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Renibacterium salmoninarum p57 antigenic variation is restricted in geographic distribution and correlated with genomic markers

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ABSTRACT: The 57 kDa protein (p57) is an important diagnostic antigen that is implicated in the pathogenesis of salmonid bacterial kidney disease. Little is known about the nature and extent of antigenic variation in p57. Previously, we reported that p57 produced by *Renibacterium salmoninarum* Strain 684 contains a mutation that disrupts monoclonal antibody (MAb) 4C11 binding. In the present study, we examined MAb binding to a panel of 23 additional *R. salmoninarum* isolates obtained from diverse geographic locations to examine the prevalence of this variant and whether additional variability exists within other p57 epitopes. Six p57-specific MAbs (4C11, 4D3, 3H1, 4H8, 4D10 and 1A1) were used to probe dot and western blots to determine the relative expression, size and cellular association of p57. Full-length p57 was produced by all isolates, and for each isolate, the protein was associated with the bacterial cell surface. The epitopes recognized by 4 MAbs, 4D3, 4H8, 3H1 and 1A1, were conserved among all strains tested. The 4C11 epitope was absent in 5 of 8 strains originating from Norway, while the 4D10 epitope was partially disrupted in one isolate from British Columbia, Canada. The 5 Norwegian antigenic-variant strains appeared to be clonally related as they shared the following characteristics: one tandem repeat in the ETRA locus, a Sequovar-4 16–23S rRNA intervening DNA sequence, a larger *Xho*I fragment in the *msa1* 5′ region, and absent *msa3* gene. These results indicate that limited antigenic and genomic variation exists between strains and this variation appears geographically restricted in distribution.

KEY WORDS: *Renibacterium salmoninarum* · *msa* gene P57 · Antigenic variation · Exact tandem repeat locus · ETR

INTRODUCTION

Renibacterium salmoninarum is a gram-positive pathogen that causes a chronic bacteremia of salmonid fish termed bacterial kidney disease (BKD). Clinical infection is often characterized by focal lesions in the viscera, particularly the kidney (Fryer & Sanders 1981). In many fish populations, the bacterium is found in a large percentage of individuals but the infection is usually subclinical in nature. In the Pacific Northwest of the United States, the prevalence of *R. salmoninarum* in juvenile Chinook salmon has been reported to reach 100% in the Columbia River Basin (Elliott et al. 1997) and 64% in the Puget Sound (Rhodes et al. 2006), but the fraction of fish with clinical symptoms of BKD is usually less than 10% (Rockey et al. 1991). This skewed distribution of infection severity suggests that there may be variation in the host response and/or pathogen virulence.

Studies to define virulence factors and to compare the virulence between strains have been difficult due to long challenge times and slow growth of this microorganism. Nevertheless, differences in virulence between isolates have been reported (Dale et al. 1997, Starliper et al. 1997), although the precise molecular differences between isolates are unknown in most cases.

The major cell surface and secreted protein of *Renibacterium salmoninarum* is termed MSA or p57

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(Wiens & Kaattari 1991, 1999). A single gene encoding p57 was originally cloned and designated *msa* for major soluble antigen (Chien et al. 1992), and subsequent investigation identified the presence of as many as 3 copies of *msa* in the bacterial genome depending on the *R. salmoninarum* isolate (Rhodes et al. 2004). Experimental reduction in gene copy number correlated with reduced virulence, thus demonstrating a crucial contribution of this protein to virulence (Rhodes et al. 2004, Coady et al. 2006). The coding regions of the *msa1* and *msa2* genes are identical, suggesting relatively recent gene duplication, and both genes are present in all characterized strains of *R. salmoninarum* (O'Farrell & Strom 1999, Wiens et al. 2002). *msa3* has not yet been fully sequenced, but the flanking regions resemble *msa1* and this gene has only been identified in a subset of strains originating from the US (Rhodes et al. 2004). Both *msa1* and *msa2* promoters are functional because integrated reporter plasmids were shown to be transcribed and translated during *in vitro* culture (Rhodes et al. 2002). Whether the *msa3* promoter is functional has not been determined.

We have previously reported antigenic variation in p57 produced by *Renibacterium salmoninarum* Strain 684 (Wiens et al. 2002). Remarkably, DNA sequencing identified that both *msa1* and *msa2* genes contained the same, single nucleotide mutation that changed Ala^{139} to Glu in the amino-terminal region of p57. This strain does not contain *msa3*. The p57 protein, isolated from *R. salmoninarum* Strain 684 culture supernatant, exhibited 2-fold greater agglutinating and binding activities compared to Strain ATCC 33209 p57. Furthermore, monoclonal antibody (MAb) 4C11 blocked the agglutinating activity of Strain 33209 p57, but not the activity of Strain 684 p57. These results indicate the Ala^{139} to Glu substitution in *R. salmoninarum* Strain 684 p57 altered both immune recognition and enhanced biological activity.

The overall aim of this work was to further examine the nature and scope of antigenic variation among *Renibacterium salmoninarum* strains and to determine whether pathogen variation correlates with geographic location of strain isolation. We specifically focused on 3 aspects of antigenic and genetic variation between strains. Firstly, we undertook a comprehensive examination of antigenic variation in p57 by testing 23 additional strains with a panel of MAbs which we have previously demonstrated to recognize distinct epitopes on p57. Secondly, we determined whether the 4C11 epitope was present or absent in 8 *R. salmoninarum* strains from Norway. Finally, we investigated whether antigenic variation correlated with genomic variation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 26 different isolates of *Renibacterium salmoninarum* from diverse geographic regions were analyzed (Table 1). Three of these strains have been subject to previous molecular analysis: the type strain ATCC 33209 (Table 1, Isolate #25), Strain 684 from Norway (Isolate #1), and Strain MT239 (Isolate #26). MT239 lacks cell-associated p57 but transcribes the *msa* genes and secretes p57 (O'Farrell & Strom 1999). *R. salmoninarum* Strains WR99C2, CAR96, Man 96, Little Goose, Gr5, SAW91, DWK90, DWK91, AK98, BQ96-01, BPS91-1, DR 143, GL64 and MT239 were obtained as a generous gift from R. Pascho (USGS Western Fisheries Research Center, Seattle, WA). Strains K28, K70, and D6 were a generous gift from C. Banner (Oregon Department of Fish and Wildlife, Corvallis, OR). Strains 1840, 4451, 667, 40922, 1185, 2649 and 4467 were part of a comprehensive collection of *R. salmoninarum* isolates obtained from salmonid culture facilities in Norway and maintained by the Norwegian Veterinary Institute. *R. salmoninarum* strains were grown as previously described (Wiens & Kaattari 1989).

Monoclonal antibodies. The generation of MAbs 4D3, 4H8, 4C11, 1A1 and 4D10 have previously been described (Wiens & Kaattari 1989, 1991). Hybridoma cells were grown in either tissue culture medium or in BALB/c mice ascites fluid. MAbs were purified using Protein A-sepharose (Bio-Rad) and stored in 50% glycerol at –20°C.

Dot and western blotting. Methods for dot and western blotting have been described previously (Wiens et al. 2002), with the exception that primary antibody was detected with a 1:1000 dilution of alkaline phosphatase-coupled goat anti-mouse kappa antiserum (Southern Biotechnology Associates).

Genomic DNA extraction and Southern blotting. Methods for genomic DNA extraction and Southern blotting have been described previously (Wiens et al. 2002).

ETR-A and intervening sequence PCR and sequencing. The exact tandem repeat locus (ETR-A) was amplified using the previously described primers 17D+95 (5′-TCG CGA ATA GCT TGG CCA TTT TGC-3′) and 17D-344 (5′-CGT AGC ACC GAA GTC AGA TAA GAG-3′) (Grayson et al. 2000). Polymerase chain reaction (PCR) amplification was carried out using high-fidelity PfuTurbo polymerase as recommended by the manufacturer (Stratagene) with 100 ng of genomic DNA as template. PCR amplification cycles (94°C for 5 min, followed by 30 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 4 min) and a final extension of 7 min at 72°C were performed with a Perkin-Elmer 9700 thermocycler. Reactions were carried out in a

Table 1. *Renibacterium salmoninarum* designation, location, phenotype and genotype. ETR-A: exact tandem repeat locus (TR1: single repeat; TR2: double repeat); 16–23S: Sequovar (SV) of the 16S–23S intergenic sequence. –: negative, +: positive, ±: weak

No.	Isolate	Location	Year	Host	4C11	4D10	*msa3*	ETR-A	16–23S
1	684/87	Aurland, Norway	1987	*Salmo trutta*	–	+	–	TR1	SV4
2	1840/85	Selöy, Norway	1985	*Salmo salar*	–	+	–	TR1	SV4
3	4451/86	Brekke, Norway	1986	*S. salar*	–	+	–	TR1	SV4
4	667/85	Tafjord, Norway	1985	*S. salar*	–	+	–	TR1	SV4
5	40922/84	Vadheim, Norway	1984	*S. salar*	–	+	–	TR1	SV4
6	WR99C2	Washington, USA	1999	*Oncorynchus kisutch*	+	+	+	TR2	SV1
7	CAR 96	Washington, USA	1996	*Oncorynchus tshawytscha*	+	+	+	TR2	SV1
8	Man96	Washington, USA	1996	*O. tshawytscha*	+	+	+	TR2	SV1
9	Little Goose	Washington, USA	1984	*O. tshawytscha*	+	+	+	TR2	SV1
10	Gr5	Montana, USA	1997	*Thymallus thymallus*	+	+	+	TR2	SV1
11	SAW91	Idaho, USA	1991	*O. tshawytscha*	+	+	+	TR2	SV1
12	DWK90	Idaho, USA	1990	*O. tshawytscha*	+	+	+	TR2	SV1
13	DWK91	Idaho, USA	1991	*O. tshawytscha*	+	+	+	TR2	SV1
14	AK 98	Alaska, USA	1998	Unknown	+	+	+	TR2	SV1
15	BQ96-01	Nanaimo, BC, Canada	1996	*O. tshawytscha*	+	+	+	TR2	SV1
16	BPS91-1	Nanaimo, BC, Canada	1991	*Oncorhynchus gorbuscha*	±	±	+	TR2	SV1
17	DR 143	Nanaimo, BC, Canada	1972	*Salvelinus fontinalis*	+	+	–	TR2	SV1
18	K28	France	Unknown	Unknown	+	+	–	TR2	SV1
19	K70	England	Unknown	Unknown	+	+	–	TR2	SV1
20	1185/87	Langöyneset, Norway	1987	*S. salar*	+	+	–	TR2	SV1
21	2649/85	Hyllestad, Norway	1985	*S. salar*	+	+	–	TR2	SV1
22	4467/86	Aurland, Norway	1986	Unknown[a]	+	+	–	TR2	SV1
23	GL64	Lake Michigan, USA	1991	*O. tshawytscha*	+	+	–	TR2	SV1
24	D6	Oregon, USA	1982	*O. kisutch*	+	+	–	TR2	SV1
25	ATCC33209	Oregon, USA	1974	*O. tshawytscha*	+	+	–	TR2	SV1
26	MT239	Scotland	1993	*S. salar*	–	–	–	TR2	SV1

[a]Isolated from either *S. salar* or *S. trutta*

total volume of 40 µl. PCR products were resolved on a 1% agarose gel (MetaPhor agarose; BioWhittaker Molecular Applications) and scored as either containing 1 or 2 repeats based on a fragment size migration.

Amplification of the 16S–23S spacer region was carried out using the previously described primers RS+1002 (5′-CCG TCC AAG TCA CGA AAG TTG GTA-3′) and ML1329 (5′-ATC GCA GAT TCC CAC GTC CTT CTT-3′) (Grayson et al. 1999). PCR conditions and cycles were identical to those used to amplify the ETR-A locus. PCR products were gel-extracted, purified and directly sequenced using the original amplification primers.

RESULTS

Cell association of P57

A total of 26 different isolates of *Renibacterium salmoninarum* from diverse geographic regions were analyzed for the production of p57 (Table 1). As previously reported, Strain MT239 lacked bacterial cell-associated p57 (O'Farrell & Strom 1999) and was thus negative by western blot utilizing cell lysates, while all other tested strains contained *R. salmoninarum* cell-associated protein that was recognized by MAb 4D3 (Fig. 1). P57 appeared to be identical in size between strains and minor amounts of proteolytic breakdown products were observed (G. D. Wiens unpubl. data). The presence of cell-associated p57 in all strains allowed us to screen strains with the remaining MAbs, using a whole *R. salmoninarum* cell dot blot as this was a more rapid screening procedure. Dot blot analysis identified 4 strains (40922, 1840, 4451, 667) in addition to Strain 684, which failed to react with MAb 4C11 (Fig. 2, highlighted with solid circles) but produced sufficient p57 to be recognized by MAb 4D3 (Fig. 2, left blot). Note that strain 1840 was independently cultured and replicated on the blot. The 4 additional 4C11 negative strains originated from Norway (Table 1); however, not all strains from Norway showed this phenotype. Three other strains from Norway (2649, 1185 and 4467; Fig. 2, highlighted with dotted circles) were strongly recognized by MAb 4C11, indicating strain heterogeneity within this geographic region. MAbs 4H8, 3H1 and 1A1 recognized p57 produced by all strains, while 4D10 recognized all strains but only bound weakly to Strain BPS91-1 obtained from British Columbia (Table 1, G. D. Wiens unpubl. data).

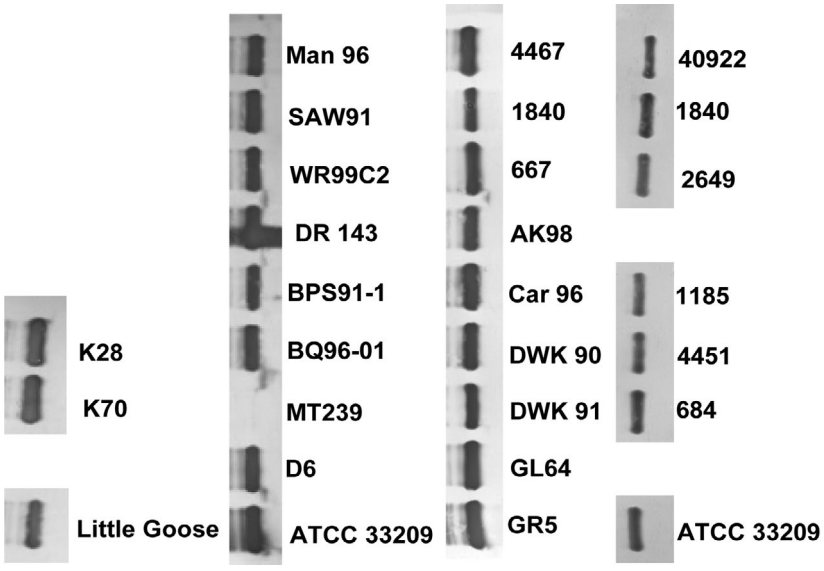

Fig. 1. *Renibacterium salmoninarum*. Western blot identifies that full-length P57 is produced by strains isolated from diverse geographic locations. Blots were probed with anti-p57 MAb 4D3. Origins of the strains are listed in Table 1. Strain MT239 is known to secrete p57, but lacks the cell-associated form and was used here as a negative control. Note that strains ATCC 33209 and 1840 are present in multiple lanes. Similar western blotting results with MAb 403 were obtained in 2 experiments

Correlation between loss of the 4C11 epitope and the 5′ *Xho*I band shift

To examine the possibility of clonal relationships between *Renibacterium salmoninarum* strains, genomic DNA was extracted, cut with *Xho*I, and probed with an internal *msa* probe (Fig. 3A). The restriction enzyme *Xho*I was used because there is only one restriction site within each *msa* gene and thus each gene is cleaved into 2 fragments. The *Xho*I fragments were demonstrated to hybridize to a single *msa* probe that overlaps both fragments, thus allowing the simultaneous examination of size length heterogeneity in both the 5′ and 3′ regions of *msa1*, *msa2*, and the putative *msa3* gene (Fig. 3A). The 5′ region of *msa2* is shortest with a length of 2.94 kb, the 5′ region of *msa1* is intermediate at 5.889 kb, and that of *msa3* is the largest fragment and estimated at approximately 8 kb (Fig. 3A). Previously, it was determined that the 5′ region DNA fragment of *msa1* from Norwegian Isolate 684 was about 700 bp larger than that of ATCC Strain 33209. This size difference was also observed in all 5 of the 4C11 epitope-loss variants from Norway: 40922, 667, 1840, 4451 and 684 (Fig. 3B). This finding is consistent with a common clonal origin of these antigenic variants. Furthermore, the shift was not observed in Norwegian Isolates 1185, 4467 and 2649 which were recognized by MAb 4C11, confirming that 2 genetically distinct variants of *R. salmoninarum* are present in Norway. Bands corresponding to both the 5′ and 3′ regions of *msa3* were observed in 11 strains, and the geographic origins of these strains ranged from the Pacific Northwest to Alaska, but were not observed in any of the strains of European or Norwegian origin (Fig. 3B and Table 1). Strain BPS91-1 is not shown in Fig. 3B, but displayed a similar Southern blot profile to BQ96-01 (G. D. Wiens unpubl. data). Some variation was observed in the migration of bands corresponding to the 3′ ends of *msa1* and *msa2*, but these differences were difficult to resolve given the large size of the restriction fragments. The bands corresponding to the 5′ region of *msa2* were identical in size between all isolates, suggesting a common evolutionary relationship between the strains included in this study.

Correlation between antigenic variation and genomic markers

In order to further examine *Renibacterium salmoninarum* strains for evidence of common clonal origins, 2 additional molecular diagnostic assays were used. First, we examined the DNA sequence between the 16S and 23S rRNA genes of which 4 sequences are known that have been designated as Sequovars 1 to 4 (Grayson et al. 1999). Sequencing of amplified product from each of the 4C11 antigenic variants matched Sequovar 4, while the amplified products from all other strains tested were identical to Sequovar 1 (Table 1).

Second, we examined the nucleotide ETR-A by PCR amplification. In this locus, *Renibacterium salmoninarum* strains have been reported to exhibit 2 different size variants, designated TR1 and TR2, that differ by a 51 bp exact repeat (Grayson et al. 2000). The precise location of the ETR-A locus was investigated by examination of the whole genome sequence of the ATCC 33209 strain (Wiens et al. 2008). A basic local alignment search tool (BLASTn) search using the 2 primer sequences identified locus ID RSal33209_ 2187, which is a 2865 bp open reading frame encoding a predicted protein of 955 amino acids assigned GenBank protein accession number YP_001625333. This open reading frame contains two 17 amino acid identical repeats (aa numbers 528–544 and 545–561; Fig. 4A, bold). Amplification of this locus using the 17D+95 and 17D-344

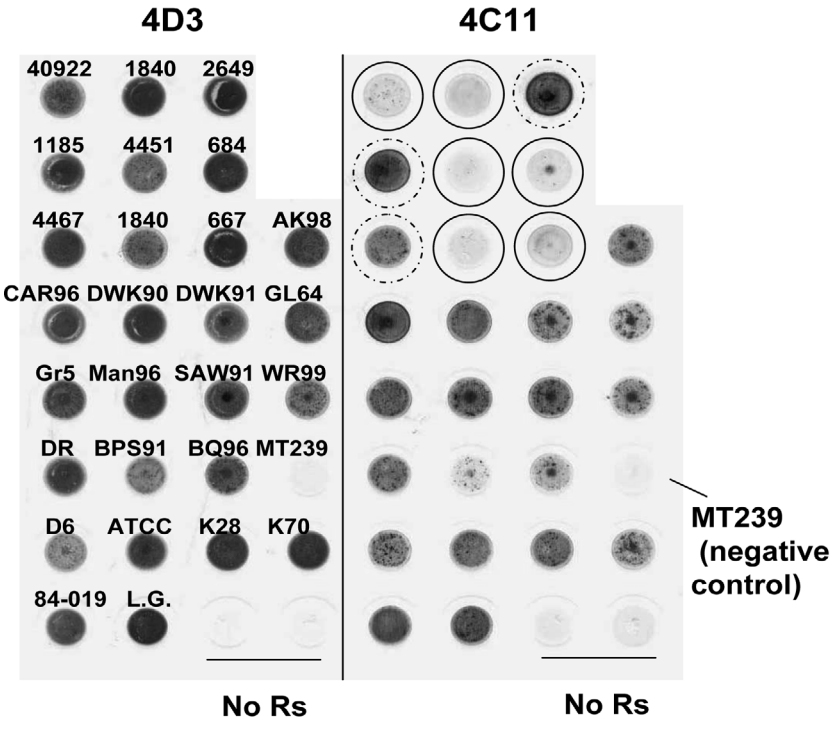

Fig. 2. *Renibacterium salmoninarum* (Rs). Identification of 4C11 binding-loss variants by dot blot. Washed cells were spotted onto nitrocellulose and probed with either MAb 4D3 or 4C11. Strain order is the same on both dot blots. Solid circles identify strains displaying impaired 4C11 binding in comparison to 4D3 binding. Note that 2 preparations of Strain 1840 were tested. Circled strains originated from Norway and the dotted lines identify 3 Norwegian strains that are 4C11 epitope positive. The 2 wells in the bottom right corner of each blot received no bacterial cells and thus served as control for background staining. Similar results were obtained in 2 dot blot experiments using 4D3, and 3 dot blot experiments using 4C11

primers identified a single band that varied in size depending on the strain tested (Fig. 4B). An approximately 300 bp-sized amplified product was obtained from Strain 684 indicating it contained only one repeat that was confirmed by sequencing (Fig. 4B, lane 2, and data not shown). Amplification of all strains that lacked the 4C11 epitope indicated that they also contained only one repeat sequence (Table 1), while all other strains, including the ATCC 33209 type strain, contained 2 tandem repeats (Table 1). In summary, data from both the 16-23S rRNA intervening sequence and the ETR-A locus support a common clonal origin of the antigenic variants isolated from Norway.

DISCUSSION

Renibacterium salmoninarum is a common pathogen of salmonid fish (Fryer & Sanders 1981, Fryer & Lannan 1993) and, as of May 2008, remains on the Group B list of reportable pathogens to the World Organization for Animal Health (OIE). Bacterial isolates have been noted for their similar serological (Getchell et al. 1985, Wiens & Kaattari 1989), biochemical (Bruno & Munro 1986), and genetic profiles (Rhodes et al. 2000). These shared features suggest either a relatively recent dispersal of the pathogen or a slow sequence divergence/recombination rate for this microorganism. We have previously described a strain that produces full-length p57 but is not recognized by the mouse MAb 4C11 (Wiens et al. 2002). In the present study, we extended our examination of antigenic variation in p57 by testing 23 additional strains originating from diverse geographic locations using a panel of MAbs previously demonstrated to recognize distinct epitopes on p57 (Wiens & Kaattari 1991, Wiens et al. 1999, Wiens & Owen 2005). We demonstrate that the epitopes recognized by 4 MAbs, 4D3, 4H8, 3H1 and 1A1, were conserved among all strains tested while the 4D10 epitope was partially disrupted in one isolate from British Columbia. We demonstrated that 5 of 8 strains originating from Norway were 4C11 negative. In addition to expressing p57 lacking the 4C11 epitope, these strains are similar by genomic marker analyses in that they show a similar shift in *msa1 5′ Xho*I fragment, possess the TR1 allele, have a 16-23S rRNA SV4 genotype, and lack *msa3*. These antigenic variants were collected between 1984 and 1987 from Atlantic salmon and brown trout (Table 1) from diverse locations in Norway (Fig. 5B). The 4C11 negative variant was not detected in any of the other strains of European or North American origin. The isolation of the variant strains occurred at a time when an increasing number of outbreaks was detected in Norway (Fig. 5A). In addition, the prevalence of these isolates overlapped in geographic distribution with 4C11 positive variants that, antigenically and genetically, resemble the ATCC 33209 type strain (Fig. 5B). These data are consistent with the clonal expansion of a 4C11 negative variant within Norway. An important caveat of the present study is the relatively small number of examined strains and the lack of more recent isolates from Norway and the Eastern US. In the future, examination of additional strains and data analyses utilizing clustering (multivariate and discriminate analyses) are necessary to quantify the observed differences in order to further understand the genetic

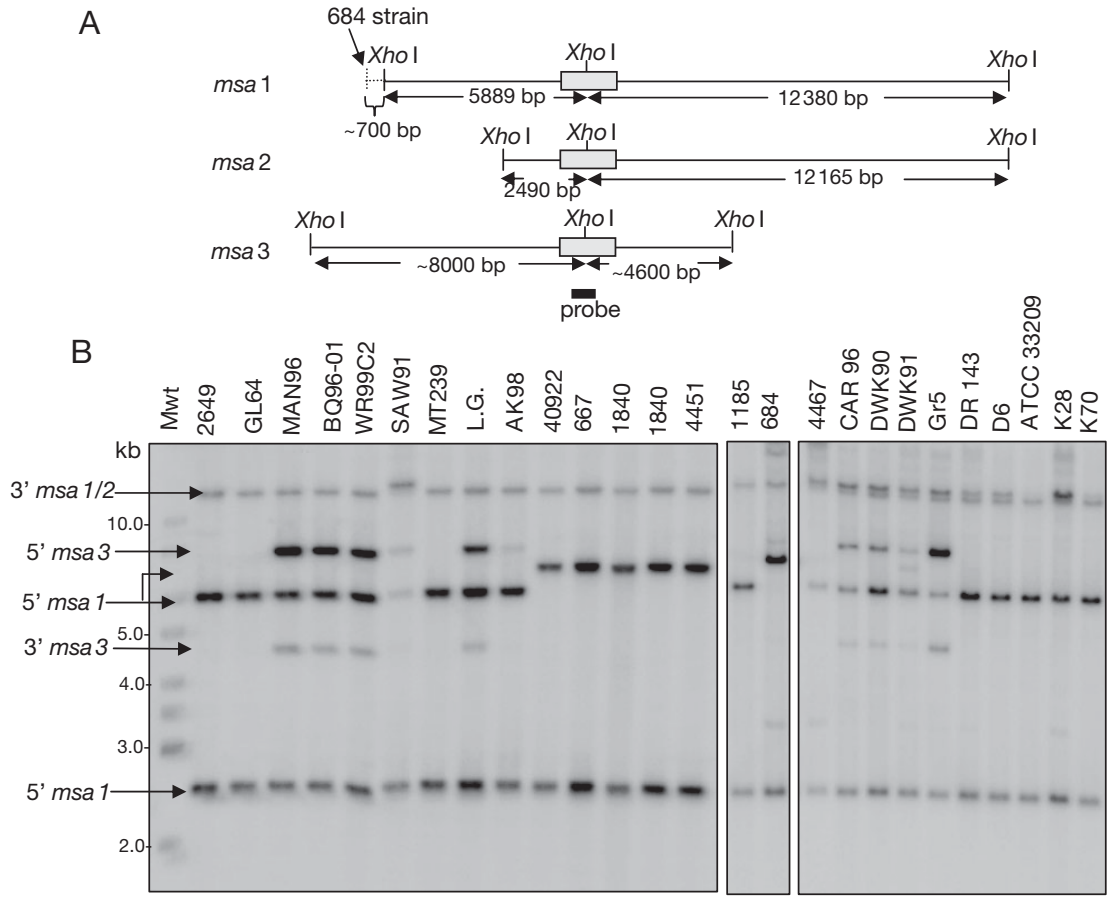

Fig. 3. *Renibacterium salmoninarum*. Most Norwegian isolates have a shifted 5′ *Xho*I site and lack *msa3*. (A) *Xho*I restriction map of *msa1*, *msa2* and *msa3*. The sizes of *msa1* and *msa2 Xho*I fragments were determined from the *R. salmoninarum* ATCC 33209 genome sequence. (B) The *msa3* fragment sizes are estimated from the Southern blot of *Xho*I digested *R. salmoninarum* genomic DNA, probed using an *msa* probe. *Xho*I cleaves once within *msa* genes and the 5′ and 3′ gene fragments can be distinguished based on size. The Southern blot was probed with a 629 nt PCR fragment obtained with primers 7 and 8 (described in Wiens et al. 2002) and labeled with [α-^{32}P]dCTP. This probe binds to both fragments of the *msa* genes. The migration positions of the molecular size (Mwt) markers (in kb) are indicated. Note that Norwegian Strains 40922, 667, 1840, 4451 and 684 have a shifted 5′ *msa1* band as compared to the ATCC 33209 strain, while Norwegian Strains 2649, 1185 and 4467 have a pattern identical to ATCC 33209. The presence of *msa3* is variable among the tested strains

diversity and molecular evolution of *R. salmoninarum*.

The relevance of the 4C11 negative variant for clinical BKD in Norway appears limited at present. BKD was a particular problem in salmonid fish in Norway between 1987 and 1993, with a peak of 61 outbreaks occurring in 1990 (Fig. 5A). A steady decline has occurred since the early 1990s that coincided with increased biosecurity measures and implementation of a MAb-based screening and rigorous culling program to establish BKD-free broodstocks. Between 1999 and 2007, the average number of BKD outbreaks has remained below 2 per year with no reported outbreaks in 2006 (Olsen et al. 2007) or 2007 (O. B. Dale et al. unpubl. data). Since 2005, farms are screened using a similar protocol to that of the European Union infectious haematopoietic necrosis/viral haemorrhagic septicaemia (IHN/VHS) program to document the maintenance of BKD-free stock status once established. The situation for restocking programs is different as the restocking is often based on feral, returning adults and these fish populations need careful monitoring because a low prevalence of BKD cannot be excluded in wild salmonids. The antigenic variants described in the present study were collected between 1984 and 1987 prior to the peak in case reports. The presence of putative clonal isolates may reflect movement of infected fish or gametes. However, management practices appear to have reduced the disease to negligible levels, so that other pathogens are currently of higher concern. In contrast to the effective control of BKD in Norwegian aquaculture, the West Coast of the US has experienced a relatively high prevalence of BKD-infected fish that may reflect the extensive runs of

A

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          A   N   S   L   A   I   L   Q   W   G   A   G   R   I
TC.GCG.AAT.AGC.TTG.GCC.ATT.TTG.CAG.TGG.GGC.GCC.GGG.CGA.ATT

528                                                         544
 D   D   A   V   A   T   A   R   I   A   V   A   A   A   P   D   L
GAC.GAC.GCC.GTA.GCC.ACG.GCC.CGG.ATT.GCC.GTC.GCC.GCA.GCG.CCG.GAC.TTA

545                                                         561
 D   D   A   V   A   T   A   R   I   A   V   A   A   A   P   D   L
GAC.GAC.GCC.GTA.GCC.ACG.GCC.CGG.ATT.GCC.GTC.GCC.GCA.GCG.CCG.GAC.TTA

 V   D   K   E   Q   A   A   V   I   G   G   L   F   G   S   L   F
GTG.GAC.AAA.GAA.CAG.GCC.GCT.GTC.ATT.GGT.GGG.CTC.TTC.GGC.AGC.CTC.TTC

 A   V   A   G   D   D   S   L   I   P   E   A   S   R   L   L   D
GCC.GTG.GCC.GGA.GAT.GAC.TCG.CTC.ATC.CCA.GAA.GCT.AGC.CGA.TTG.CTC.GAC

 A   T   L   A   V   D   T   V   P   A   S   Y   L   T   S   V   L
GCA ACA CTA GCC GTC GAC ACG GTG CCC GCC TCT TAT CTG ACT TCG GTG CTA.CG
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B

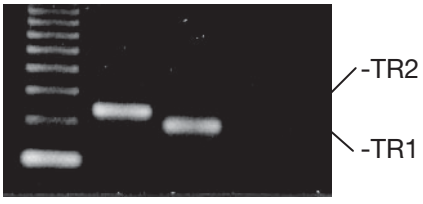

Fig. 4. *Renibacterium salmoninarum*. PCR amplification and genome analysis of the exact tandem repeat ETR-A locus from the 33209 genome. (A) Identification of an open reading frame in the *R. salmoninarum* ATCC 33209 genome sequence matching the sequence from ETR-A. Underlined sequence represents primer locations and the repeated region nucleotide and translated protein sequence is highlighted in bold font. (B) Representative PCR showing the 2 variants obtained. Lane 1: *R. salmoninarum* ATCC 33209; Lane 2: *R. salmoninarum* Strain 684; Lane 3: Control (water)

infected wild fish or a lack of effectiveness of control efforts (Elliott et al. 1989).

Interestingly, we identified 11 strains in the US and Canada that possess a putative third copy of *msa*, termed *msa3*. This finding confirms and extends a previous report that identified *msa3* in 19 of 26 strains (Rhodes et al. 2004). The high prevalence of strains containing *msa3* and the previous correlation with increased virulence suggests that diagnostic assays identifying this variant may have relevance, particularly for regions outside of North America that are negative for this variant. The presence of variable hybridization intensity in Southern blots indicates that *msa3* likely resides on a low-copy number extra-

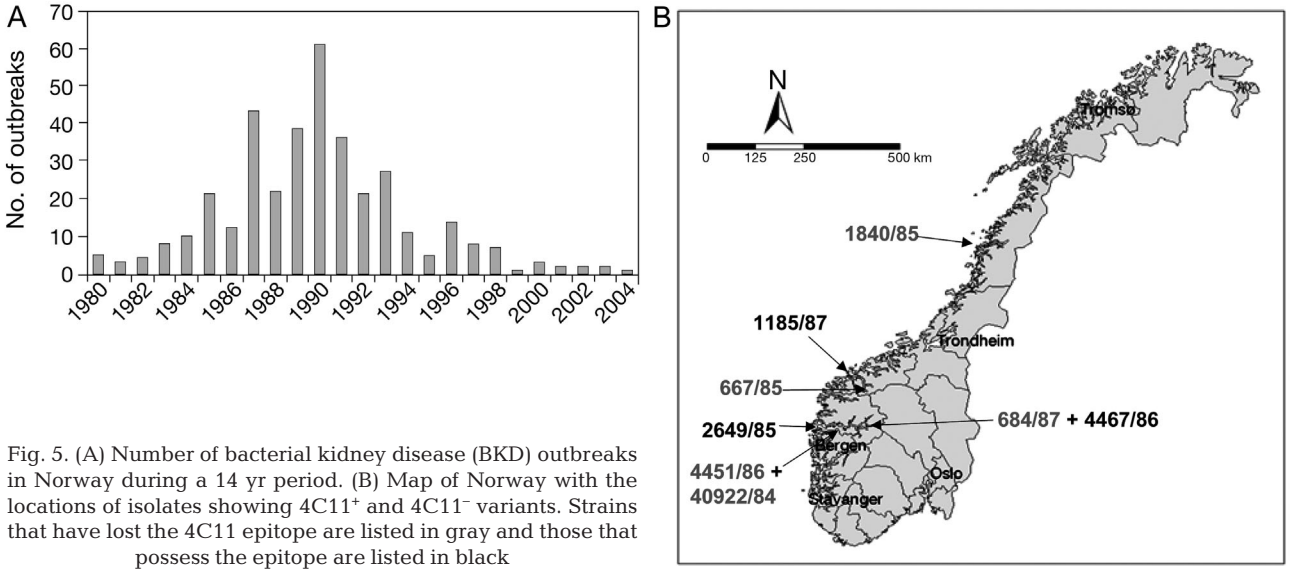

Fig. 5. (A) Number of bacterial kidney disease (BKD) outbreaks in Norway during a 14 yr period. (B) Map of Norway with the locations of isolates showing $4C11^+$ and $4C11^-$ variants. Strains that have lost the 4C11 epitope are listed in gray and those that possess the epitope are listed in black

chromosomal element. The presence of *msa3* residing on a plasmid is not unexpected as the closely related genome-sequenced microorganisms *Arthrobacter aurescens* TC1 and *Arthrobacter* sp. FB24 contain multiple large-sized, low-copy number plasmids (Mongodin et al. 2006). The presence of numerous IS994 elements in the genome and also flanking the *msa1* and *msa2* genes suggests that recombination may be involved in the putative gene duplication (O'Farrell & Strom 1999, Rhodes et al. 2000, Wiens et al. 2008). However, further DNA sequence and transcriptional analyses are needed to resolve the origin and relationship of the putative third *msa* gene to the *msa1* and *msa2* genes, and whether *msa3* is functionally active or a pseudogene.

The ability to discriminate the presence or absence of the 4C11 epitope adds to a growing number of molecular techniques for typing *Renibacterium salmoninarum*. These include using randomly amplified polymorphic DNA and rRNA intergenic spacer sequences (Grayson et al. 1999), the ETR-A loci (Grayson et al. 2001), and IS994 profiles (Rhodes et al. 2000, Alexander et al. 2001). The availability of the complete genome sequence of the ATCC 33209 strain (GenBank accession number NC_010168) now provides a starting point for understanding the molecular basis of some of these differences (Wiens et al. 2008). In the present study, we identified the ETR as part of an open reading frame, designated RSal33209_2187, encoding a 955 aa protein with an unknown function. The open reading frame encoding RSal33209_2187 is located immediately upstream of the 2 DNA-directed RNA polymerase β-chain genes, which are genes that are highly conserved in all microorganisms. The gene downstream of RSal33209_2187 is a chaperone encoding gene, *htp*G, a gene organization which is found in both *Streptomyces coelicolor* A3 and *Thermomonospora fusca* YX. Interestingly, homologues of the *R. salmoninarum* RSal33209_2187 protein were not identified in either of the closely related and completely sequenced *Arthrobacter aurescens* TC1 and *Arthrobacter* sp. FB24 genomes (Mongodin et al. 2006). The functional role of the RSal33209_2187 protein and the contribution of the 17 aa exact duplication remains to be determined.

In summary, we demonstrate a number of molecular differences between *Renibacterium salmoninarum* strains. It is important to note that the strains used in the present study were isolated in the 1980s and 1990s and further efforts are needed to characterize more recent isolates. The availability of a completed genome sequence now provides a template for full genome comparisons that will provide the basis for the next generation of technologies for strain discrimination. We also stress the importance of strain isolation and documentation of BKD outbreaks and the long-term storage of such strains either as −80°C frozen or lyophilized cultures. Such isolates will allow future reconstruction of *R. salmoninarum* evolution and possible connections with fish transport. This information may have practical relevance to fish health workers responsible for developing policies to control BKD. The example of the successful reduction of BKD outbreaks in Norway suggests that management of this disease is possible under some conditions. The event of rapid genome sequencing also opens up the opportunity of much deeper understanding of both the adaptive and non-adaptive evolutionary forces constraining the salmonid host and bacterial pathogen interaction.

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