Vol. 27: 89–94, 1996 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published November 14

Factors influencing the clearance of a genetically attenuated (Δ*aroA*) strain of *Aeromonas salmonicida* from rainbow trout *Oncorhynchus mykiss*

M. J. Marsden*, E. Collins, C. J. Secombes**

Department of Zoology, University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ, United Kingdom

ABSTRACT: Clearance of an *aro*A deletion mutant of *Aeromonas salmonicida* from tissues of rainbow trout *Oncorhynchus mykiss* was studied under a number of conditions. Following intraperitoneal injection into trout, the bacteria were rapidly found in all tissues/body fluids examined (kidney, spleen, liver, heart, muscle, blood and peritoneal fluid), with highest recoveries in the spleen and peritoneal fluid. Whilst in some tissues numbers increased over the first few days post-injection, in general clearance had been effected within 7 to 9 d at 16°C regardless of the inoculum dose. However, with the highest dose (2×10^9 bacteria per fish) used some fish mortalities occurred. Temperature was shown to influence clearance, with low water temperatures significantly delaying clearance. Whole body homogenates confirmed that some increase in bacterial numbers occurred *in vivo*, after an initial and immediate dramatic drop in bacterial viability. Finally, a 2 h crowding stress which significantly elevated blood glucose levels had no effect upon clearance. These results are discussed with respect to the possible use of this deletion mutant as a live vaccine in aquaculture.

KEY WORDS: Genetically attenuated vaccine · *Aeromonas salmonicida* · Furunculosis · Rainbow trout · Clearance · Δ*aroA*

INTRODUCTION

With the success of many vaccines in aquaculture has come an interest in more novel vaccines, such as recombinant subunit vaccines, peptide vaccines and live, attenuated vaccines. Each has a number of advantages over whole, killed vaccines (Brown et al. 1993), but have yet to have a major impact in fish vaccine design. With respect to bacterial vaccines, several recent successes with prototype live vaccines (Thornton et al. 1991, Vaughan et al. 1993, Norqvist et al. 1994) have focussed attention on this particular approach. Live vaccines are known to be effective stimulators of cell-mediated immune responses, especially important for intracellular pathogens, and are able to elicit responses at mucosal surfaces via oral administration (Dougan et al. 1987). In addition, it is apparent that many novel molecules on pathogens are preferentially expressed *in vivo*, as with the salmonid bacterial pathogen *Aeromonas salmonicida* (Garduno et al. 1993a, b, Thornton et al. 1993, Hirst & Ellis 1994). Live vaccines clearly have the potential to express such molecules *in vivo*, including any yet to be characterised, before elimination by the host immune system.

One problem associated with the use of live vaccines has been the concern over the possible reversion to virulence, through changes in the bacteria or compromising conditions in the host. For example, a live vaccine may be cleared by a healthy host but may overcome a stressed or otherwise immunologically compromised host. This is a particular concern with naturally attenuated mutants, where it is often not known exactly how πthe attenuation has been achieved. However, ad-

*Present address: Veterinary Medicines Directorate, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, UK
**Addressee for correspondence.
E-mail: c.secombes@abdn.ac.uk

vances in molecular biological techniques allow the production of genetically attenuated mutants, as with the *aro*A deletion mutant (Δ*aroA*) of *Aeromonas salmonicida* developed by Vaughan et al. (1990) where an *aro*A::kanamycin resistance mutation was introduced into the chromosome of *A. salmonicida* by allele replacement using a suicide plasmid delivery system. No revertants were detectable among 10^{11} bacteria examined (Vaughan et al. 1993), and doses of up to 10^9 bacteria per fish, injected intramuscularly (i.m.) into brown trout *Salmo trutta* or Atlantic salmon *Salmo salar* had no adverse effect on fish health, confirming that this mutant was attenuated. In contrast, the wild type bacterium from which this strain was derived killed all salmon within 3 d of an i.m. injection of 10^6 bacteria per fish. Thus, deletion of genes resulting in severe metabolic deficiency appears to be a most rational approach to attenuation.

Recently a mark II strain of the Δ*aroA* mutant of *Aeromonas salmonicida* (Brivax II) has been developed (Marsden et al. 1996a). This strain contains a complete deletion of the *aro*A gene, constructed by allele replacement, and thus lacks kanamycin resistance. The present study was carried out to investigate in detail the clearance of this strain from rainbow trout *Oncorhynchus mykiss* using a variety of temperature and dose regimes. Such data is essential to understand what factors may influence the ability of this strain to stimulate the fish immune system, prior to its development as a commercial vaccine.

MATERIALS AND METHODS

Fish. Rainbow trout *Oncorhynchus mykiss*, weighing approximately 50 g, were obtained from local fish farms and maintained in aerated fibreglass tanks supplied with a continuous flow of recirculating water at 10 to 16°C. Fish were fed twice daily on commercial trout food (EWOS). Fish vaccinated with the live Brivax bacterium were placed in the pathogen containment aquarium facility at the Department of Zoology, University of Aberdeen, where the outflow water was sterilised by contact with hypochlorite.

Bacterial passaging. Brivax II were inoculated from storage at −70°C into 3% tryptic soy broth (TSB, Gibco). Following an initial 24 h incubation of cultures at 20°C, bacteria were sub-cultured and grown for a further 24 to 48 h at this temperature prior to their use for vaccination.

Clearance of Brivax II. Groups of 20 to 30 rainbow trout, acclimated to 10, 13 or 16°C, were anaesthetised using ethyl-4-aminobenzoate (Benzocaine, Merck) at 25 μg ml^{-1} water. The fish were then vaccinated intraperitoneally (i.p.) with Brivax II at a concentration of 2×10^7, 2×10^8 or 2×10^9 bacteria per fish. In one set of experiments, fish were given a 2 h crowding stress, according to Thompson et al. (1993), prior to injection to study clearance in immunocompromised animals. To confirm that stress had an effect *in vivo*, serum samples were taken from fish in this experiment at the time of vaccination, for determination of serum glucose levels as described by Thompson et al. (1993).

Levels of bacteria present in the tissues of vaccinated fish were assessed every other day for 7 to 12 d after vaccination, dependent on the nature of the experiment. At each sampling time 5 fish were taken and either homogenised in a commercial blender (Jencons) containing 50 ml 0.15 M phosphate-buffered saline pH 7.2 (PBS, Gibco), or bled and then dissected and selected tissues (kidney, spleen, liver, dorsal muscle, heart) removed for analysis and weighed. In addition, peritoneal washes were collected by flushing the peritoneal cavity with 200 μl PBS following removal of the organs. Prior to use, the whole body homogenates and 200 μl whole blood samples were diluted 1:10 and 1:5, respectively, in PBS. For preparation of the tissue samples, the surfaces of each tissue were initially washed with PBS to remove potentially contaminating surface bound Brivax, especially important for samples from within the peritoneal cavity. Subsequently, washed tissue samples were pushed through a nylon mesh (100 μm) with 10 ml PBS. Five-fold dilutions of each of the prepared whole body, blood, peritoneal washes or tissue suspensions were then made and plated onto tryptic soy agar (TSA, Gibco) plates. Plates were incubated for 48 h at 20°C, the resultant *Aeromonas salmonicida* colony forming units (cfu) counted and the mean number of bacteria present per fish, per 0.1 g tissue or per 200 μl body fluid (blood and peritoneal fluid) estimated. The sensitivity of the assay was estimated to be 83 cfu per sample.

Data were analysed by 1- and 2-way analysis of variance (ANOVA) for the time, dose and temperature experiments, and by 1-way ANOVA for the stress experiment.

RESULTS

Clearance of Brivax II

As seen in Fig. 1, following i.p. injection of 2×10^8 Brivax II into rainbow trout acclimated to 16°C, there was a rapid dissemination to all tissues examined. That Brivax II was found within the blood during the first few days post-injection suggests that it becomes systemic. Highest cfu were recorded in the spleen and peritoneal fluid, sites potentially influenced by the route of injection. However, the numbers of cfu recov-

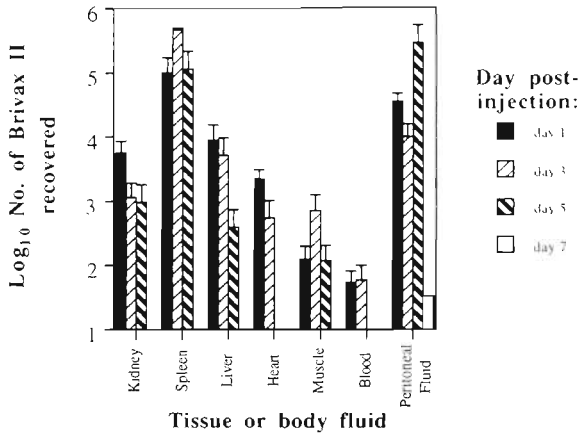

Fig. 1. *Oncorhynchus mykiss*. Tissue and body fluid distribution of Brivax II over time in rainbow trout maintained at 16°C and injected with 2×10^8 bacteria per fish. Data are means + SE of cfu per 0.1g tissue or 200 µl body fluid. N = 5. No positive values below 10 cfu were recorded at any time

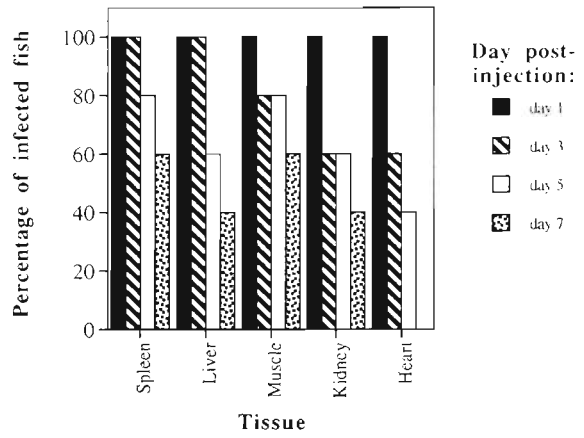

Fig. 2. *Oncorhynchus mykiss*. Percentage of tissues infected with Brivax II over time in rainbow trout maintained at 16°C and injected with 2×10^8 bacteria per fish. Data are means + SE. N = 5

ered from the heart and dorsal muscle were significant and well removed from the injection site. Whilst in some tissues highest numbers of cfu were found on Day 1 (kidney, liver, heart), this was not always the case and numbers also peaked on Day 3 (spleen, muscle) or Day 5 (peritoneal fluid). Nevertheless, in all cases numbers had dramatically fallen by Day 7 ($p < 0.001$ relative to values present at Day 1), and were only detectable in the peritoneal fluid. At Day 9 no cfu were detected in any samples in this experiment.

In a separate experiment performed using the same conditions as above, the percentage of fish with infected tissues was calculated. As seen in Fig. 2, on Day 1 post-injection all tissues were infected in all fish. However, by Day 3, 1 fish had cleared the infection in the muscle, kidney and heart, and 2 others had cleared the Brivax II from a single tissue (heart or kidney). By Day 5, again 1 fish had cleared the infection from all tissues examined, and all the others had cleared 1 or more tissues. Finally, at Day 7 all fish had cleared the heart, but low numbers of bacteria were still present in several tissues of some fish, which had not entirely cleared the infection at this time despite recoveries of Brivax over the first few days essentially similar to those seen in the previous experiment.

Since the numbers of bacteria present in some tissues had increased with time, a further experiment was performed to investigate what was happening at the whole animal level. Following injection, fish were homogenised and the numbers of cfu per fish calculated. It was clear that within minutes (the time taken to inject and then homogenise the fish, etc.) there was a dramatic reduction in cfu to around 24% of the starting inoculum (Fig. 3). This value decreased a little further during the first day *in vivo* (to 15%) but then increased approximately 3-fold over the next 2 d, before finally being cleared.

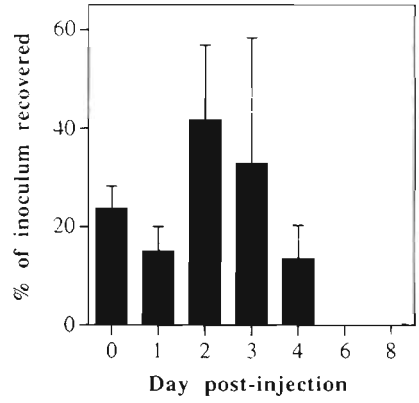

Fig. 3. *Oncorhynchus mykiss*. Whole body persistence of Brivax II with time in rainbow trout maintained at 16°C and injected with 2×10^8 bacteria per fish. Data are means expressed relative to the inoculum dose (2×10^8 bacteria). N = 5

Effect of dose on clearance

To examine the effect of inoculum dose on tissue persistence, 3 groups of trout were injected with 2×10^7, 2×10^8 or 2×10^9 bacteria at 16°C. As seen in Fig. 4, fish injected with 2×10^7 bacteria rapidly cleared the infection from the kidney, and by Day 3 no bacteria were detectable. As previously, highest numbers of cfu were found in the kidney on Day 1 using 2×10^8 bacteria per fish, with significantly lower ($p < 0.01$) numbers present on Days 3 and 5, and none being detectable by

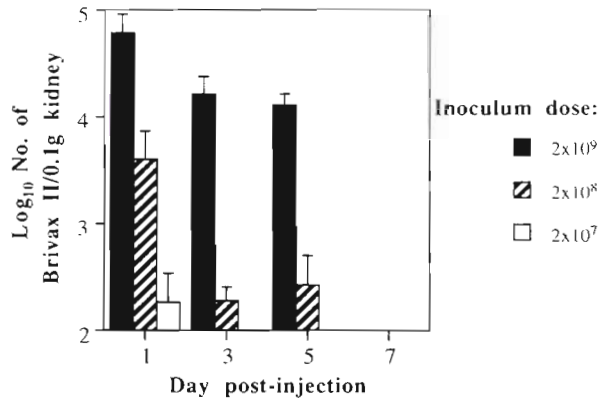

Fig. 4. *Oncorhynchus mykiss*. Effect of inoculum dose on persistence of Brivax II in the kidney of rainbow trout maintained at 16°C. Data are means + SE. N = 5. No positive values below 100 cfu were recorded at any time

Day 7. Using a dose of 2×10^9 was more problematical, in that approximately 50% of individuals died within 4 to 7 d post-injection. In the surviving fish, high levels of bacteria were found in the kidney on Day 1, and the levels remained relatively high for the next 2 samplings but had been cleared by Day 7. Comparison of the Day 1 data showed a clear and significant ($p < 0.001$) dose effect, with each log increase in inoculum dose resulting in an approximately 10-fold increase in bacteria recovered per 0.1 g kidney. Subsequently, this relationship was less clear, possibly as a result of some multiplication of the bacteria *in vivo* using the higher inoculum doses.

Effect of temperature on clearance

To examine the effect of temperature on tissue persistence, 3 groups of trout were acclimated to 10, 13 or 16°C and injected with 2×10^8 bacteria per fish. As seen in Fig. 5, fish injected at 16°C showed similar results to those above, in that bacteria were detectable in the kidney (Fig 5a), liver (Fig. 5b) and spleen (Fig. 5c) during the first week post-injection but were cleared, in general, by Day 9. At 13°C, bacteria persisted for longer than at 16°C in the liver and spleen, and were still detectable, although at low levels, at Day 12, the last time examined. Bacterial numbers were also significantly higher ($p < 0.01$) overall than in tissues from fish kept at 16°C, indicating a faster clearance rate in the latter. At 10°C, whilst bacterial numbers significantly decreased ($p < 0.01$) with time in all tissues examined, the rate of clearance was much reduced relative to the other 2 temperatures and bacteria were still detectable at relatively high numbers on Day 12.

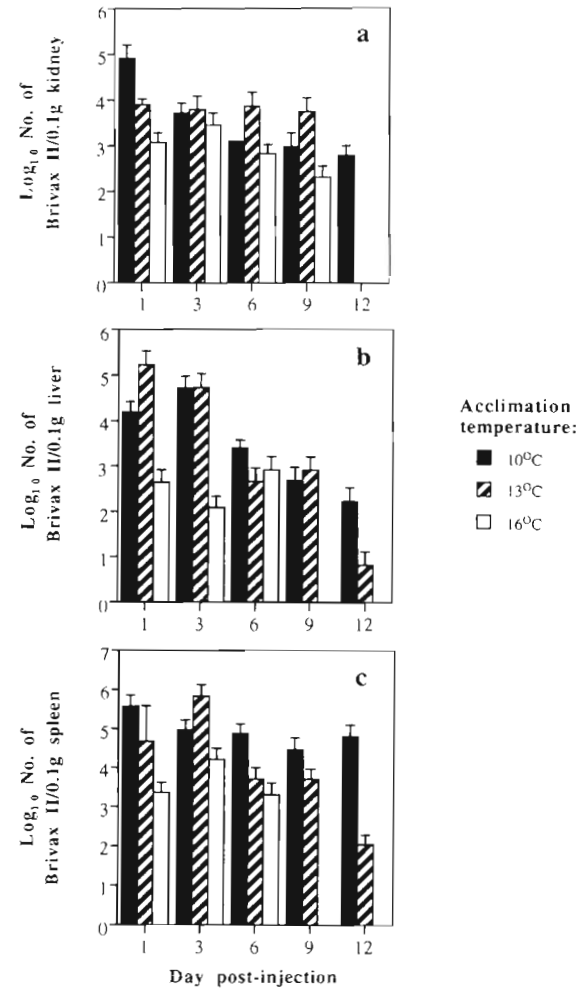

Fig. 5. *Oncorhynchus mykiss*. Effect of acclimation temperature on persistence of Brivax II in (a) kidney, (b) liver and (c) spleen of rainbow trout injected with 2×10^8 bacteria per fish. Data are means + SE. N = 5

Effect of stress on clearance

Following a 2 h crowding stress, previously shown to dramatically compromise head kidney leucocyte bactericidal and respiratory burst activity (Thompson et al. 1993), no impact on clearance rate was detectable relative to control fish, following injection with 2×10^8 bacteria per fish at 16°C (Fig. 6). At both Days 1 and 3 post-injection, there were no significant differences in the numbers of bacteria recovered in either the kidney or spleen of control and stressed fish. That the 2 h stress did indeed elicit an effect was assessed by monitoring glucose levels in serum samples taken from the 2 groups at the time of vaccination. Stressed fish had significantly higher ($p < 0.05$) serum glucose levels (163.9 ± 13.1 mg dl^{-1}) compared with control fish (97.9 ± 7.7 mg dl^{-1}).

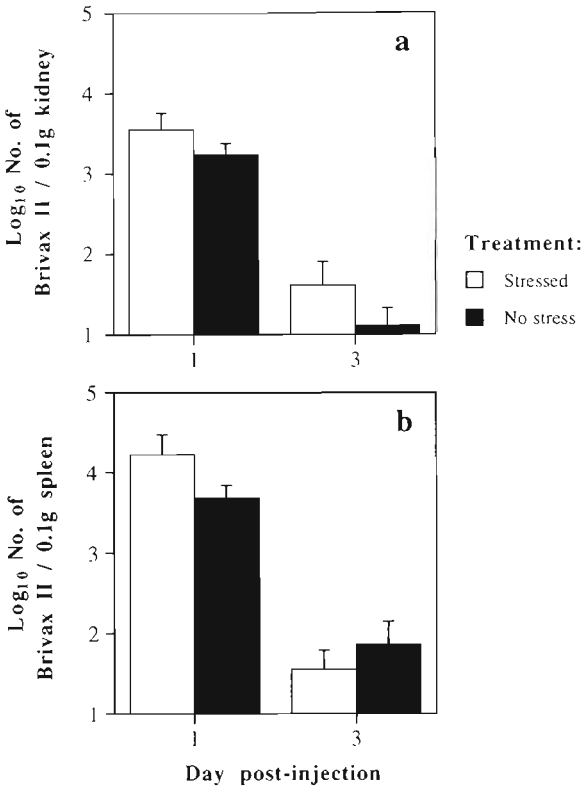

Fig. 6. *Oncorhynchus mykiss*. Effect of crowding stress on persistence of Brivax II in (a) kidney and (b) spleen of rainbow trout maintained at 16°C and injected with 2×10^8 bacteria per fish. Data are means + SE. N = 5

DISCUSSION

Analysis of the clearance of a genetically attenuated (Δ*aroA*) strain of *Aeromonas salmonicida* (Brivax II) from rainbow trout tissues and body fluids revealed that post-injection the bacteria were rapidly found in all tissues examined, and were typically cleared within a week at 16°C regardless of the inoculum dose. Since the bacteria became systemic (i.e. were found in the blood), they were probably distributed to the different tissues via the circulation, and well-vascularised tissues such as the spleen had relatively higher recoveries on a weight:weight basis than less vascularised tissue such as the muscle. High recoveries were also found in the peritoneal cavity, presumably as a consequence of the injection route used.

That the metabolic deficiency resulting from the *aro*A deletion resulted in attenuation of Brivax II was shown by the survival of the trout. Doses as high as 2×10^9 per fish had to be used before any mortalities were seen, similar to the situation using Brivax I in brown trout and Atlantic salmon where doses up to 10^9 bacteria per fish had no observable effects on fish health (Vaughan et al. 1990). The mortalities seen in the present study may have resulted from high levels of proteases/toxins (Price et al. 1989, Lee & Ellis 1990, Ellis in press) released from the bacteria prior to clearance, with the relatively small fish used in the present study being more susceptible than larger fish at this dose (authors' pers. obs.). However, a dose of 2×10^9 bacteria per fish is some 40 times higher than the dose of Brivax I already shown to effectively induce protection in brown trout, and 100 times higher (i.e. 2×10^7 bacteria per fish) than doses considered optimal for priming immune responses such as specific lymphocyte proliferation and antibody production in rainbow trout (Marsden et al. 1996a). Thus, a dose of 2×10^9 bacteria per fish would never be used in practice to vaccinate fish in aquaculture. Interestingly, at this high dose differences in survival between individual fish were present, as were differences in the speed of clearance from tissues using lower doses. Such differences may relate to genetic differences in the immune system, which is known to have polymorphic components (Bjering Jensen & Koch 1991, Wiegertjes 1995) which can be selected to enhance disease resistance traits in aquaculture (Chevassus & Dorson 1990, Lund et al. 1995).

The Δ*aroA* mutant is unable to synthesise chorismate, which in turn results in a block in folate biosynthesis. The latter deficiency is thought to be primarily responsible for the attenuation of aromatic-dependent bacteria (Hoiseth & Stocker 1981, Vaughan et al. 1990, 1993). Thus, such mutants would be expected to die after their folate reserves are exhausted, and this may result in the clearance observed. Nevertheless, in some tissues/fluids bacterial numbers increased over the first few days post-injection, as seen in the spleen, muscle and peritoneal fluid. Since muscle in particular represents a large total tissue mass, the overall number of bacteria in the fish will potentially increase during this period. This was confirmed using homogenates of whole fish, where following an initial and immediate decline in viability numbers rose over the next few days before being cleared. Similar results have been seen using virulent strains of *A. salmonicida* within a 1 ml chamber fitted with 0.45 µm filters, surgically implanted into the peritoneal cavity of trout (Garduno et al. 1993a, b). Here, following a rapid and precipitous 'kill-off' during the first 24 h some bacteria were able to survive and replicate in the host, eventually exceeding the inoculum dose over the following 48 h. Survival was correlated with the appearance of novel antigens, such as a carbohydrate capsule, that conferred protection against bacteriolytic and phagocytic mechanisms (Garduno et al. 1993a, Thornton et al. 1993). Whilst similar antigens may be induced in Brivax II post-injection, allowing the bacteria to replicate *in vivo*, it is also possible that the metabolic deficiency prevents this process occurring, allowing cellular and humoral

defences to kill the pathogen (Secombes & Olivier in press).

Both inoculum dose and temperature had a major impact on bacterial persistence. Whilst at first sight fast clearance may seem desirable, in fact this could limit the time available to stimulate an effective immune response. Studies to examine this further are on-going but are complicated by the temperature sensitivity of the fish immune system (Bly & Clem 1992). Thus, at low temperatures the increased bacterial persistence may give the immune system longer to be stimulated but the response may take longer to develop.

A 2 h crowding stress, which significantly elevated serum glucose values to levels seen in other stress studies (Barton & Iwama 1991) and which is known from previous studies to result in suppression of several immune parameters (Thompson et al. 1993), had no effect on clearance. This suggests that the Δ*aroA* attenuation is sufficient to allow clearance even in immunocompromised hosts, although clearly further studies are warranted to confirm this.

In conclusion, the clearance data in this study combined with previous immunological studies (Marsden et al. 1996a, b) show that Brivax II is attenuated and able to effectively stimulate the salmonid immune system. The relative degree of protection conferred on fish immunised with Brivax II versus fish immunised with killed vaccine preparations has still to be established, but such trials are clearly worthwhile. The use of Brivax II as a carrier of heterologous antigens also has potential for priming against other diseases (Marsden et al. 1996b) and such studies are likely to extend to attenuated viral vaccines over the next few years.

Acknowledgements. This work was supported by a contract from the EC (No. AIR1-CT92-0441). Thanks go to Daniel Clarke, Mike van der Laak and Peter Axton for technical assistance with the clearance studies, and to Dr Larry Vaughan (BioResearch Ireland) for critically reading the manuscript.

LITERATURE CITED

Barton BA, Iwama GK (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann Rev Fish Dis 1:3–26

Bjering Jensen L, Koch C (1991) Genetic polymorphism of component C3 of rainbow trout (*Oncorhynchus mykiss*) complement. Fish Shellfish Immunol 1:237–242

Bly JE, Clem LW (1992) Temperature and teleost immune functions. Fish Shellfish Immunol 2:159–171

Brown F, Dougan G, Hoey EM, Martin SJ, Rima BK, Trudgett A (1993) Vaccine design. Wiley, Chichester

Chevassus B, Dorson M (1990) Genetics and resistance to disease in fishes. Aquaculture 85:83–107

Dougan G, Hormaeche CE, Maskell DJ (1987) Live oral *Salmonella* vaccines: potential use of attenuated strains as carriers of heterologous antigens to the immune system. Parasite Immunol (Oxf) 9:151–160

Ellis AE (in press) The extracellular toxins of *Aeromonas salmonicida* subsp. *salmonicida*. In: Bernoth EM, Olivier G, Midtlyng PJ, Ellis AE, Smith P (eds) Furunculosis in fish—a multidisciplinary review. Academic Press, London

Garduno RA, Thornton JC, Kay WW (1993a) *Aeromonas salmonicida* grown *in vivo*. Infect Immun 61:3854–3862

Garduno RA, Thornton JC, Kay WW (1993b) Fate of the fish pathogen *Aeromonas salmonicida* in the peritoneal cavity of rainbow trout. Can J Microbiol 39:1051–1058

Hirst ID, Ellis AE (1994) Iron-regulated outer membrane proteins of *Aeromonas salmonicida* are important protective antigens in Atlantic salmon against furunculosis. Fish Shellfish Immunol 4:29–45

Hoiseth SK, Stocker BAD (1981) Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature 291:238–239

Lee KK, Ellis AE (1990) Glycerophospholipid: cholesterol acyltransferase complexed with lipopolysaccharide (LPS) is a major lethal exotoxin and cytolysin of *Aeromonas salmonicida*: LPS stabilizes and enhances toxicity of the enzyme. J Bacteriol 172:5382–5393

Lund T, Gjedrem T, Bentsen HB, Eide DM, Larsen HJS, Roed KH (1995) Genetic variation in immune parameters and associations to survival in Atlantic salmon. J Fish Biol 46: 748–758

Marsden MJ, DeVoy A, Vaughan L, Foster TJ, Secombes CJ (1996b) Use of a genetically attenuated strain of *Aeromonas salmonicida* to vaccinate salmonid fish. Aquacult Int 4:55–66

Marsden MJ, Vaughan L, Foster TJ, Secombes CJ (1996a) A live (Δ*aroA*) *Aeromonas salmonicida* vaccine for furunculosis preferentially stimulates enhanced T cell responses relative to B cell responses in rainbow trout (*Oncorhynchus mykiss*). Infect Immun 64:3863–3869

Norqvist A, Bergman A, Skogman G, Wolf-Watz H (1994) A field trial with the live attenuated fish vaccine strain *Vibrio anguillarum* VAN1000. Bull Eur Ass Fish Pathol 14: 156–158

Price NC, Stevens L, Duncan D, Snodgrass M (1989) Proteases secreted by strains of *Aeromonas salmonicida*. J Fish Dis 12:223–232

Secombes CJ, Olivier G (in press) Host-pathogen interactions in salmonids. In: Bernoth EM, Olivier G, Midtlyng PJ, Ellis AE, Smith P (eds). Furunculosis in fish—a multidisciplinary review. Academic Press, London

Thompson I, White A, Fletcher TC, Houlihan DF, Secombes CJ (1993) The effect of stress on the immune response of Atlantic salmon (*Salmo salar* L.) fed diets containing different amounts of vitamin C. Aquaculture 114:1–18

Thornton JC, Garduno RA, Carlos SJ, Kay WW (1993) Novel antigens expressed by *Aeromonas salmonicida* grown *in vivo*. Infect Immun 61:4582–4589

Thornton JC, Garduno RA, Newman SG, Kay WW (1991) Surface disorganised, attenuated mutants of *Aeromonas salmonicida* as furunculosis vaccines. Microb Pathog 11 85–89

Vaughan L, Smith P, Foster TJ (1990) Aromatic-dependent mutants of *Aeromonas salmonicida*. Res Microbiol 141: 941–943

Vaughan LM, Smith PR, Foster TJ (1993) An aromatic-dependent mutant of the fish pathogen *Aeromonas salmonicida* is attenuated in fish and is effective as a live vaccine against the salmonid disease furunculosis. Infect Immun 61:2172–2181

Wiegertjes GF (1995) Immunogenetics of disease resistance in fish. PhD thesis, Wageningen Agricultural University, Wageningen

Responsible Subject Editor: D. W. Bruno, Aberdeen, United Kingdom

Manuscript first received: March 4, 1996
Revised version accepted: May 27, 1996