattered at different levels within the brush border from the tips to bases of microvilli. In addition, one bacterial profile (arrowhead) is seen to be contained in an internalized, membrane-bound endocytic vacuole. $\times 15000$. After Ringø et al. (2001).

factor to be considered. At the time of hatching, the digestive tract of fish is an undifferentiated straight tube which is morphologically and physiologically less elaborate than that of the adult (Govoni et al., 1986). However, endocytosis was demonstrated in pyloric caeca (fig. 6), midgut (fig. 7) and hindgut (fig. 8) of adult Arctic charr (Ringø et al., 2002).

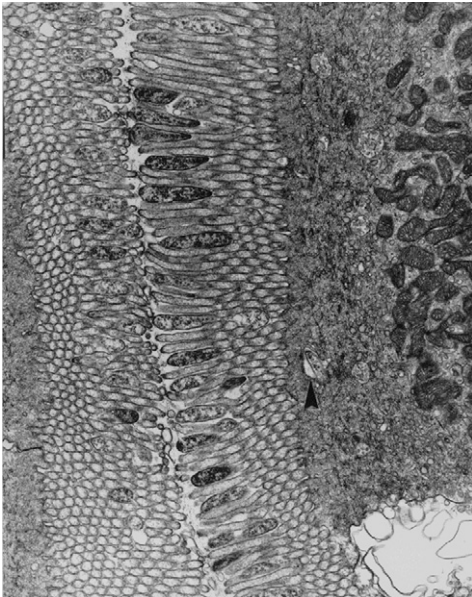


Fig. 7. High power transmission electron micrograph of the midgut of adult Arctic charr (*Salvelinus alpinus* L.). The opposed surfaces of two enterocytes are shown. Both cells have appreciable numbers of bacterial profiles between their microvilli. Note the internalized bacterium in the subapical cytoplasm (arrowhead). $\times 15000$. After Ringø et al. (2001).

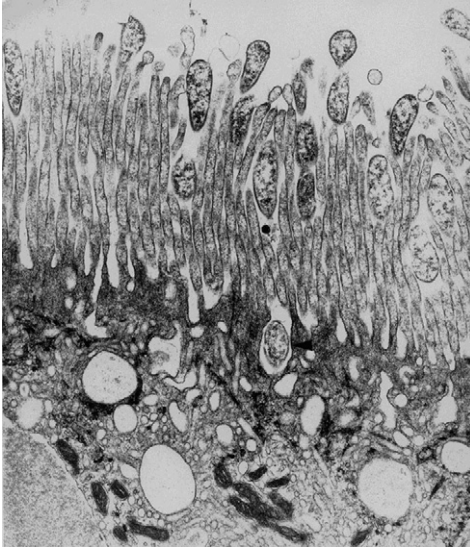


Fig. 8. Transmission electron micrograph showing bacteria associated with the microvilli of enterocytes in the hindgut of adult Arctic charr (*Salvelinus alpinus* L.). Enterocytes in this region show endocytic activity and are characterized by large numbers of intracytoplasmic vacuoles (V) with contents of varying electron density. An internalized bacterium is discernible (arrowhead). $\times 15000$. After Ringø et al. (2002).

5. INVASION OF HOST CELLS

Entry into host cells is a specialized strategy for survival and multiplication utilized by a number of pathogens which can exploit existing eukaryotic internalization pathways (Finlay and Falkow, 1989, 1997; Sansonetti, 1993). Three general mechanisms are recognized by which bacteria can invade epithelial cells. The most common method, as employed by *Yersinia*, *Shigella* and *Salmonella*, is by inducing rearrangement of the actin cytoskeleton of the epithelial cell. Enteropathogenic *Yersinia* spp. induce uptake into endocytic vacuoles of epithelial cells following close contact of the bacteria at many points to the cell surface (zippering). This involves three adhesins – the invasin, Ail and YadA proteins – and interaction between invasin and its cell-surface receptor, $\alpha_5\beta_1$ integrin induces actin cytoskeleton rearrangement via a protein tyrosine kinase signalling system (Cornelius and Wolf-Watz, 1997; Lloyd et al., 2001). Invasion by *Shigella* is dependent upon possession of a 220 kb plasmid encoding 32 invasion-associated genes (Menard et al., 1996), including those for a type III secretion system which directly secretes *Shigella* proteins into the cytoplasm of the epithelial cell; this induces actin cytoskeleton rearrangement and pseudopodia formation to internalize the bacterial cell. Once internalized, lysis of the vesicle is mediated by a *Shigella* protein releasing the organism into the cytoplasm where it can multiply and spread through the cytoplasm propelled by an actin “tail” (Menard et al., 1996). Inhibitors of actin polymerization, such as cytochalasin D, block entry of such pathogens into cells. However, invasion of epithelial cells by *Campylobacter jejuni* is unaffected by cytochalasin D but is sensitive to the microtubule depolymerizing drug colchicine,

indicating an actin-independent, microtubule-dependent pathway of entry (Russell and Blake, 1994; Biswas et al., 2000). A direct invasive mode of entry is utilized by rickettsiae, which bind to the phospholipid cell membrane and gains entry via expression of phospholipase A (Silverman et al., 1992). For fish pathogens, examples are known of invasion of epithelial cells. Although none has been characterized to the extent of human pathogens, current knowledge is summarized below.

5.1. *Aeromonas*

Aeromonas salmonicida is the causative agent of furunculosis, a disease which caused very serious losses in European aquaculture in the early 1990s (Munro and Hastings, 1993) and which had previously caused major epizootics in wild fish (Mackie et al., 1930). All salmonid species are affected, but Atlantic salmon and brook trout appear to be more susceptible to infection than rainbow trout or Pacific salmon (Cipriano, 1983). The intestine has long been considered a route of infection for *A. salmonicida* as Plehn (1911) found inflammation of the gut to be a common characteristic of furunculosis. However, there is still debate about the route of entry of this pathogen (Bernoth et al., 1997). The presence of *A. salmonicida* in the intestine of Atlantic salmon has been demonstrated using an enzyme-linked immunosorbent assay by Hiney et al. (1994), who suggested that the intestine could be the primary location of *A. salmonicida* in stress-inducible infections. O'Brien et al. (1994) also detected *A. salmonicida* in faeces using a species-specific DNA probe, in conjunction with a polymerase chain reaction (PCR) assay. Although the organism can be detected in the intestinal tract in the above assays, McCarthy (1977) failed to infect brown trout (*Salmo trutta*) either by administering food pellets soaked in a culture of *A. salmonicida*, or by direct intubation into the stomach. In the latter case, 10^5 – 10^6 *A. salmonicida* were recovered per ml homogenized stomach within 12 h of introduction, no organisms could be recovered by 48 h and no mortalities occurred, despite recovery of low numbers of organisms from homogenates of kidney within 5 h. Despite failing to cause disease by the intestinal route the organism killed five of six fish exposed for 5 days to an aqueous suspension of the bacteria (10^6 cells/ml).

In experimental infections of turbot (*Scophthalmus maximus* L.) and halibut (*Hippoglossus hippoglossus* L.) yolk sac larvae with *A. salmonicida* subsp. *salmonicida*, Bergh et al. (1997) failed to re-isolate the pathogen from halibut larvae, but using immunohistochemical techniques showed the bacteria to be present in the intestinal lumen of some turbot larvae, but not associated to mucus or gut microvilli.

A recent study by Lødemel et al. (2001) clearly demonstrated that *A. salmonicida* subsp. *salmonicida* could be detected within enterocytes of the midgut of Arctic charr (*Salvelinus alpinus* L.).

In summary, although *A. salmonicida* can be detected in the intestine of infected fish there is still doubt that this is the principal route by which systemic infection

occurs, although translocation of organisms from stomach to kidney has been demonstrated (McCarthy, 1977).

A range of freshwater fish are susceptible to motile aeromonad septicaemia caused by *A. hydrophila* (Thune et al., 1993) and this organism is capable of binding to collagen and fibronectin (Ascencio et al., 1991), and invading EPC (epitheliosum papillosum of carp) tissue culture cells (Tan et al., 1998). Studies with inhibitors of tyrosine kinase, protein kinase C and protein tyrosine phosphatase indicated that the organism initiated a signalling cascade involving tyrosine kinase, leading to actin microfilament reorganization involving actin "clouds" (Tan et al., 1998).

5.2. *Edwardsiella*

Two species of this genus, *E. ictaluri* and *E. tarda* are serious pathogens of fish (Plumb, 1993), causing distinctly different diseases in a range of fish species.

Edwardsiella septicaemia, which affects warm water fish is widely distributed in the environment and can cause severe losses in farmed catfish, *Ictalurus punctatus* (Plumb, 1993). Although Darwish et al. (2000) found no histological lesions in the intestine of catfish during experimentally induced infections, a different type of study by Ling et al. (2000) employing green fluorescent protein (GFP)-labelled bacteria showed that 3 days after intramuscular injection of 1.2×10^5 *E. tarda* into blue gourami approximately 10^6 bacteria were recovered from the intestine, although the highest concentrations of bacteria were found in the muscle and liver. However, *E. tarda* is not considered a pathogen with significant involvement of the gut in infection. Nevertheless, it has a pronounced capacity to invade both human and fish tissue culture cells (Janda et al., 1991; Ling et al., 2000). The invasion of both tissue culture cell types by *E. tarda* was sensitive to cytochalasin D (Janda et al., 1991; Ling et al., 2000), and in fish cells was also dependent on protein tyrosine kinase activity (Ling et al., 2000).

The second species, *E. ictaluri*, causes enteric septicaemia of catfish which can result in high mortalities. Two disease conditions are known with infection of brain via the olfactory organ or the intestine (Shotts et al., 1986; Francis-Floyd et al., 1987). Doses of 5×10^9 bacteria were intubated into the stomach of fingerling catfish and within 2 weeks fish developed enteritis and other chronic lesions. Horizontal transmission occurred to cohabiting fish, which also developed lesions beginning in the intestine (Shotts et al., 1986). As yet, the invasion pathway from the gut to other tissues and organs has not been established.

5.3. *Photobacterium damsela* subsp. *piscicida*

This organism was found to adhere to tissue sections of intestine from sea bream *Sparus aurata*, sea bass *Dicentrarchus labrax*, and turbot *Scophthalmus maximus* at concentrations of 10^4 – 10^5 per gram by Magarinos et al. (1996).

Evaluation of the invasive capacities of the *Photo. damsela* subsp. *piscicida* on different poikilothermic cell lines indicated that according to the Janda index (Janda et al., 1991), the strains studied were weakly or moderate invasive, with the number of intracellular bacteria ranging from 10^1 to 10^3 . *Photo. damsela* subsp. *piscicida* was able to invade CHSE-214 tissue culture cells and to remain viable for at least 2 days inside the infected cells.

5.4. *Piscirickettsia salmonis*

Piscirickettsia salmonis is an obligate, intracellular, Gram-negative organism and such fastidious bacteria have been increasingly detected as emerging pathogens in a range of fish species in different geographic locations (Fryer and Mauel, 1997). In 1990 it was recognized that the causative agent responsible for the loss of 1.5 million coho salmon in the previous year (Cvitanich et al., 1990, 1991; Fryer et al., 1990; Branson and Diaz-Munoz, 1991; Garces et al., 1991) was a rickettsial agent of a new genus and species (Fryer et al., 1992). Whereas the Rickettsiaceae are members of the α -Proteobacteria, *P. salmonis* is assigned to the γ -Proteobacteria. The disease was termed salmonid rickettsial septicaemia because of the systemic nature of the disease (Cvitanich et al., 1991). Several organs were affected in diseased fish, including the intestine, which was severely damaged with necrosis and inflammation of the lamina propria and sloughing off of epithelial cells (Branson and Diaz-Munoz, 1991). The route of infection was studied by Smith et al. (1999) who investigated various routes as possible portals of entry for the pathogen. Subcutaneous injection of *P. salmonis* (10^4 TCID₅₀) resulted in 100% cumulative mortality of fish by day 33 post injection. Application to the skin or gills of patches soaked in *P. salmonis* ($10^{4.2}$ TCID₅₀ per patch) resulted in 52 and 24% mortalities, respectively, whereas 24 and 2% cumulative mortalities occurred following intestinal or gastric intubation (10^4 TCID₅₀ administered in both cases). The authors concluded that rickettsia could infect the fish directly through the skin or gills and that the intestinal route was not the normal route of infection.

5.5. *Vibrio anguillarum*

Vibrio anguillarum is an important pathogen of marine and estuarine fish species and is the causative agent of vibriosis. This disease is one of the major bacterial diseases affecting fish, as well as bivalves and crustaceans (Austin and Austin, 1999), and vibriosis can cause substantial losses to the aquaculture industry. Vibriosis is characterized by deep focal necrotizing myositis and subdermal haemorrhages, with the intestine and rectum becoming swollen and filled with fluid (Horne et al., 1977; Munn, 1977). The GI tract of fish appears to be a site of colonization and amplification for pathogenic *Vibrio* species (Horne and Baxendale, 1983; Ransom et al., 1984; Olsson et al., 1996), and Olsson et al. (1998) recently demonstrated that orally

ingested *V. anguillarum* can survive passage through the stomach of feeding juvenile turbot (*Scophthalmus maximus* L.). *Vibrio anguillarum* and *V. ordalii* have been found primarily in the pyloric caeca and the intestinal tracts of three species of Pacific salmon (chum salmon, *Oncorhynchus keta*; coho salmon, *Oncorhynchus kisutch*; and chinook salmon, *Oncorhynchus tshawytscha*) (Ransom et al., 1984). In addition, Olsson (1995) and Olsson et al. (1996) demonstrated that the GI tract can serve as a portal of entry for *V. anguillarum* and it can utilize intestinal mucus as its sole nutrient source (Olsson et al., 1992; Garcia et al., 1997). Although some evidence indicates that *V. anguillarum* can invade fish either via the skin or the GI tract, Grisez et al. (1996) showed that the organism is transported across the intestinal epithelium by endocytosis. Chemotactic motility mediated by a single polar sheathed flagellum is essential for virulence as bacteria deficient in this activity were unable to infect fish when administered by immersion in bacteria-containing water but were virulent when given by intraperitoneal injection (O'Toole et al., 1996). These findings imply that *V. anguillarum* responds chemotactically to certain fish-derived products in a manner that promotes the infection process prior to penetration of the fish epithelium.

Recently, it was shown that *V. anguillarum* exhibited a stronger chemotactic response towards intestinal mucus than towards skin (O'Toole et al., 1999). Of the free amino acids identified in the intestinal mucus, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, serine and threonine, and carbohydrates such as fucose, glucose, mannose and xylose behaved as chemoattractants, while the lipid components identified, bile acid, taurocholic acid and taurochenodeoxycholic acid induced only a weak chemotactic response. A combination of all individual chemoattractants identified from mucus reconstituted a high level of chemotactic activity similar to that present in the intestinal mucus homogenate. On the basis of these results, the authors proposed that multiple chemoattractants in rainbow trout mucus indicated a strong relationship between chemotaxis and bacterial virulence.

The invasion mechanism of vibrios for fish cells has been investigated recently by Wang et al. (1998) in a comparison of 24 isolates of seven different species. Thirteen isolates were invasive for gruntfin (GF) and EPC tissue culture cell lines including all five *V. vulnificus* and both *V. harveyii* isolates. Of the 11 *V. anguillarum* isolates tested, three were invasive, two of which adhered strongly to EPC cells. Cytochalasin D inhibited invasion by both strains although one was also sensitive to inhibition by vincristin, a microtubule depolymerizing agent, indicating different routes of invasion for the two strains. This difference was confirmed by the difference in response of the strains to inhibitors of the signalling molecules protein kinase C and tyrosine kinase.

5.6. Streptococcosis

Streptococcosis is a septicæmic disease that affects freshwater and marine fish in both farmed and wild populations. Among commercially important fish species, this

disease has been reported worldwide in yellowtail (*Seriola* spp.), eels (*Anguilla japonica*), menhaden (*Brevoortia patronus*), striped mullet (*Mugil cephalus*), striped bass (*Morone saxatilis*) and turbot. Romalde et al. (1996) demonstrated the capacity of *Enterococcus* sp. to overcome adverse conditions in the stomach when associated with food or faecal materials, since the pathogen was able to establish an infective state and to produce mortalities after 16 to 20 days post ingestion.

6. VIRUSES

With the availability of effective vaccines against many major bacterial fish pathogens (Gudding et al., 1997) agents such as infectious pancreatic necrosis (IPN) virus, infectious salmon anaemia (ISA) virus, viral haemorrhagic septicaemia (VHS) virus, infectious haematopoietic necrosis (IHN) virus and nodavirus have emerged as more prominent threats to aquaculture. In mammals, the enteroviruses, rotaviruses, coronaviruses and Norwalk virus group are important causes of diarrhoeal disease transmitted by the faecal–oral route (Mims et al., 2000). For poliovirus the initial replication in the GI tract can be followed by invasion of the bloodstream and penetration of the blood–brain barrier to cause paralytic poliomyelitis (Mims et al., 2000).

In salmonids, IPN virus is a serious pathogen causing major losses in Atlantic salmon aquaculture in Norway, Scotland and Chile (Smail and Munro, 2001). As its name implies this virus causes significant necrosis of the pancreas in salmonids but other organs, including the intestinal tract, may also be affected (Wolf, 1988). Pathological changes in the intestinal tract have also been shown in larval sea bass (Bonami et al., 1983) and larval halibut (Biering et al., 1994). In the latter study, focal necrosis was observed in the intestinal tract with sloughing off of epithelial cells, and the GI tract was considered the most likely route of entry and replication for the virus (Bergh et al., 2002). However, there was no evidence of damage to the pancreas in larval halibut.

Viral encephalopathy and retinopathy (VER), caused by nodaviruses, is a recently recognized serious disease of Atlantic halibut which poses a serious threat to larval culture of this fish (Grotmol et al., 1995, 1997; Munday and Nakai, 1997). Although pathology is largely restricted to lesions in the brain, spinal chord and retina (Grotmol et al., 1995), experimental infection models indicate that the intestinal epithelium is the probable route of entry for this virus into the larval fish (Grotmol et al., 1999). However, as with IPN virus, little is known of the pathogenic mechanisms involved in invasion from the intestinal tract to the sites where significant pathological damage is caused, and this awaits further investigation.

7. THE EFFECT OF DIET ON DISEASE RESISTANCE

Intensive fish production has increased the risk of infectious diseases. Therefore, there is a growing need to find alternatives to antibiotic treatments for disease control, as indiscriminate use of antibiotics in many parts of the aquaculture industry

has led to the development of antibiotic resistance in bacteria. Nutritional status is considered an important factor in determining disease resistance. The complex relationship between nutritional status, immune function and disease resistance has been documented for higher vertebrates in several comprehensive reviews and books (Gershwin et al., 1985; Chandra, 1988; Bendich and Chandra, 1990). The influence of dietary factors on disease resistance in fish has been extensively reviewed (Lall, 1988; Landolt, 1989; Blazer, 1992; Lall and Olivier, 1993; Waagbø, 1994; Olivier, 1997), and micronutrients such as vitamins have received particular attention. Studies on the essential fatty acid, vitamin and trace element requirements of several warm and cold water fish have demonstrated their integral role in the maintenance of epithelial barriers of skin and the gastrointestinal tract. Although there is some information on the relationship between disease resistance and dietary lipid (Salte et al., 1988; Erdal et al., 1991; Obach et al., 1993; Waagbø et al., 1993; Li et al., 1994; Bell et al., 1996; Thompson et al., 1996), there is a lack of information about the functional role of dietary lipid on intestinal microbiota, their antagonism and disease resistance. However, a recent study showed clear differences in the gut microbiota of fish fed different oils (post and prior to challenge) and the ability of the gut microbiota to inhibit growth of three fish pathogens (*A. salmonicida* subsp. *salmonicida*, *V. anguillarum* and *V. salmonicida*) (Ringø et al., 2002). Also, Lødemel et al. (2001) clearly demonstrated that survival of Arctic charr after challenge with *A. salmonicida* subsp. *salmonicida* was improved by dietary soybean oil. These results are in agreement with those reported by Hardy (1997) that replacement of dietary fish oil with plant- or animal-derived fats increases resistance of catfish (*Ictalurus punctatus*) to disease caused by experimental challenge with *E. ictaluri*.

8. FUTURE PERSPECTIVES

Bacterial and viral diarrhoeal diseases are major causes of mortality and morbidity in mammals but no equivalent diseases are recognized in fish, presumably because dramatic fluid loss does not occur so readily in an aquatic environment. However, the GI tract still presents a route of infection, especially for opportunistic bacteria present on ingested food particles, and there is clear evidence for this as a route for invasion to affect other organs and tissues.

The main reasons why studies on fish pathogenic bacteria have lagged behind those of mammalian pathogens is because intensive aquaculture has developed quite recently as a significant industry, several of the pathogens are novel, and there has been a relatively small research effort in this field, in comparison with human and veterinary medicine. The past decade has seen major developments in methodology for studying microbial pathogenicity, and the techniques applied to human pathogens are only now being applied to fish pathogens (O'Toole et al., 1996, 1999; Tan et al., 1998; Ling et al., 2000; Mathew et al., 2001). Undoubtedly, the most

significant development in microbiology for 50 years has been the genome sequence determination for many prokaryotes. Since the first complete sequence was published in 1995, a total of 56 genome sequences have been completed to date and a further 210 are in progress (see www.tigr.org and www.integratedgenomics.com). Those in progress include genomes for the fish pathogens *A. salmonicida* and *P. salmonis* and this will provide unique insights into the potential pathogenic mechanisms of these bacteria, including evolutionary distances between *P. salmonis* and *Rickettsia prowazekii* and whether pseudogenes are also prevalent in *P. salmonis* (Andersson et al., 1998; Andersson and Andersson, 1999).

Other methods, including the use of expressed markers such as green fluorescent protein and laser confocal microscopy (e.g. Ling et al., 2000), will provide more definitive analysis of pathways of invasion by pathogens taken up via the GI tract. One area in which there is particular deficiency at present is in the nature of fish-cell-surface colonization by bacteria and any downstream signalling which occurs. This would be of considerable practical value in designing pathogen prevention strategies using probiotic bacteria to prevent colonization by pathogens.

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