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Characterization of attenuated *Renibacterium salmoninarum* strains and their use as live vaccines

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ABSTRACT: Two nutritionally mutant strains of *Renibacterium salmoninarum* (Rs) were isolated that grew on tryticase soy agar (Rs TSA1) or brain heart infusion agar (Rs BHI1). These 2 strains could be continuously cultured on these media, whereas typical *R. salmoninarum* would only grow on KDM-2 agar. We determined no other phenotypic difference that could be used to distinguish them from wild-type *R. salmoninarum*. Both strains were found to be avirulent when 5×10^6 bacteria were intraperitoneally (IP) injected into Atlantic salmon. Rs TSA1, Rs BHI1, and Rs MT-239 (a *R. salmoninarum* strain previously shown to be attenuated) were tested as live vaccines in 2 separate trials. The best protection was seen with Rs TSA1. Vaccinated Atlantic salmon had relative percent survival (RPS) of 50 at 74 d post-challenge in Trial 1 and 76 at 60 d post-challenge in Trial 2. In both trials, 100% of the control salmon died from bacterial kidney disease (BKD) (within 40 d for Trial 1 and 50 d for Trial 2) after IP challenge with 5×10^6 live cells of the virulent isolate Rs Margaree.

KEY WORDS: Bacterial kidney disease · *Renibacterium salmoninarum*

INTRODUCTION

Bacterial kidney disease (BKD), caused by the Gram-positive bacterium *Renibacterium salmoninarum*, is a serious disease of salmonid fish that is transmitted both horizontally and vertically. In clinically diseased fish large numbers of viable bacteria are typically found within macrophages. The presence of the bacterium within macrophages, and the production of granulomatous lesions found with BKD, are distinctive features of facultative, intracellular pathogens. Despite extensive research, successful chemotherapy or immunotherapy protocols are not available to efficiently prevent or cure BKD (Young & Chapman 1978, Evelyn 1993, Fryer & Lannan 1993, Griffiths et al. 1998).

Renibacterium salmoninarum has been shown to survive and perhaps multiply within macrophages *in vitro*, as well as the RTG-2 and *epithelioma papillosum cyprini* (EPC) teleost cell lines (Gutenberger 1993, Evelyn 1996, MacIntosh et al. 1997). Thus *R. salmoninarum* can accurately be described as an intracellular pathogen. Unlike bacterial pathogens such as *Listeria* or *Salmonella*, the mechanisms responsible for *R. salmoninarum* intracellular survival have not been forthcoming. The most important renibacterial protein that has been extensively studied is the p57 haemagglutinin protein. This protein has been shown to have numerous *in vitro* properties that include haemagglutination, leucoagglutination, and spermagglutination abilities. It also makes the bacterium hydrophobic and has been suggested to be a major virulence factor, since strains missing this protein are less virulent (Daly & Stevenson 1987, 1989, 1990, Bruno 1988, Weins & Kaattari 1991).

Renibacterium salmoninarum requires specialized media and long periods of time for growth *in vitro*. Typically the bacterium is grown on the serum and L-cysteine containing agar medium, KDM-2 (Evelyn 1977). However, in agar-containing media, the serum can be effectively replaced with charcoal and to a

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lesser extent starch (Daly & Stevenson 1985). In broth media, serum and charcoal are not necessary for the growth of large quantities of the bacterium (Daly & Stevenson 1985, 1993). This suggests that it may be possible to select for bacterial mutants that grow on agar media without the requirement for serum or charcoal. In this study, trypticase soy agar (TSA) and brain heart infusion agar (BHIA) were chosen for this purpose since these 2 media are typically used for growing less fastidious bacteria. Both of these media have previously been shown not to support the growth of the bacterium due to their lack of L-cysteine (required at an amount of 0.05 or 0.1% (Daly & Stevenson 1993), and serum or charcoal (Daly & Stevenson 1985).

Another atypical strain that was characterized in this study was *Renibacterium salmoninarum* MT239 (Rs MT-239) which has previously been reported to have reduced virulence (Bruno 1988). Rs MT-239 is unique because it has a reduced expression of the p57 on the bacterial cell surface (Bruno 1988, Daly & Stevenson 1990, Senson & Stevenson 1999). The protein is, however, excreted into broth culture media (Senson & Stevenson 1999).

Currently there are no vaccines available to prevent BKD. It is generally recognized that the best means to successfully immunize against intracellular pathogens is to develop cellular immunity as a means of increasing the bacterial killing activity of the macrophages. One means that may direct the host to develop cellular immunity is to immunize the animal with a live, attenuated strain of the bacterium. As a first step in producing cellular immunity in salmon towards *Renibacterium salmoninarum*, we have developed nutritionally mutant strains of *R. salmoninarum* which are avirulent. These and the previously described, attenuated strain Rs MT-239 have been characterized and tested as live vaccines.

MATERIALS AND METHODS

Media. Unless stated otherwise, *Renibacterium salmoninarum* strains were grown at 18°C on agar plates of either KDM-2 agar (Evelyn 1977) or KDM-2 agar plus antibiotics (SKDM; Austin et al. 1983).

Renibacterial isolates. *Renibacterium salmoninarum* strains Margaree and Miramichi were typical, virulent *R. salmoninarum* isolated from Atlantic salmon *Salmo salar* in eastern Canada. *R. salmoninarum* MT239 is an attenuated isolate that has reduced concentrations of the p57 protein on its surface, is relatively hydrophilic but has the typical growth and nutrient requirements of *R. salmoninarum* (Bruno 1988, Senson & Stevenson 1999). It was previously obtained from Dr David Bruno, FRS Marine Laboratory, PO Box 101, Victoria Rd., Aberdeen AB11 9DB, Scotland, UK.

Nutritionally mutant strains of *Renibacterium salmoninarum* capable of growth on either TSA (Difco) or BHI agar (Difco) were selected by growing *R. salmoninarum* Margaree on KDM-2 agar for 2 wk, washing the bacteria in peptone saline (0.1% peptone in 0.85% NaCl) and plating 10^8 bacteria ml^{-1} onto either TSA or BHI agar. After 4 to 5 wk growth at 18°C, isolated colonies were randomly selected and re-plated onto their respective isolation medium. After 3 further passages, 2 strains, 1 that grew on TSA (designated Rs TSA1) and 1 that grew on BHI agar (designated Rs BHI1), were randomly selected for further passage and characterization.

Isolate characterization. All strains were tested for typical *Renibacterium salmoninarum* characteristics. It was determined whether each strain was composed of Gram-positive rods, whether they were hydrophobic according to the salt aggregation test (Daly & Stevenson 1987), whether they required KDM-2 agar for growth, and whether they reacted with a monoclonal antibody (4D3) against *R. salmoninarum* p57 (Diag-Xotics, Inc.) which was detected with a fluorescein labeled goat anti-mouse antibody (Bio-Rad Laboratories). All strains were also characterized for their haemolytic and proteolytic abilities. β-haemolytic activity was assessed by growth on either KDM-2 (Rs Margaree, Rs Miramichi and Rs MT-239 strains), TSA (Rs TSA1) or BHI (Rs BHI1) agars containing either 5% sheep or 5% rainbow trout *Oncorhynchus mykiss* erythrocytes. Proteolytic activity was assessed by growth on either KDM-2 plus casein (Rs Margaree, Rs Miramichi and Rs MT-239), TSA plus casein (Rs TSA1) or BHI plus casein (Rs BHI1) (Daly & Stevenson 1985). Further enzymatic activity of all strains was assayed using the API-ZYM system (Bio-Merieux) at 15°C by using whole live cells of individual bacterial strains incubated for 24 h. The results were compared with previously described *R. salmoninarum* API-ZYM profiles (Bruno & Munro 1986, Bandin et al. 1991).

Growth of Rs TSA1 and Rs BHI1 on various bacteriological agars. The ability of strains Rs Margaree, Rs TSA1, and Rs BHI1 to grow on KDM-2 agar (typically used to grow *Renibacterium salmoninarum*) was compared with their ability to grow on media that would typically not provide optimal nutritional components: TSA, BHIA and KDM-2 without L-cysteine or serum. An end point dilution method was used to compare growth on the different agar media (Daly & Stevenson 1985). Briefly, serial 10-fold dilutions of bacteria suspended in peptone saline were prepared, and 25 µl drops of each dilution were spotted onto duplicate plates of each medium. After 30 d of incubation at 15°C, the highest dilution of the culture at which colonies were observed on spot plates was recorded as the end point dilution.

Fish challenges. The virulence of *Renibacterium salmoninarum* strains TSA1, BHI1 and MT-239 was compared with that of known virulent strains Rs Margaree and Rs Miramichi. This was assessed using 50 to 70 g Atlantic salmon *Salmo salar* that were held at 13°C in tanks with flow-through, dechlorinated fresh water. For each strain, 25 salmon were anaesthetized with 2-phenoxyethanol (1:7000; v/v) and injected intraperitoneally (IP) with 0.1 ml of peptone saline containing either 10^8 or 5×10^6 viable bacteria as determined by plate counts on SKDM agar (Austin et al. 1983). Similarly, 25 negative and positive control fish were injected with either peptone saline or freshly isolated virulent strains of either Rs Margaree or Rs Marimichi. (These strains had previously been isolated from Atlantic salmon with clinical BKD. Bacterial virulence was regularly assessed in Atlantic salmon because the authors have previously observed that *R. salmoninarum* can spontaneously become avirulent after culture on KDM-2 agar.) After challenge, fish mortalities were removed twice daily, and aseptically excised kidney tissue was streaked onto SKDM, TSA or BHIA, and incubated for up to 5 wk at 18°C. Any resultant bacterial cultures were identified as *R. salmoninarum* by their Gram-positive diplobacilli morphology and by the fluorescent antibody technique (FAT) using a fluorescein isothiocyanate conjugated rabbit anti *R. salmoninarum* serum. At the time of death, a kidney smear was made from each fish which was stained by FAT for further confirmation that *R. salmoninarum* was the agent of mortality.

Vaccination of Atlantic salmon with attenuated strains. Fifty gram Atlantic salmon, with no prior history of BKD, were anaesthetized with 2-phenoxyethanol (1:7000; v/v) and immunized with a single IP dose of 1 of the following: (1) 10^8 formalin (0.5%)-killed Rs Margaree in peptone saline and Complete Freund's adjuvant (Difco Inc., Detroit, USA), (2) 5×10^6 live cells of Rs TSA1 in peptone saline, (3) 5×10^6 live cells of Rs BHI in peptone saline, (4) 5×10^6 live cells of Rs MT239 in peptone saline or (5) peptone saline alone. Each group of 25 fish was specifically finclipped for later identification. The salmon were held in 1 tank containing fresh water at 13 to 15°C. After vaccination, the fish were allowed to rest for 1 mo. At this time, all fish of each group were again anaesthetized with 2-phenoxyethanol (1:7000; v/v) and individually challenged with 5×10^6 live cells of Rs Margaree in peptone saline. After challenge with an IP injection, the fish were observed twice daily for mortalities for 75 d in vaccination Trial 1 and 60 d in vaccination Trial 2. If a fish died, a portion of the kidney was excised and cultured on either SKDM, TSA or BHI to determine the presence of *Renibacterium salmoninarum*, or some other pathogen. The identification of *R. salmoninarum* colonies was confirmed by staining resulting bacteria with fluorescein isothiocyanate conjugated rabbit anti-*R. salmoninarum* serum. The efficacy of the vaccine was calculated as relative percent survival (RPS) (Amend 1981).

RESULTS

Isolation and characterization of nutritionally mutant strains

Two strains of *Renibacterium salmoninarum* designated as Rs TSA1 and Rs BHI1 were isolated that were capable of repeated growth on either TSA (Rs TSA1) or BHI agar (Rs BHI1). Cell suspensions of the typical, virulent Rs Margaree wild-type strain which showed end point dilutions of 10^8 on KDM-2 would rarely grow as distinct colonies on either TSA or BHI, even when 10^8 CFU ml^{-1} was spot-inoculated. In comparison, both the Rs TSA1 and Rs BHI1 strains gave similar CFUs when diluted and grown on KDM-2 or their host medium (TSA for Rs TSA1 or BHIA for Rs BHI1) (Table 1). On KDM-2 agar without serum or L-cysteine, both strains produced similar CFUs to those found on KDM-2 or TSA (in the case of Rs TSA1) or BHIA (in the case of Rs BHI1). Although both strains grew quicker on serum containing agar, the serum was not required for their growth and did not cause the formation of a greater number of bacterial colonies. After growth on KDM-2 agar, both Rs TSA1 and Rs BHI1 did not revert back to their wild-type nutritional requirements. After 25 passages on either TSA (Rs TSA1) or BHIA (Rs BHI1), both strains continued to grow well.

All *Renibacterium salmoninarum* strains, both wild-type and mutants were proteolytic on casein-containing agar, and were haemolytic on agar containing either sheep or trout erythrocytes. The proteolytic and

Table 1. *Renibacterium salmoninarum.* Growth of wild-type and nutritionally mutant strains on various agar media. NT: not tested

Medium	Growth end point dilution[a]		
	Rs Margaree	Rs TSA1	Rs BHI1
KDM-2	10^{-8}	10^{-8}	10^{-8}
BHI	10^{0}	NT	10^{-8}
TSA	10^{0}	10^{-8}	NT
Basal KDM[b]	10^{0}	10^{-8}	10^{-8}

[a]The end point indicates the highest dilution of the culture which produced visible growth. Plates were read after 30 d growth at 18°C. The experiment was conducted on 3 separate occasions, all of which gave similar results

[b]Basal KDM is KDM-2 (Evelyn 1977) without serum

β-haemolytic zones surrounding strains Rs MT-239, Rs TSA1 and Rs BHI1 were similar to those seen with the wild-type Rs Margaree strain. In addition, both Rs TSA1 and Rs BHI1 produced the typical API-ZYM patterns described previously (Bruno & Munro 1986, Bandin et al. 1991) and they were Gram-positive with typical *R. salmoninarum* size and shape. Most importantly, when examined at 1000× with an UV-microscope, both Rs TSA1 and Rs BHI1 gave typical fluorescent reactions when reacted with the anti-*R. salmoninarum* p57 monoclonal antibody.

Virulence of *Renibacterium salmoninarum* mutant strains

The 3 mutant *Renibacterium salmoninarum* strains Rs TSA1, Rs BHI1 and Rs MT-239 were avirulent for 50 g Atlantic salmon after a challenge dose of 5×10^6. After 5 mo there were no mortalities from any of these 3 strains. Furthermore, none of the fish became lethargic or diseased. At this time, all of the bacteria appeared to have been cleared from the salmon, as *R. salmoninarum* was not cultured from the kidney. At dosages of 10^8 bacteria per fish, Rs BHI1 caused no mortalities after 5 wk whereas both RS TSA1 and Rs MT-239 each killed 8% (2 of 25) of the challenged salmon within this period. In all cases, the bacteria that were isolated had phenotypes similar to the strain that was injected. In contrast, the 2 wild-type strains, Rs Margaree and Rs Miramichi, killed 100% of the salmon within 15 d with a challenge dose of 10^8 per fish and in 40 to 50 d with a challenge dose of 5×10^6 (Fig. 1; see also Figs. 2 & 3 for additional Rs Margaree results).

Vaccination of Atlantic salmon with attenuated *Renibacterium salmoninarum*

The use of Rs TSA1, Rs BHI1 and Rs MT-239 as live vaccines were assessed in 2 separate experimental trials. In the first experimental trial (Fig. 2) formalin-killed Rs Margaree bacterin plus adjuvant, and live Rs Mt-239 provided no protection against BKD. Some limited protection was provided by live Rs BHI1 (RPS of 30), whereas Rs TSA1 protected 50% of the salmon from disease (RPS of 50) 74 d after challenge (Fig. 2). In the second experimental trial (Fig. 3), all treatments provided protection during the 60 d post-challenge. Fish vaccinated with killed bacterin plus adjuvant had a RPS of 36, Rs MT-239 vaccinated fish had a RPS of 44, Rs BHI1 vaccinated fish had a RPS of 60, whereas those vaccinated with Rs TSA1 had a RPS of 76 (Fig. 3).

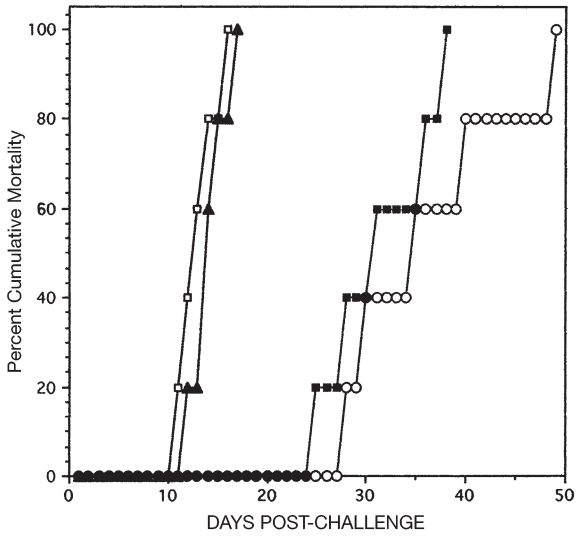

Fig. 1. Intraperitoneal challenge of Atlantic salmon *Salmo salar* with *Renibacterium salmoninarum* strains Rs Margaree and Rs Miramichi. Twenty-five salmon were injected intraperitoneally (IP) with 0.1 ml of peptone saline containing either 10^8 viable Rs Margaree (□) , 10^8 viable Rs Miramichi (▲), 5×10^6 viable Rs Margaree (■) or 5×10^6 viable Rs Margaree (○)

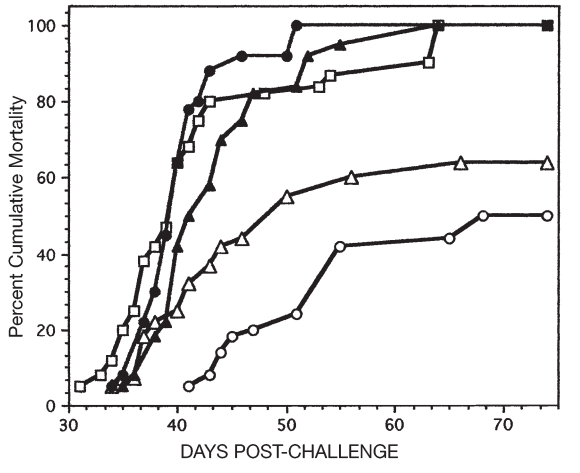

Fig. 2. Intraperitoneal (IP) challenge of vaccinated Atlantic salmon *Salmo salar* with Rs Margaree, Trial one. Fifty gram Atlantic salmon, with no prior history of BKD, were anaesthetized with 2-phenoxyethanol and immunized with a single IP dose of one of the following: (1) 10^8 formalin (0.5%)-killed Rs Margaree in peptone saline containing Complete Freund's adjuvant (●) (Difco), (2) 5×10^6 live cells of Rs TSA1 in peptone saline (○), (3) 5×10^6 live cells of Rs BHI in peptone saline (△), (4) 5×10^6 live cells of Rs MT239 in peptone saline (▲) or (5) peptone saline alone (□). The salmon were held in 1 tank containing fresh water at 13 to 15°C. All groups contained 25 fish. After vaccination, the fish were allowed to rest for 1 mo. At this time, all fish of each group were again anaesthetized with 2-phenoxyethanol (1:7000; v/v) and individually challenged with 5×10^6 live cells of Rs Margaree in peptone saline. After being challenged, the fish were observed twice daily for mortalities for 75 d

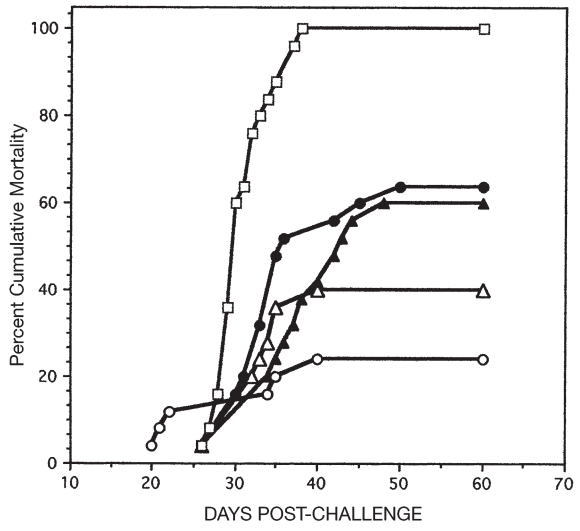

Fig. 3. Intraperitoneal (IP) challenge of vaccinated Atlantic salmon *Salmo salar* with Rs Margaree, Trial 2. Fifty gram Atlantic salmon, with no prior history of BKD, were immunized with a single IP dose of 1 of the following: (1) 10^8 formalin (0.5%)-killed Rs Margaree in peptone saline containing Complete Freund's adjuvant (●) (Difco), (2) 5×10^6 live cells of Rs TSA1 in peptone saline (○), (3) 5×10^6 live cells of Rs BHI in peptone saline (△), (4) 5×10^6 live cells of Rs MT239 in peptone saline (▲) or (5) peptone saline alone (□). The salmon were held in one tank containing fresh water at 13 to 15°C. All groups contained 25 fish. After vaccination, the fish were allowed to rest for 1 mo. At this time, all fish of each group were again anaesthetized with 2-phenoxyethanol (1:7000; v/v) and individually challenged with 5×10^6 live cells of Rs Margaree in peptone saline. After being challenged, the fish were observed twice daily for mortalities for 60 d

DISCUSSION

To date, both the Rs TSA1 and Rs BHI1 strains have been cultured on either TSA or BHI agar for more than 25 bacterial transfers. Both strains continue to remain hydrophobic and still produce the p57 cell surface antigen (data not shown; Griffiths et al. 1998), and we have never observed them to revert back to their original nutritional wild type. Recently, it has also been independently shown by Grayson et al. (1999) that Rs TSA1 produces the *msa*, *hly* and *rsh* genes. Under molecular analysis, this strain did not appear to be different from the other wild-type *Renibacterium salmoninarum* strains that were examined (Grayson et al. 1999). Except for the fact that Rs TSA1 and Rs BHI1 grow on TSA and BHIA (unlike their parental strains), and therefore do not have the absolute L-cysteine requirement necessary for the typical virulent strains, we know of no other phenotypic difference that can be used to distinguish them from wild-type *R. salmoninarum*. Furthermore, we do not know why they are avirulent. On a number of occasions, from a number of different isolates, we have isolated *R. salmoninarum* that can grow on TSA.

Previously Bruno (1988) has shown that Rs MT-239 has reduced virulence in rainbow trout *Oncorhynchus mykiss* and has suggested that the loss of p57 (or bacterial cell hydrophobicity) was associated with reduced virulence. Recent data have shown that Rs MT-239 does produce p57. However the protein shows little attachment (assembly?) to the bacterium's cell surface. Thus, cell-associated p57 is not detected in the typical amounts seen with other isolates of *Renibacterium salmoninarum* (Senson & Stevenson 1999). The data presented here for Rs TSA1 and Rs BHI1 demonstrate that nutritionally mutant strains can produce p57 on their surface, and yet be avirulent. This suggests that although p57 may be an important virulence factor, another as of yet uncharacterized factor must also be present. Thus, *R. salmoninarum* must have multiple virulence factors that must be expressed in order for the bacterium to cause disease in salmon.

Until recently, researchers have had limited success in the development of a BKD vaccine. Piganelli et al. (1999) have had success protecting coho salmon with an oral vaccine consisting of formalin-killed *Renibacterium salmoninarum* with the p57 antigen removed by heat treatment. Griffiths et al. (1998) have shown some protection against *R. salmoninarum* in Atlantic salmon using both live *Arthrobacter* and live Rs TSA1 vaccines. These authors demonstrated that after 14 wk only 31% of the live Rs TSA1 vaccinated Atlantic salmon (80 g) were culture positive for *R. salmoninarum*, whereas in the unvaccinated fish, 85% were culture positive. However in their study, the challenge strain of *R. salmoninarum* failed to kill either their non-vaccinated or their vaccinated groups of salmon. In order to extrapolate protection, Griffiths et al. (1998) were forced to compare relative tissue levels of the bacterium in non-vaccinated groups versus vaccine groups. The data presented in our study strengthen and confirm the findings of Griffiths et al. (1998), and clearly demonstrate that vaccinating Atlantic salmon with the live Rs TSA1 reduces mortalities from a lethal challenge. Our data also demonstrate that different attenuated *R. salmoninarum* do not provide the same level of protection against a lethal challenge of *R. salmoninarum*. Although the protection that is seen after the vaccination with live, attenuated Rs TSA1 is not complete, it is encouraging for those that hope to develop a vaccine to prevent this salmonid disease. Further investigations into the mechanisms for the avirulence of Rs TSA1 may permit the development of genetically defined mutants leading to their potential use as vaccines.

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