Colonisation and transmission of *lux*-marked and wild-type *Aeromonas salmonicida* strains in Atlantic salmon (*Salmo salar* L.)

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Abstract

Colonisation and transmission of *Aeromonas salmonicida* in Atlantic salmon was investigated using wild-type and *lux*-marked strains of *A. salmonicida*. An initial intra-peritoneal (i.p.) challenge showed that *lux*-marked cells were virulent only when injected at concentrations $\geq 10^9$ cfu ml^{-1} and significantly less infective than wild-type MT463. The low virulence of *A. salmonicida* MT463 *lux*AB was probably due to loss of the proteinaceous A-layer, which is an important virulence factor involved in both intra- and inter-cellular *A. salmonicida* interactions. During the i.p. challenge, all fish were held in one tank enabling assessment of transfer of *lux*-marked *A. salmonicida* between fish. *Lux*-marked cells shed from moribund and dead fish survived in the water column and cross-infected cohabitant fish. Cross-infection by *A. salmonicida* MT463 *lux*AB was investigated further by carrying out a cohabitation challenge. *Lux*-marked cells were recovered in low numbers from gill tissue and skin/mucus of cohabitant fish. Poor adhesion of cells may be due to loss of the A-layer protein. During a second cohabitation challenge using A-layer$^+$ and virulent wild-type strain MT432, between 10^2 and 10^7 cells g^{-1} of fish gill tissue or skin/mucus were isolated. This result confirmed the preliminary observations obtained using *lux*-marked *A. salmonicida* MT463 and suggested that the gill and skin/mucus regions of fish were the main sites for attachment of *A. salmonicida*. None of the *A. salmonicida* strains was recovered from fish intestine samples during cohabitation challenges. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Typical strains of *Aeromonas salmonicida* are the causative agent of furunculosis in salmonids and outbreaks of disease have significantly restricted the production of farmed Atlantic salmon [1]. Disease transmission occurs via contact with infected individuals [2–5], but little is known about the mechanisms involved in attachment and penetration of host tissue by *A. salmonicida*. One reason for this is a lack of selective medium for isolation of the pathogen. In

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fact it is often difficult to isolate *A. salmonicida* from the aquatic environment on artificial medium, even during outbreaks of disease [6]. This is possibly due to co-culture of inhibitory microorganisms such as *Pseudomonas* spp. [7].

In a previous paper, we described genetic marking of *A. salmonicida* MT463 with *lux*AB and *tet* genes, encoding bacterial luciferase and tetracycline resistance, respectively [8]. Luminescence in the *lux*-marked strain was inducible and required exogenous addition of fatty aldehyde substrate, allowing differentiation from indigenous microorganisms including naturally luminescent bacteria. Introduction of the antibiotic resistance gene provided an additional means for selecting the target strain. The *lux*-based detection system could therefore be used to study survival of *A. salmonicida* cells in non-sterile aquatic environments and mechanisms of dispersal and transmission.

There have been few laboratory-based studies aimed at determining the sites on fish for attachment of *A. salmonicida* and those which exist have been largely artificial due to the methods used to expose fish to the pathogen, for example, challenging fish with *A. salmonicida* by gastric intubation [2,3,9]. This may cause artefactual results from inadvertent damage to fish oesophagus and stomach [2]. In addition, the surface characteristics of in vivo and in vitro grown *A. salmonicida* cells differ [10,11]. A cohabitation challenge would provide a better system for modelling environmental exposure to the pathogen.

A. salmonicida cells isolated from fish are characteristically hydrophobic and autoaggregate in liquid culture [12] due to the presence of the surface A-layer protein. Since these cells readily colonise surfaces, *A. salmonicida* may become part of the indigenous skin/mucus microflora of non-infected fish following release from other, infected fish. Attached cells could gain entry into the host tissue following breakdown of this barrier [2]. Equally, cells attached to lipid rich food pellets [4] could become ingested, but not digested due to protection conferred by the surface A-layer. *A. salmonicida* cells present in the water column may also colonise fish gill surfaces and become concentrated in high numbers during respiration.

The aim of this study was to determine the survival of *A. salmonicida* in freshwater, to assess its ability to colonise different regions of Atlantic salmon parr and to determine the relative importance of these factors in transmission of *A. salmonicida* between fish. The study involved an initial comparison of the infectivity of *lux*-marked and wild-type *A. salmonicida* MT463 following intra-peritoneal (i.p.) challenge of Atlantic salmon parr. All fish were held in one tank, enabling assessment of transfer of *lux*-marked *A. salmonicida* between fish. Sites for attachment of in vivo grown *A. salmonicida* were investigated by carrying out cohabitation challenges using both strains [6]. Transmission of disease was monitored by screening fish skin/mucus, gill, intestine and kidney tissue for culturable *A. salmonicida* cells. The study also provided an assessment of the use of *lux*-based marker systems in investigating the ecology of microorganisms in natural environments.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A. salmonicida subsp. *salmonicida* MT463 and MT432 (Scottish Office Agriculture, Environment and Fisheries Department Marine Laboratory, Aberdeen, UK) were isolated from separate Atlantic salmon with clinical furunculosis in 1988. Construction of chromosomally *lux*-marked *A. salmonicida* subsp. *salmonicida* MT463 *lux*AB was described previously [8]. All strains were routinely cultured at 22°C on tryptone soya agar (TSA, Unipath) containing the appropriate antibiotics (5 μg ml^{-1} oxolinic acid for growth of *A. salmonicida* MT463 (OA), 12.5 μg ml^{-1} tetracycline and 5 μg ml^{-1} oxolinic acid for MT463 *lux*AB (TOA) and 15 μg ml^{-1} oxytetracycline for *A. salmonicida* MT432 (TOT)). The characteristics of all strains were consistent with those described by Popoff [13].

2.2. Maintenance of fish

Atlantic salmon (*Salmo salar* L.) parr were obtained from an out-bred population provided by the SOAEFD experimental fish production unit (Aultbea, Wester Ross, UK). The fish were main-

tained at 14 ($\pm$1)°C in 1-m^3 tanks with a continuous flow of temperature-controlled freshwater (4 l min^{-1}). Fish which were exposed to *lux*-marked *A. salmonicida* were approximately 15 g and those exposed to *A. salmonicida* MT432 were approximately 40 g.

2.3. Intra-peritoneal challenge with lux-marked and wild-type A. salmonicida MT463

Prior to i.p. challenges, *A. salmonicida* strains were grown on TSA supplemented with antibiotics at 14°C for 48 h. Cells were harvested using a sterile 10-µl inoculating loop and resuspended in 10 ml sterile phosphate-buffered saline (PBS; 8.5 g NaCl, 1.07 g Na_2HPO_4, 0.39 g $NaH_2PO_4 \cdot 2H_2O$, dissolved in 1 l distilled water; pH 7.0) to an OD_{540nm} of 1.0, corresponding to 10^9 cfu ml^{-1} [5]. Cell suspensions of each strain were centrifuged ($4000 \times g$ at 4°C for 30 min) and pellets were resuspended in PBS to a final cell concentration of 10^{10} cfu ml^{-1}. Serial dilutions of each strain were prepared in sterile PBS.

Fish were anaesthetised by bathing in 2 mg ml^{-1} 3-aminobenzoic acid ethyl ester (Sigma) in a temporary holding tank. Following sedation, 20 fish were removed and i.p. injected with 100 µl of *A. salmonicida* MT463 *lux*AB at a concentration of 10^{10} cfu ml^{-1}. These fish were marked with alcian blue (Sigma) [14] to enable identification. This procedure was repeated with suspensions containing 10^9, 10^8, 10^7 and 10^6 *A. salmonicida* MT463 *lux*AB cfu ml^{-1}. Similarly, groups of 20 fish were injected with *A. salmonicida* MT463 at concentrations in the range 10^{10}– 10^6 cfu ml^{-1}. A control group of 20 fish was injected with 100 µl sterile PBS. All fish were maintained together in a 1 m^3 tank as described above. Mortalities were removed daily from the tank and stored at −20°C. Fish which had not succumbed to *A. salmonicida* infection at 3 weeks post challenge were killed by immersion in a concentrated solution of anaesthetic followed by a blow to the head.

Samples were removed from the head kidney of i.p. challenged fish with a sterile 1-µl loop and used to inoculate selective and non-selective media. Bacteria isolated on different media (TSA $\pm$ antibiotics) from one fish in each group were screened for lumi-

nescence activity using a nitrogen-cooled, slow-scanning type charge-coupled device (CCD) camera encased in a light-tight box [15]. Luminescence was detected following the addition of 1 µl *n*-decyl aldehyde (Sigma) to the lid of the Petri dish and dark field images were observed after 2 min exposure. The bacteria isolated on TSA were characterised further by comparing their API 2OE diagnostic profiles (BioMérieux) with those of *lux*-marked and wildtype *A. salmonicida* MT463 cultures.

2.4. Cohabitation challenge

A sub-group of 25 Atlantic salmon parr, removed from a population of 250 fish, were anaesthetised as described above and i.p. injected with 100 µl of *A. salmonicida* MT463 *lux*AB at a concentration of 10^{10} cfu ml^{-1}. Injected fish were placed into a second 1-m^3 tank for 24 h then returned to the original 1-m^3 tank containing the remaining 225 non-injected fish. Similarly, a group of 25 control fish were removed from a second population consisting of 250 fish and i.p. injected with 100 µl of sterile PBS. These fish were also maintained overnight in a separate tank before being returned to the remaining control population. The cohabitation challenge was repeated as described above using strain MT432, but the total number of fish exposed to *A. salmonicida* was reduced to 108 and 10 fish were i.p. injected with 100 µl of 10^5 cfu ml^{-1}.

At designated time intervals, five and ten fish challenged with *A. salmonicida* MT432 or *A. salmonicida* MT463 *lux*AB, respectively were removed from the holding tank and killed by overdosing with benzocaine. Individual fish were placed into separate polythene bags and struck on the head to ensure death. Triplicate skin/mucus samples from fish exposed to *A. salmonicida* MT463 *lux*AB were obtained by passing a sterile 1-µl inoculating loop along the surface of fish. The mucus was spread directly over the surface of TSA supplemented with appropriate antibiotics. The gill arches were aseptically dissected from fish and added to 500 µl sterile PBS. Gill samples were mixed by vortexing for 1 min. Triplicate 10-µl samples were removed with a sterile inoculating loop and these were used to inoculate selective media. Triplicate kidney samples were recovered from fish corpses as described above. The fish intestine was

severed as close to the anus as was possible and the contents collected in a sterile Bijou bottle. A suspension of each intestine sample was prepared in 500 µl sterile PBS by vortexing for 1 min. Triplicate 10-µl samples were removed from the suspension using a sterile inoculating loop and were used to inoculate selective medium.

All samples collected from fish exposed to strain MT432 were collected following the order and method described above. During this challenge however, the mass of mucus, gill and intestine samples was determined and samples were suspended in 2 ml sterile PBS. Gill samples were homogenised for 2 min at 800 rpm using a Heidolph RZR 2100 homogeniser. A suspension of the intestinal contents was prepared by vortexing for 1 min. Triplicate 100-µl samples removed from all tissue samples were used to spread on to triplicate selective agar plates. In addition, serial dilutions were prepared in sterile PBS for gill, intestine and mucus samples and triplicate 20-µl aliquots were used to inoculate selective medium by the drop plate technique [16]. Samples were removed from the head kidney of fish exposed to strain MT432 as described above.

Identity of *A. salmonicida* strains recovered during each cohabitation challenge was confirmed using the API20E system. In addition, cells isolated during the challenge with strain MT463 *lux*AB were screened for bioluminescence [8].

Tanks containing control fish exposed to a sub-population which had been injected with sterile PBS were routinely monitored for the presence of mortalities. None of the control fish died during either cohabitation challenge and, therefore, none was killed and screened for presence of *A. salmonicida*.

2.5. Detection of A-layer protein in A. salmonicida strains

A. salmonicida strains MT463 *lux*AB, MT463 and MT432 were incubated overnight in Tryptone Soya Broth (TSB) at 22°C and then serially diluted in sterile PBS. Triplicate 100-µl samples from 10^{-5}, 10^{-6} and 10^{-7} dilutions were used to inoculate TSA plates containing 30 µg ml^{-1} Congo red. Cultures were incubated at 22°C for 48 h and then examined for the presence of red colonies [17].

3. Results

3.1. Virulence of wild-type and lux-marked A. salmonicida

Cumulative mortalities were determined following i.p. injection of wild-type and *lux*-marked strains of *A. salmonicida* MT463. Statistical analysis of survival curves (performed by Kaplan-Meier estimations) showed that the appearance of mortalities and the time taken to reach 100% mortality were related to the concentration and strain of cells injected. Wild-type *A. salmonicida* strain was more virulent at all doses applied. Injection of either strain at the highest dose (10^{10} cfu ml^{-1}) resulted in significant mortality within 2 days of injection but maximum mortality was achieved within 6 days for the wild-type strain and 14 days for the *lux*-marked strain. Maximum mortalities of fish injected with the wild-type strain at concentrations of 10^7, 10^8 and 10^9 cfu ml^{-1} occurred after 7, 7 and 12 days, respectively. *Lux*-marked cells did not cause death within 12 days when injected at concentrations $\leq 10^8$ cfu ml^{-1}, while a dose of 10^9 cfu ml^{-1} resulted in 75% mortality on day 12.

Mortalities appeared in the PBS-injected fish on day 12 of the challenge. All fish were held in one tank and the wild-type MT463 strain was significantly more virulent than the *lux*-marked strain. Death of these control fish was therefore, likely to have resulted from infection by wild-type *A. salmonicida* cells which had been shed from dying fish.

3.2. Re-isolation of A. salmonicida MT463 from i.p. injected fish

Analysis of the API and antibiotic resistance profiles obtained showed that *lux*-marked and wild-type *A. salmonicida* MT463 strains were the only bacteria recovered from the head kidney of i.p. injected fish. The API profiles of *lux*-marked and wild-type *A. salmonicida* strains were identical, but the two strains could be differentiated on the basis of antibiotic resistance and by the bioluminescence activity of *A. salmonicida* MT463 *lux*AB. *A. salmonicida* MT463 *lux*AB was re-isolated from fish injected with this organism at concentrations of 10^{10}, 10^9 and 10^8 cfu ml^{-1}. However, the bacteria isolated from fish in-

jected with 10^7 and 10^6 *A. salmonicida* MT463 *lux*AB cfu ml^{-1} were sensitive to tetracycline and were non-luminescent, indicating that mortalities in these fish could have been due to the wild-type strain *A. salmonicida.* Further evidence for this is that these fish started to die at the same time as fish injected with PBS (control fish).

Both wild-type and *lux*AB-marked strains were recovered on TSA from fish injected with the wild-type strain. In addition both *A. salmonicida* strains were isolated from the control fish which died on day 12, but only the wild-type organism was recovered from the PBS injected fish which died on days 14, 15 and 21 (data not shown). Both *lux*-marked and wild-type *A. salmonicida* MT463 strains were therefore recovered from fish regardless of the primary infectious agent.

3.3. Cross-infection by A. salmonicida MT463 luxAB during a cohabitation challenge

A. salmonicida was shed from i.p. injected fish and on day 1 of the cohabitation challenge (48 h after initial injection) the water contained 1.1×10^3 cfu ml^{-1} (Table 1). By this time, 23 of the 25 i.p. injected fish had died and were removed. Analysis of material from the head kidney confirmed the presence of *A. salmonicida* MT463 *lux*AB. Following mass mortality of i.p. injected fish, the number of *A. salmonicida* MT463 *lux*AB cells recovered from the water column remained at or below the limit of detection $(3 \times 10^2$ cfu ml$^{-1})$ for the remainder of the experiment.

Infection of non-injected fish was determined by analysis of mucus, gill, head kidney and intestinal samples. *A. salmonicida* MT463 *lux*AB was recovered from the mucus of all cohabitation challenged fish which were killed on days 1 and 2, but was isolated from only 20% of mucus samples on day 3 (Table 1). The strain was not recovered on day 5 of the cohabitation challenge but reappeared in mucus from all fish sampled on day 7, after death of the final two i.p. injected fish. A similar trend was observed in the recovery of *A. salmonicida* MT463 *lux*AB from fish gill tissue, but the frequency of detection was reduced (Table 1). *A. salmonicida* MT463 *lux*AB was not recovered in samples of head kidney or intestine from non-injected fish killed at any time.

3.4. Cross-infection by A. salmonicida MT432 during a cohabitation challenge

During this cohabitation study, eight of 10 fish injected with *A. salmonicida* MT432 died by day 2 of the experiment, 3 days after injection, and the remainder died by day 4 (Fig. 1). On day 2, *A. salmonicida* was recovered from the water column $(1.05 \times 10^3$ cells ml$^{-1})$ and from all five gill samples of cohabitant fish (Fig. 2), but was not detected in mucus samples (Fig. 3). By day 4, cells could not be detected in the water column (detection limit, 3×10^7 cfu ml^{-1}), remained at high concentrations in all gill samples and were detected in three mucus samples. By day 6, 23 of the non-injected fish had died and flesh removed from fish corpses had been lost due to

Table 1
Recovery of *A. salmonicida* MT463 *lux*AB from tank water and Atlantic salmon parr (approximately 15 g fish) during a cohabitation challenge at 14°C in freshwater

Day	Number of dead i.p. injected fish	Detection of *A. salmonicida* MT463 *lux*AB		
		Water column (mean cfu $\times 10^3$ ml^{-1})	Mucus	Gill
1	23	1.10	+ (10/10)	+ (5/10)
2	0	−	+ (10/10)	+ (2/10)
3	0	−	+ (2/10)	−
5	2	−	−	−
7	0	−	+ (10/10)	+ (4/10)
9	0	−	−	−

(+) positive and (−) negative isolation of *A. salmonicida* MT463 *lux*AB cells. The number of fish which harboured *A. salmonicida* MT463 *lux*AB is given in parentheses. The standard errors of viable cell concentrations in tank water were not greater than 15% of the mean.
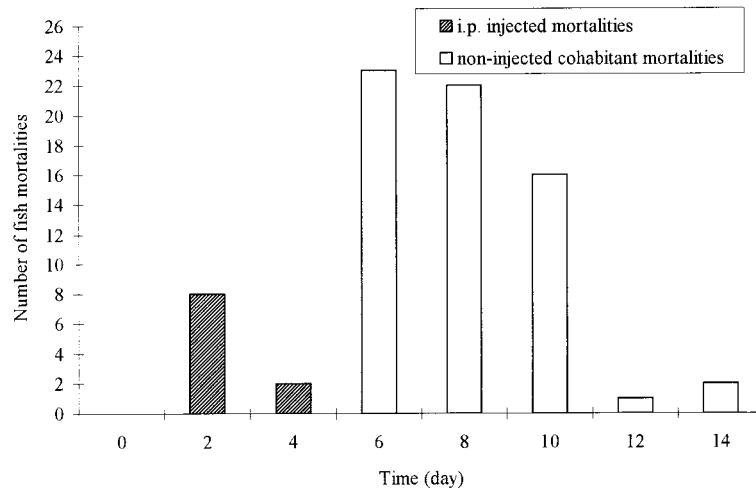
Fig. 1. Number of Atlantic salmon mortalities observed on each sample day during a cohabitation challenge with *A. salmonicida* MT432 in freshwater at 14°C. Ten fish were i.p. injected with a 100 μl suspension of *A. salmonicida* MT432 containing 10^5 cfu ml^{-1}. Following a 24-h incubation period, these fish were added to a tank containing 98 non-injected, cohabitant fish.

either decomposition or ingestion by cohabitant fish. Subsequent mortalities on days 8–14 (Fig. 1) were removed as they were discovered. Cells were again detected in the water column on day 6, remained present in all gill samples and were detected at high concentration in one of five mucus samples.

On day 8, *A. salmonicida* cells were recovered in high numbers from the water column (8.8×10^2 cells ml^{-1}) but were not detected in any subsequent water samples, even though further mortalities occurred.

Cells were detected in gill tissue from the majority of fish sampled between days 8 and 12, but were not present on day 14 (Fig. 2). A similar pattern was seen in mucus, with cells detected in most samples taken on days 8–12, but absent from mucus from fish sampled on day 14.

A. salmonicida MT432 was recovered as a pure culture from all fish which had died due to infection, from the head kidney of all dead fish and from fish killed on days 4, 6, 8 and 12. It was not isolated from
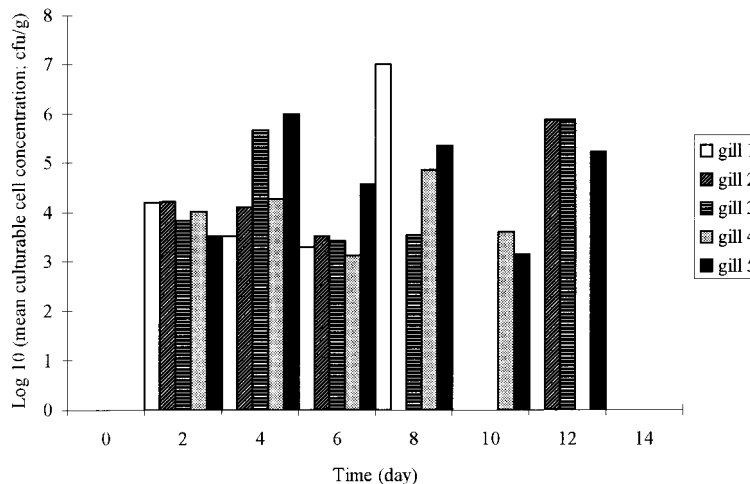

Fig. 2. Recovery of *A. salmonicida* MT432 from the gill tissue of killed Atlantic salmon (five replicates) during a cohabitation challenge at 14°C in freshwater (Fig. 1). Results are plotted as means of triplicate samples and the standard error was not greater than 28% of the mean. The minimum detection limit was 1.27×10^3 cfu g^{-1} gill tissue.
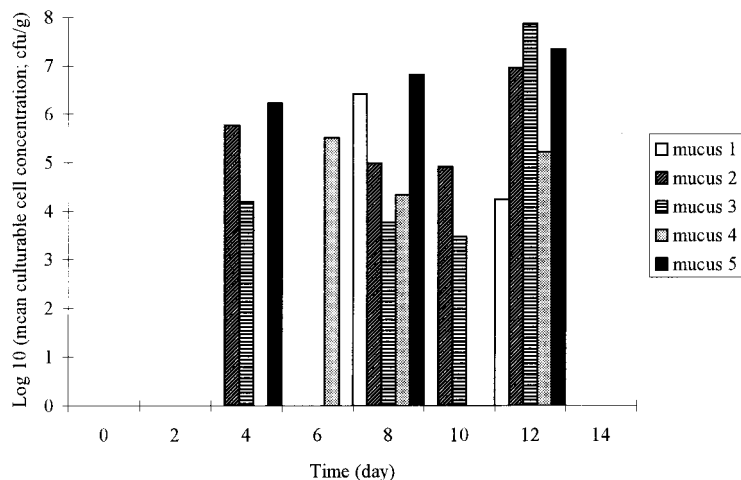
Fig. 3. Recovery of *A. salmonicida* MT432 from the mucus of killed Atlantic salmon (five replicates) during a cohabitation challenge at 14°C in freshwater (Fig. 1). Results are plotted as means of triplicate samples and the standard error was not greater than 21% of the mean. The minimum detection limit was 5.67×10^3 cfu g^{-1} mucus.

the intestine of any killed fish. *A. salmonicida* was present at high concentrations in the gill arches and mucus of all killed fish (Figs. 2 and 3) and was isolated as a pure culture at concentrations greater than 1.27×10^3 cfu g^{-1} gill tissue and 5.67×10^3 cfu g^{-1} mucus.

Recovery of *A. salmonicida* MT432 from fish samples and from the water column during the cohabitation challenge was confirmed using API20E diagnostic strips. The *A. salmonicida* colonies isolated from both water and fish tissue grew slowly and pigment production took up to 5 days, making it difficult to distinguish *A. salmonicida*, present at low concentrations, from other bacterial isolates.

3.5. Detection of A-layer in A. salmonicida strains MT463 luxAB, MT463 and MT432

Colonies of *A. salmonicida* MT432 and MT463 appeared red on A-layer selective medium but colonies of *A. salmonicida* MT463 *lux*AB were orange pigmented, indicating lack of A-layer.

4. Discussion

Genetic marking of *A. salmonicida* with *lux*AB and *tet* genes represents a novel approach to study transmission of the pathogen between fish. Antibi-

otic resistance of the marked *A. salmonicida* strain allowed detection in the presence of indigenous micro-organisms, while the introduction of *lux* genes increased sensitivity of viable cell enumeration. However, this study showed that the *lux*-marked strain was significantly less virulent than the wild-type organism, which may have been due to disruption of virulence genes through introduction of the marker genes. The most likely explanation for the reduced virulence of *A. salmonicida* MT463 *lux*AB is loss of the proteinaceous A-layer, which is an important virulence factor involved in bacterial cell association with host cells [10,12]. This was not detected in *A. salmonicida* MT463 *lux*AB, although conclusive evidence requires electron microscopy or serological tests. Loss of virulence may, however, have resulted from repeated subculture of this strain [18], rather than gene disruption, as the A-layer is frequently lost during culture maintenance, indicating an addition potential disadvantage of the use of marker genes for environmental studies. Despite loss of the A-layer, the *lux*-marked strain was sufficiently virulent to facilitate cross-infection studies.

Release of *A. salmonicida* cells into the water column from dead and moribund fish can account for transmission of disease [3,4], although the decrease in viable cell concentrations below detection limits during the course of infection indicates that survival of planktonic cells is poor and unlikely to lead to long-

term dispersal. Analysis of bacteria isolated from i.p. injected fish for bioluminescence and antibiotic sensitivity revealed that fish which had been injected with *A. salmonicida* MT4632 *lux*AB harboured the wild-type strain and vice versa. Cross-infection occurred throughout the experiment and fish were effectively exposed to two *A. salmonicida* strains. Competition between the two *A. salmonicida* strains will have occurred [19] and the more aggressive wild-type strain caused clinical symptoms. There is typically a 5-day incubation period between infection of fish with *A. salmonicida* via a water-borne route and manifestation of disease. Fish mortalities during the initial 7 days of the experiment (i.e. 5 days after the first mortality) were therefore primarily due to the effects of the i.p. injected bacterium, enabling a comparison of virulence caused by the two strains.

Results presented here clearly demonstrate that *A. salmonicida* MT463 *lux*AB was much less virulent than the wild-type strain. Nevertheless, results indicated that *lux*-marked *A. salmonicida* could persist in the water column for a sufficient period to facilitate uptake by surviving fish and cause secondary infection. This was investigated further by carrying out a cohabitation challenge experiment which showed that fish mucus and gills were the main sites for attachment of both *A. salmonicida* strains, agreeing with results obtained from bath challenge studies [9] and field observations [20].

Due to the low virulence of *A. salmonicida* MT463 *lux*AB, it was hypothesised that few cells would be isolated from fish tissue during the cohabitation and its presence/absence was assessed. In comparison, *A. salmonicida* MT432 was A-layer$^+$ and recovery of this strain ranged from 10^2–10^7 cfu g^{-1} tissue.

A. salmonicida MT463 *lux*AB was not isolated from either the intestine or head kidney of cohabitant fish at any time during the experiment. In contrast, strain MT432 was recovered from the head kidney of cohabitant fish killed on days 4, 6, 8 and 12. It is probable that *A. salmonicida* infection was by this stage systemic and that these fish were moribund. *A. salmonicida* MT432 was not recovered from the intestine of any fish during the challenge, despite the possibility of cohabitant fish feeding from corpses. This observation agrees with the results of McCarthy [2], but contradicts those of Lavelle [21].

The latter study was based on an *in vitro* system and use of tissue culture. Whilst this is an innovative method to study inter- and intra-cellular associations, it is an over simplification of a complex environment. Persistence of *A. salmonicida* within fish intestine would depend on a number of factors, including competition with, and/or inhibition by the resident micro-organisms [22,23], cellular resistance to digestive enzymes and bile salts, and penetration of the mucus layer. The absence of these factors from tissue culture systems may have influenced Lavelle's results.

Neither of the *A. salmonicida* strains was recovered from fish tissue at the end of the cohabitation challenges. Although both wild-type and *lux*-marked *A. salmonicida* were detected in killed fish at the end of the i.p. challenge, recovery efficiency was low (i.e. few fish harboured *A. salmonicida* cells). This suggests that the pathogen was either washed out of the tanks, or was present on or in fish tissue in numbers below the detection limit of plate counts. Persisting *A. salmonicida* cells may have caused latent infections, but this was not investigated.

Cahill [24] debated whether the organisms isolated from fish surfaces were true colonisers or were loosely associated and reflected the bacterial assemblage of water. In the present study both *A. salmonicida* strains were isolated from fish gills and/or skin mucus when the plate counts obtained from water samples had fallen below the limit of detection, suggesting that both strains had colonised these surfaces.

McCarthy [2] failed to infect rainbow trout (*Onchorynchus mykiss* Walbaum) with *A. salmonicida* following an immersion challenge without prior abrasion of the fish skin surface with sandpaper. This indicated that *A. salmonicida* could not persist within the mucosal layer of rainbow trout and/or could not traverse the fish epidermis. Austin and McIntosh [25] have subsequently shown that rainbow trout mucus is inhibitory to the growth of *A. salmonicida*. This phenomenon has also been reported for a number of other fish species [26,27]. Results described in this study and those presented by others [6,20] show that Atlantic salmon does not produce inhibitory compounds and *A. salmonicida* can survive when attached to skin/mucus tissue. Thus, the pathogen could potentially infect Atlantic

salmon by this route. No obvious lesions were observed on the surface of fish during the cohabitation challenges described here. It is more likely that *A. salmonicida* formed acute infection by penetrating the delicate gill tissue. During oxygen exchange, large volumes of water are passed over this surface. This action could enable significant numbers of *A. salmonicida* cells to concentrate within the fine filaments of the gill lamellae and result in the formation of microcolonies. Attachment of *A. salmonicida* and formation of microcolonies could induce expression of virulence genes (such as genes encoding GCAT/LPS and protease [28]) enabling the pathogen to invade host cells. Degradation of gill tissue may result in respiratory failure leading to fish death. Alternatively, *A. salmonicida* may enter the fish circulatory system by, for example, phagocytosis and be transported to and localised within the major organs.

The *lux*-based detection system was an effective method for tracking movement of *A. salmonicida* MT463 *lux*AB. The study successfully provided information on the sites within fish which provide the most favourable environments for colonisation and demonstrated the relative importance of survival of vegetative planktonic cells and colonisation of fish in the dispersal of this organism. A limitation of marker systems was highlighted in the reduction in virulence of the luminescence-marked strain, suggesting that construction of a virulent, A-layer^{+} *lux*-marked *A. salmonicida* strain would further increase the usefulness of this approach in determining cell dispersal in aquatic environments. The approach also has potential for investigation of other forms of dispersal, e.g. through use of luminometry and CCD microscopy to investigate cell association with zooplankton.

Acknowledgments

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