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A Single Ala^{139}-to-Glu Substitution in the *Renibacterium salmoninarum* Virulence-Associated Protein p57 Results in Antigenic Variation and Is Associated with Enhanced p57 Binding to Chinook Salmon Leukocytes

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The gram-positive bacterium *Renibacterium salmoninarum* produces relatively large amounts of a 57-kDa protein (p57) implicated in the pathogenesis of salmonid bacterial kidney disease. Antigenic variation in p57 was identified by using monoclonal antibody 4C11, which exhibited severely decreased binding to *R. salmoninarum* strain 684 p57 and bound robustly to the p57 proteins of seven other *R. salmoninarum* strains. This difference in binding was not due to alterations in p57 synthesis, secretion, or bacterial cell association. The molecular basis of the 4C11 epitope loss was determined by amplifying and sequencing the two identical genes encoding p57, *msa1* and *msa2*. The 5′ and coding sequences of the 684 *msa1* and *msa2* genes were identical to those of the ATCC 33209 *msa1* and *msa2* genes except for a single C-to-A nucleotide mutation. This mutation was identified in both the *msa1* and *msa2* genes of strain 684 and resulted in an Ala^{139}-to-Glu substitution in the amino-terminal region of p57. We examined whether this mutation in p57 altered salmonid leukocyte and rabbit erythrocyte binding activities. *R. salmoninarum* strain 684 extracellular protein exhibited a twofold increase in agglutinating activity for chinook salmon leukocytes and rabbit erythrocytes compared to the activity of the ATCC 33209 extracellular protein. A specific and quantitative p57 binding assay confirmed the increased binding activity of 684 p57. Monoclonal antibody 4C11 blocked the agglutinating activity of the ATCC 33209 extracellular protein but not the agglutinating activity of the 684 extracellular protein. These results indicate that the Ala^{139}-to-Glu substitution altered immune recognition and was associated with enhanced biological activity of *R. salmoninarum* 684 p57.

Renibacterium salmoninarum is a gram-positive bacterial pathogen that causes significant losses of salmonid fish (reviewed in references 22 and 50). This bacterium is prevalent throughout most regions of the world where salmon live wild or are cultured. *R. salmoninarum* causes a chronic bacteremia termed bacterial kidney disease that is characterized by focal lesions in the viscera, particularly the kidney (22, 50). Bacterial kidney disease is difficult to control as *R. salmoninarum* is a slowly growing, facultatively intracellular pathogen (27, 54) that is transmitted from parent to progeny via intracellular infection of eggs (8, 10, 18), as well as from fish to fish during cohabitation (32). Successful control of bacterial kidney disease has been achieved in part through extensive chemotherapy combined with culling of infected broodstock (reviewed in reference 16).

Diagnosis of *R. salmoninarum* infection in salmon is often based on detection of the 57-kDa (p57) protein, mRNA, or DNA (6, 11, 13, 34, 37, 41). p57 is a good marker of active infection as this protein is the predominant cell surface and secreted protein produced by *R. salmoninarum* (23, 26, 51). However, little is known about whether antigenic variation exists in p57 or how variation may affect the biological functions of p57.

The gene encoding p57 has been cloned and designated *msa* (major soluble antigen) (12), and two copies of the *msa* gene have been identified in the *R. salmoninarum* genome (36). The *msa1* and *msa2* genes have identical coding regions and are both present in all strains of *R. salmoninarum* that have been examined (36). The *msa1* and *msa2* gene sequences diverge 40 bp 5′ to the open reading frame, while sequences 3′ to the open reading frame are essentially identical for at least 225 bp, except for a single base substitution 37 nucleotides (nt) downstream of the stop codon (36).

While the precise role of p57 in pathogenesis is unclear, we and other workers have demonstrated that secreted p57 has both agglutinating and immunomodulatory activities (7, 21, 42, 47, 52). p57 concentrated from bacterial culture supernatant binds and agglutinates salmonid leukocytes, as well as red blood cells from a number of mammalian species (14, 52). p57 does not agglutinate salmonid erythrocytes or mouse leukocytes, suggesting that binding occurs via a specific receptor(s). Two murine monoclonal antibodies (MAbs), MAbs 4C11 and 4H8, block the leukocyte-agglutinating activity, while the same antibodies, as well as MAb 4D3, also block p57-mediated agglutination of rabbit red blood cells (52). Furthermore, MAbs 4C11, 4H8, and 4D3 recognize epitopes on the amino-terminal portion of p57 since these antibodies bind a recombinant amino-terminal fragment but do not bind a recombinant carboxy-

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terminal fragment of p57 (48). These data suggest that the leukocyte-binding domain(s) may be associated with the amino-terminal portion of p57. At present it is unclear if these antibodies inhibit one leukocyte-binding site or multiple binding sites.

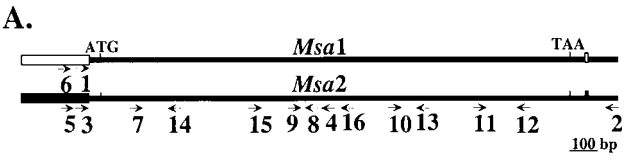
In this study we screened natural isolates of *R. salmoninarum* using MAbs to determine whether antigenic variation occurs in p57 and to facilitate characterization of the precise epitopes recognized by the agglutination-blocking MAbs. We describe a Norwegian *R. salmoninarum* isolate, strain 684, that produces p57 but lacks the 4C11 epitope. Full-length p57 is produced, and only a single nucleotide substitution was identified in the coding regions of both the *msa1* and *msa2* genes. p57 isolated from 684 has an increased capacity to bind leukocytes, indicating that the mutation, in addition to altering an antigenic epitope, is also associated with enhanced functional activity. To our knowledge, this is the first molecular characterization of antigenic variation produced by mutations in the *msa* genes of *R. salmoninarum*.

MATERIALS AND METHODS

Animals. Chinook salmon (*Oncorhynchus tshawytscha*) were maintained in sand-filtered, UV-treated lake water at the Western Fisheries Research Center, Seattle, Wash.

***R. salmoninarum* strains and culture.** *R. salmoninarum* isolates K50, K70, K28, Little Goose, and D6 were provided by C. Banner (Department of Fish and Wildlife, Corvallis, Oreg.) and are recognized by anti-p57 MAbs 4D3 and 2G5 (51). *R. salmoninarum* strains ATCC 33209 and ATCC 33739 were obtained from the American Type Culture Collection (Manassas, Va.). *R. salmoninarum* isolate 684 was provided by O. B. Dale (National Veterinary Institute, Oslo, Norway). Isolate 684 was isolated from a clinically diseased brown trout (*Salmo trutta*) that was obtained from a hatchery in Aurland Sognefjord, Norway. The hatchery had experienced previous clinical outbreaks of bacterial kidney disease, and the Gram stain results, growth characteristics, and API-ZYM test results for isolate 684 were consistent with classification of this organism as *R. salmoninarum* (O. B. Dale, personal communication). Frozen (−70°C) aliquots of *R. salmoninarum* strains were initially cultured in KDM-2 medium containing 10% fetal bovine serum (17). For large-scale culture, starter cultures of ATCC 33209 and 684 were first prepared from freezer stocks and grown in KDM-2 broth medium modified so that it contained 0.05% (wt/vol) cysteine and no fetal bovine serum. The cultures were stirred continuously while they were incubated at 15°C for 10 days. Bacteria were enumerated by using the membrane filtration-fluorescent antibody technique (15), and equivalent numbers of *R. salmoninarum* ATCC 33209 and 684 were inoculated into separate 1.25-liter volumes of the modified KDM-2 broth medium. In the first experiment (preparation 1), the initial inoculum densities were 1×10^5 cells ml^{-1}, and in the second experiment (preparation 2), the initial inoculum densities were 8×10^3 cells ml^{-1}. Cultures were grown for an additional 12 days (preparation 1) or 14 days (preparation 2), and the numbers of viable bacteria were determined by plate counting. To harvest cultures, cells were pelleted by centrifugation at 5,000 × *g*, and both cell pellets obtained at 4°C and supernatants were frozen at −70°C.

Chromosomal DNA isolation and Southern blotting. Genomic DNA was prepared by using a modified procedure described in the Molecular Biology Protocols (U.S. Department of Commerce/NOAA/NMFS/NWFSC website at http://www.nwfsc.noaa.gov/protocols/GramPosDNA.html) that was originally adapted from the work of Flamm et al. (20). Briefly, 100 μl of a 500-mg (wet weight) ml^{-1} suspension of bacterial cells was added to 0.9 ml of 0.01 M sodium phosphate-buffered saline (PBS) containing 20% sucrose, 2.5 mg of lysozyme (Sigma), and 1 mM EDTA and incubated overnight at 37°C. Nine milliliters of lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10 mg of proteinase K [Gibco BRL, Grand Island, N.Y.] ml^{-1}, 1% sodium dodecyl sulfate [SDS]) was added, and the preparation was incubated for 3 h at 37°C. DNA was extracted with phenol-chloroform, ethanol precipitated, isolated by using a glass rod, and resusupended in 10 mM Tris-HCl–1 mM EDTA. For Southern blotting, genomic DNA was cut with *Bam*HI, *Hin*dIII, *Eco*RI, and *Xho*I restriction endonucleases for 6 h at 37°C. Digested DNA (7 μg) was resolved on a 0.75% agarose gel for 21 h. DNA was transferred to a nitrocellulose membrane and heated for 45 min at 80°C as described previously (43). A probe comprising a 629-nt PCR fragment obtained by using primers 7 and 8 (Fig. 1) was labeled with [α-^{32}P]dCTP by using a random hexamer labeling kit (Roche) according to the manufacturer's directions (19). Southern hybridization and washes of the blot were performed by standard methods (43).



B.

Name	Sequence (5' to 3')
1. MSA#1A-For	GTT.GAA.CGA.GCG.GAG.ATG.G
2. MSA#1B-Rev	CGG.CGT.TGC.CGT.CTT.ACC
3. MSA#2A-For	GTT.TTC.GAC.AAC.GGG.ATG.G
4. MSA#1C-Rev	CGT.CGA.ATA.GGT.TGC.CGC.C
5. MSA#2B-For	ATC.CGG.CAG.CAA.TCC.CGG.CC
6. MSA#1D-For	GTC.TCC.GCT.CGT.TGC.AGA.GC
7. RS EcoRI-For	GAA.TCT.TCC.ACT.TCA.ACA.G
8. RS EcoRI-Rev	AGT.TGC.TGT.CGG.GGC.GCT
9. RS Seq #1For	AAC.CCC.GGT.GGG.GAG.ACG
10. RS Seq #2For	GGT.AAA.TGG.TGG.TCT.GGC
11. RS Seq #3For	GCC.AAT.CCG.CCT.GTT.TCC
12. RS Seq #4Rev	CTC.ACG.ATG.TAA.CCC.AAC
13. RS Seq #5Rev	GGG.ATT.ACC.AAA.AGC.AAC
14. RS Seq #6Rev	CCC.TTT.TTA.CCT.AAA.TCA.CC
15. RS Seq #7For	GGA.TGG.CAG.CAA.CCT.ATT.CG
16. RS Seq#8Rev	GTC.TCC.GAA.GGT.AAC.TCT.CG

FIG. 1. (A) Linear depiction of the *msa1* and *msa2* genes of *R. salmoninarum* ATCC 33209. The 5′ region and the 3′ single nucleotide that are different in the ATCC 33209 *msa1* and *msa2* genes are indicated by boxes. (B) Primers used for PCR and sequencing of p57 *msa1* and *msa2*. Primers 1, 3, 5, and 6 are specific for either *msa1* or *msa2*, while all other primers do not distinguish between the known *msa* gene sequences. Translational start (ATG) and stop (TAA) sites are indicated.

ECP preparation. Extracellular protein (ECP) was concentrated from medium by ultrafiltration followed by ammonium sulfate precipitation as previously described (52). The predominant components of ECP are intact p57 and associated proteolytic degradation fragments, as determined by SDS-polyacrylamide gel electrophoresis (PAGE) (42, 52). Total protein was determined by using the Bradford protein assay (Pierce, Rockford, Ill.) with bovine serum albumin as the standard. p57 was filter sterilized (pore size, 0.22 μm) and stored at −70°C or kept at 4°C for immediate use.

Amplification and sequencing of *msa1* and *msa2*. The entire coding sequences of the *msa1* and *msa2* genes were amplified from *R. salmoninarum* genomic DNA by using 5′ region forward primer 1, 3, 5, or 6 in combination with 3′ reverse primer 2 (Fig. 1). PCR amplification was carried out by using high-fidelity *PfuTurbo* polymerase as recommended by the manufacturer (Stratagene, La Jolla, Calif.). PCR amplification cycles (95°C for 5 min, followed by 30 cycles of 95°C for 40 s, 58°C for 40 s, and 72°C for 4 min) and final extension for 10 min at 72°C were performed with a Perkin-Elmer 9700 thermocycler. Reactions were carried out in a total volume of 50 μl. PCR products were resolved on a 1% agarose gel (Metaphor agarose; BioWhittaker Molecular Applications, Rockland, Maine) and were purified by QIAquick gel extraction (Qiagen Inc., Valencia, Calif.). Four 5′ primers (primers 1, 3, 5, and 6) and an internal 3′ region reverse primer (primer 4) were used to sequence the *msa1* and *msa2* PCR amplicons directly from the 5′ region through 726 nt of the coding region. Subsequently, the entire coding sequence of 684 *msa1* and *msa2* was determined by sequencing both strands of cloned *msa1* and *msa2* genes with primers 1 to 4 and 7 to 15 (Fig. 1). Nucleotide sequencing was performed by using an ABI Prism terminator cycle sequencing kit and AmpliTaq DNA polymerase according to the manufacturer's directions (Applied Biotechnology, Inc., Foster City, Calif.). Reactions were performed with an Applied Biosystems 377 PRISM automated DNA sequencer (PE Applied Biosystems, Foster City, Calif.) at the Nucleic Acid Core Facility of the Department of Molecular Microbiology and Immunology at Oregon Health and Science University, Portland.

Cloning of *msa1* and *msa2* from strain 684. *msa1* and *msa2* PCR products were ligated into the pT7Blue-1 vector (Novagen, Madison, Wis.) and transformed into NovaBlue Singles Competent *Escherichia coli* according to the manufacturer's directions (Novagen).

MAbs. MAbs 3H1, 4D3, 4C11, and 4H8 bind p57 and have been described previously (52). Cells were grown in BALB/c mice as ascites, and antibody was purified by using protein A-Sepharose.

Gel electrophoresis. SDS-PAGE was performed as previously described (53).

Dot and Western blotting. Frozen *R. salmoninarum* cells were thawed, pelleted, washed in 10 mM PBS (pH 7.4), and finally resuspended in an equal volume of PBS. For dot blotting, bacterial cells were diluted 1:500 in PBS, and 100 μl of the suspension was applied to a dot blot apparatus and incubated overnight at 17°C. Wells were blocked with 3% bovine serum albumin–Tris-buffered saline (pH 8.0) for 1 h at room temperature. Wells were washed three times with 150 μl of Tris-buffered saline, 100 μl of a 10-μg ml^{-1} solution of primary antibody was added, and the preparation was incubated for 1 h at room temperature. Wells of the dot blot were washed three times, and the nitrocellulose was removed. Dot blots were incubated in a 1:500 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin (HyClone, Logan, Utah) for 1 h at room temperature. After three washes, blots were incubated with a 4-chloro-1-naphthol substrate (Bio-Rad Laboratories, Hercules, Calif.).

Leukocyte agglutination assays. Salmonid anterior kidney leukocytes were isolated and separated by using 51% Percoll as previously described (1). About 1×10^6 cells were applied per well in 10% fetal bovine serum in a total volume of 100 μl. Leukocytes were incubated with twofold dilutions of p57 (200 to 1.6 μg ml^{-1}). While agglutination occurred rapidly, the minimum concentration of p57 that exhibited agglutinating activity was determined after 24 h by microscopic examination. For inhibition experiments, protein A-purified MAb 4C11 or control antibody PCG1-1 [immunoglobulin G2b(κ) (49)] was preincubated with ECP prior to addition of cells.

p57 leukocyte binding assay. Peripheral blood or anterior kidney leukocytes (5×10^6 cells) in tissue culture medium containing 20% fetal bovine serum were incubated with 25 μg of p57 for 1 h at 4°C in a total volume of 150 μl. The cells were washed three times with tissue culture medium (1 ml) and then lysed with a buffer containing 1% bovine serum albumin, 0.05% Tween 20, and 0.25% NP-40 in PBS for 30 min. The lysates were heated for 5 min at 85°C to release bound p57, and the insoluble material was pelleted by centrifugation ($14,000 \times g$ for 2 min). A quantitative enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of soluble p57 in the supernatant.

Quantitative ELISA for p57. A quantitative ELISA for p57 was performed as described previously (41), with the following modifications. Briefly, half-area microplate wells were coated with 50 μl of a 5-μg ml^{-1} MAb 3H1 solution, and following the antigen incubation step wells received 50 μl of a 1-μg ml^{-1} biotinylated MAb 4D3 solution. The biotinylated MAb was detected by using a 1:500 dilution of streptavidin-alkaline phosphatase (Southern Biotechnology, Birmingham, Ala.) and incubation with a *p*-nitrophenylphosphate substrate (Sigma).

Nucleotide sequence accession numbers. Sequences of the cloned strain 684 *msa1* and *msa2* genes have been deposited in the GenBank database under accession numbers AF458101 and AF458102.

RESULTS

Epitope variation in *R. salmoninarum*. We examined whether antigenic variation exists in bacterial cell-associated p57 by probing bacteria with four MAbs that recognize spatially separate epitopes. Eight strains of *R. salmoninarum*, obtained from geographically diverse locations, were reacted with MAbs 3H1, 4D3, 4H8, and 4C11 by using the dot blot assay (Fig. 2). MAb 3H1 recognizes an epitope localized in the central region of p57 (52), while MAbs 4D3, 4H8, and 4C11 recognize distinct epitopes located in the amino-terminal region of p57 (48). Notably, Norwegian *R. salmoninarum* strain 684 lacked the 4C11 epitope, while all other strains contained epitopes recognized by the 3H1, 4D3, 4H8, and 4C11 MAbs. To determine if the loss of the 4C11 epitope was due to a large deletion in p57 or due to occlusion with other *R. salmoninarum* cellular constituents, we conducted Western blot experiments in which bacterial lysates were first resolved by using reducing SDS-PAGE and then probed with MAbs 4D3, 4H8, and 4C11 (Fig. 3). p57 produced by strain 684 exhibited the same relative migration as p57 produced by ATCC 33209, indicating that full-length protein was produced by strain 684 (Fig. 3A and B). Furthermore, the quantities of 684 p57 produced appeared to be similar to the quantities of ATCC 33209 p57 produced, as judged by the intensities of MAb 4D3 and 4H8 immunoreactivities (Fig. 3A and B) and Coomassie blue protein staining (data not shown). In agreement with the dot immunoblotting results, MAb 4C11 failed to bind 684 p57 as determined by Western blotting, while MAb 4C11 bound ATCC 33209 p57 (Fig. 3C). The failure of MAb 4C11 to bind 684 p57 under reducing and denaturing conditions suggested that the 4C11 epitope was directly altered rather than occluded by noncovalently associated *R. salmoninarum* cellular constituents.

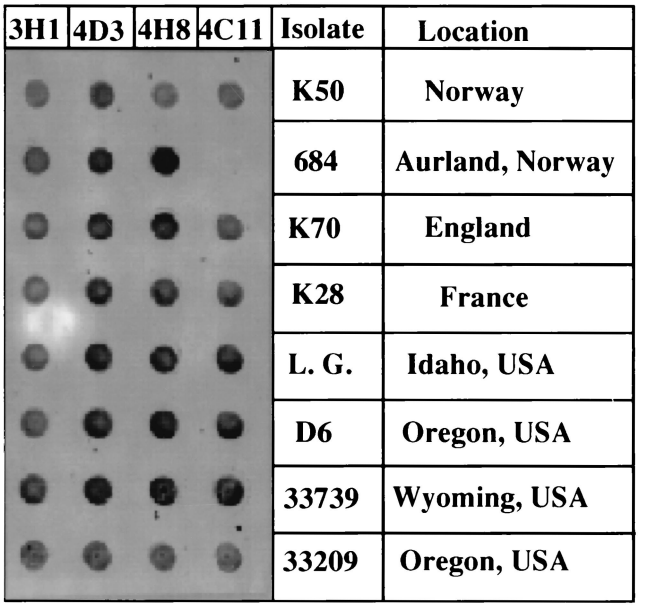


FIG. 2. Screening *R. salmoninarum* isolates with MAbs 3H1, 4D3, 4H8, and 4C11. Washed bacterial cells were dot blotted onto nitrocellulose and individually probed with 10 μg of each MAb ml^{-1}. Binding was detected with peroxidase-conjugated goat anti-mouse immunoglobulin. The designation and origin of each isolate are indicated on the right.

Both the *msa1* and *msa2* genes are present in strain 684. All strains of *R. salmoninarum* that have been examined contain two identical copies of the *msa* gene (36). At present, it is not known whether one or both of the *msa* genes are transcribed. Antigenic variation could be due to exclusive expression of one mutated gene. Alternatively, the same mutation may be present in both genes. To distinguish between these possibilities, we first examined whether the *msa1* and *msa2* genes were present in the genome of strain 684 by performing PCR amplification with gene-specific primers. The 5′ regions of *msa1* and *msa2* diverge 40 nt upstream of the transcription start site (36); thus, primers were designed that specifically hybridize to the 5′ region of either *msa1* (primers 1 and 6) (Fig. 1) or *msa2* (primers 3 and 5) (Fig. 1). Because only a single nucleotide difference in the noncoding region is known to exist between the *msa1* and *msa2* genes (36), a 3′ reverse primer (primer 2) (Fig. 1) that recognizes both *msa1* and *msa2* was used in all

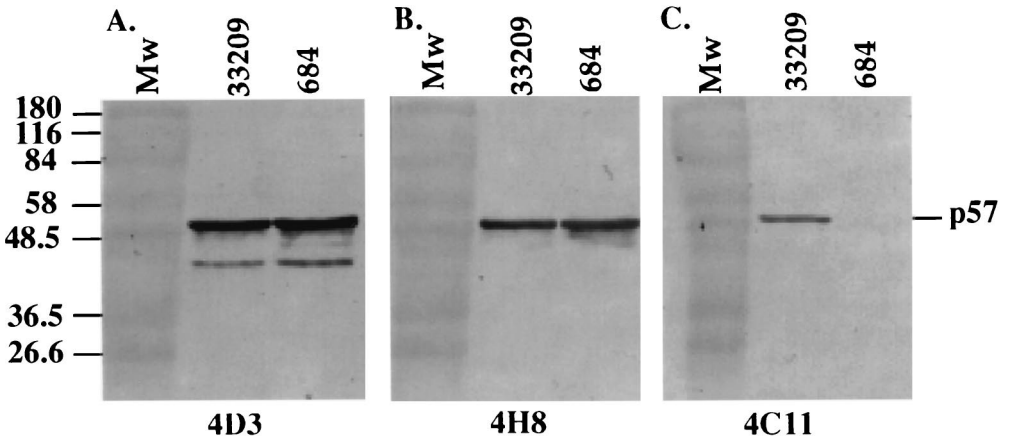


FIG. 3. *R. salmoninarum* strains 684 and ATCC 33209 express similar amounts of p57 with indistinguishable electrophoretic migration positions as determined by Western blotting with MAbs 4D3 (A) and 4H8 (B). p57 from strain 684 lacks the 4C11 epitope (C). Equal amounts of *R. salmoninarum* cells were boiled in reducing SDS-PAGE sample buffer, separated on an SDS–12% PAGE gel, transferred to nitrocellulose overnight, and subjected to immunoblotting with 5 μg of MAb 4D3, 4H8, or 4C11 ml^{-1}. Peroxidase-conjugated goat anti-mouse immunoglobulin was used to detect MAb binding. The molecular masses (in kilodaltons) of prestained markers (lanes Mw) are indicated on the left. The results are representative of one of three experiments.

amplifications. Genomic DNA from either ATCC 33209 or 684 was subjected to 30 cycles of PCR amplification with the high-fidelity *TurboPfu* polymerase. A PCR product corresponding to the predicted 1.87-kb fragment was generated from both *R. salmoninarum* ATCC 33209 and 684 DNA with either *msa1*-specific primers (Fig. 4A) or *msa2*-specific primers (Fig. 4B), indicating that both genes are present in both strains. The specificity of the primers for either *msa1* or *msa2* was demonstrated by the fact that *msa1* primers amplified cloned *msa1* but not cloned *msa2*, while *msa2* primers amplified cloned *msa2* but not the *msa1* gene when a range of input plasmid concentrations was used (Fig. 4C). To confirm the presence and examine the genomic organization of the *msa* genes, Southern blotting was performed with a 5′ region *msa* gene probe. Genomic DNA from ATCC 33209 and 684 were cut with restriction enzymes *Bam*HI, *Hin*dIII, *Eco*RI, and *Xho*I and probed with a 629-nt probe amplified from ATCC 33209 by using primers 7 and 8 (Fig. 1). In agreement with the data of O'Farrell and Strom (36), *Bam*HI digestion of ATCC 33209 genomic DNA produced 7.2-kb (*msa2*) and 5.3-kb (*msa1*) hybridizing bands. *Bam*HI digestion of 684 genomic DNA produced bands with migration positions identical to those of ATCC 33209 bands. Similarly, *Hin*dIII digestion produced 5.4-kb (*msa2*) and 2.8-kb (*msa1*) hybridizing bands that were present in both ATCC 33209 and 684 digests. *Eco*RI digestion of ATCC 33209 and 684 DNA produced a single 1.8-kb band known to contain the entire coding sequence of both *msa1* and *msa2* except for 92 nt of the 5′ coding sequence. These data confirm that both full-length *msa1* and full-length *msa2* are present in the genome of *R. salmoninarum* 684, in agreement with PCR data. While the restriction enzyme digestion patterns for the genomic DNA are similar for the two strains, they are not identical, as digestion of ATCC 33209 DNA with *Xho*I produced a 6.2-kb band, while digestion of 684 DNA produced a 6.9-kb hybridizing band. A third band, at ~14 kb, was likely a product of cross-hybridization of the 5′ probe to the second A repeat present in both the *msa1* and *msa2* genes, as a 3′ probe amplified from ATCC 33209 by using primers 11 and 2 hybridized only with the ~14-kb band (Wiens, unpublished data). Taken together, these data demonstrate that the *msa1* and *msa2* genes are present in isolate 684 and that the flanking DNA sequence of the *msa1* and *msa2* genes in isolate 684 is similar, but not identical, to the flanking DNA sequence in ATCC 33209, as judged by restriction enzyme analysis.

Genetic basis of the loss of the 4C11 epitope. The molecular basis of the loss of the 4C11 epitope was determined by directly sequencing PCR-amplified *msa1* and *msa2* genes. Since the location of the 4C11 epitope was previously demonstrated to be in the first 243 amino acids (48), the sequences of the 5′ noncoding regions through amino acid 243 were determined by direct sequencing of 684 *msa1* and *msa2* PCR amplicons (Fig. 5 and data not shown) obtained by using four independent primers unique to the divergent 5′ region of each gene (primers 1, 3, 5, and 6) in combination with the 3′ region primer (primer 2) that binds to a sequence common to the two genes. (Fig. 1). A single C-to-A nucleotide difference in the coding region was identified that resulted in an Ala^{139}-to-Glu amino acid substitution in both the *msa1* and *msa2* gene products (Fig. 5). The unique sequence in the 5′ region of each PCR product confirmed the identity as either the *msa1* or *msa2* gene. Amplification and direct sequencing of the ATCC 33209 *msa1* and *msa2* genes that were amplified in parallel with the 684 *msa* genes did not reveal any differences from the reported sequences, indicating the fidelity of the amplification and sequencing process. The presence of the single mutation in both the *msa1* and *msa2* genes from strain 684 was confirmed by cloning the full-length 684 *msa1* and *msa2* genes and sequencing the entire coding regions, as well as the immediate 5′ and 3′ flanking regions. The single C-to-A mutation was present in both cloned genes, while the 5′ region and the rest of the coding sequence were identical to those of the corresponding ATCC 33209 *msa1* and *msa2* genes. Interestingly, the single nucleotide difference between the ATCC 33209 *msa1* and *msa2* genes, located 37 nt downstream of the stop codon (Fig.

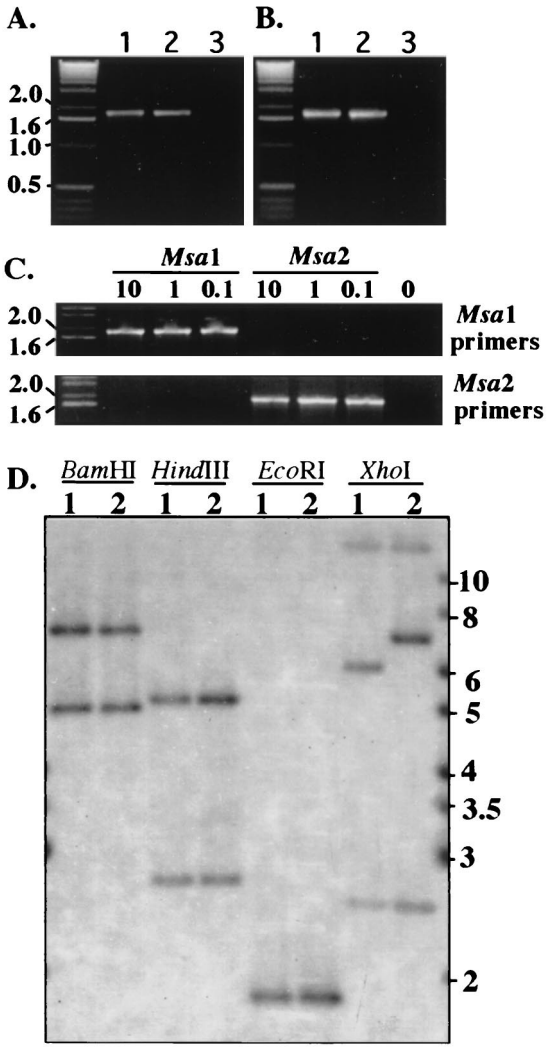


FIG. 4. Strain 684 contains both *msa1* and *msa2* as determined by PCR (A to C) and Southern blotting (D). (A and B) PCR was performed as described in Materials and Methods by using primers 1 and 2 to amplify *msa1* (A) and primers 3 and 2 to amplify *msa2* (B). The target DNA in PCR amplifications were as follows: lanes 1, 10 ng of ATCC 33209 genomic DNA; lanes 2, 10 ng of 684 genomic DNA; lanes 3, control (no DNA). (C) PCR amplification performed with either *msa1*- or *msa2*-specific primers and 0.1 to 10 ng of target plasmid DNA containing either a cloned *msa1* gene or a cloned *msa2* gene. (D) Southern blotting of genomic DNA from strain ATCC 33209 (lane 1) or strain 684 (lane 2) that was probed with a 629-nt PCR fragment obtained with primers 7 and 8 and labeled with [α-^{32}P]dCTP. The migration positions of the molecular size markers (in kilobases) are indicated on the left in panels A through C and on the right in panel D.

1), was not present in 684. The isolate 684 *msa1* and *msa2* genes both contained a C at this position, like the ATCC 33209 *msa2* gene. A cytosine at this position in both *msa1* and *msa2* of isolate 684 was also confirmed by direct sequencing of PCR products (data not shown).

Agglutinating activity of p57 from 684. To investigate whether p57 from strain 684 has altered biological activity, we prepared two independent ECP preparations from strains 684 and ATCC 33209 grown under identical conditions. Both protein preparations were tested for binding and agglutinating activities by using salmonid leukocytes and rabbit red blood cells. Surprisingly, ECP from strain 684 exhibited an approximately twofold increase in agglutinating activity for both chinook salmon anterior kidney leukocytes (Table 1) and rabbit erythrocytes (data not shown). The minimum leukocyte-agglutinating concentration of 684 ECP was 12.5 μg ml^{-1}, while the minimum leukocyte-agglutinating concentration of ATCC 33209 ECP was 25 μg ml^{-1}. To confirm these results, a quantitative binding assay was performed. In this assay, ECP was incubated with leukocytes, unbound proteins were removed by washing, and the amount of bound p57 was determined by a quantitative ELISA that specifically detects p57. Significantly more strain 684 p57 (89 $\pm$ 11 ng; $n = 3$) than strain ATCC 33209 p57 (60 $\pm$ 6 ng; $n = 3$) was bound per 1×10^6 leukocytes in 1 h (Fig. 6A). A twofold increase in strain 684 binding activity to rabbit red blood cells was also observed with this assay (Fig. 6B). The difference in p57 binding activity was not due to differences in the amounts of p57 in the extracellular preparations as measured by a p57-specific ELISA (Fig. 6C) and by SDS-PAGE (data not shown). In addition, ELISA analysis confirmed the lack of the 4C11 epitope on p57 isolated from strain 684 and the presence of the 4C11 epitope on p57 from ATCC 33209 (Fig. 6D). Binding and agglutination were specific to p57 as MAb 4C11 inhibited leukocyte agglutination mediated by ATCC 33209 ECP, while MAb 4C11 did not inhibit agglutination mediated by 684 ECP (Table 2). This indicates that the loss of the 4C11 epitope on p57 resulted in a functional inability of MAb 4C11 to block agglutinating activity. A control MAb, MAb PCG1-1, did not inhibit leukocyte agglutination mediated by either ATCC 33209 or 684 ECP, indicating that the inhibition by MAb 4C11 of ATCC 33209 ECP was specific. These data indicate that p57 produced by strain 684 has greater binding activity per microgram of protein than an equivalent amount of p57 produced by ATCC 33209 and suggest that the enhanced activity is due to the single Ala^{139}-to-Glu substitution.

DISCUSSION

R. salmoninarum infects salmonid fish over a wide geographic area, including North America, South America, Europe, and Japan. However, isolates from these diverse locations have been quite similar, as determined by serological, biochemical, and genetic analyses (9, 23, 26, 45, 51). Here we report the occurrence of antigenic variation in p57 produced by Norwegian *R. salmoninarum* strain 684. Strain 684 p57 lacks the 4C11 epitope and contains an Ala^{139}-to-Glu mutation in the products of both the *msa1* and *msa2* genes. Cook and Lynch (13) have reported a single nucleotide change in the *msa* gene of *R. salmoninarum* strain K_2A_2. Interestingly, the mutation in strain K_2A_2 was identical to that in strain 684 (B. Lynch, personal communication); however, whether the mutation occurred in both *msa* genes of strain K_2A_2 was not determined. The presence the same mutation in both strain K_2A_2 and strain 684 suggests that these isolates may have a common origin or that the second nucleotide of the Ala^{139} codon is a hot spot for mutation. A common origin would be surprising as strain K_2A_2 was isolated from an Atlantic salmon on the Margaree River in Nova Scotia, while strain 684 was isolated from a brown trout

TABLE 1. ECP from isolate 684 agglutinates salmonid leukocytes with twofold higher activity than ECP from ATCC 33209

ECP (μg ml^{-1})	Agglutination					
	Fish 1		Fish 2		Fish 3	
	ATCC 33209	684	ATCC 33209	684	ATCC 33209	684
500	+[a]	+	+	+	+	+
100	+	+	+	+	+	+
50	+	+	+	+	+	+
25	+	+	+	+	+	+
12.5	−	+	−	+	−	+
6.25	−	−	−	−	−	−
0 (PBS)	−	−	−	−	−	−

[a] +, agglutinating activity; −, no agglutinating activity. The data are data from one of two experiments that yielded similar results.

in Norway. Recently, genetic analysis of an exact tandem repeat locus (ETR-A) and the sequence of the 16S-23S rRNA intergenic spacer have provided a method to determine the relatedness of *R. salmoninarum* isolates (24–26). We carried out an analysis of these loci in strain 684 and determined that strain 684 possesses only one copy of the ETR-A locus (TR1) and that the sequence of the 16S-23S rRNA intergenic spacer belongs to sequevar 4 (Wiens, unpublished data). The TR1 and sequevar 4 genotypes resemble those of other *R. salmoninarum* strains isolated from Norway, Iceland, and Scotland and differ from those of most United States, England, Wales, mainland Europe, and Canadian strains, which belong to sequevar 1 and contain the TR2 allele (25, 26). Taken together, these data argue for a Scandinavian origin of strain 684 and may implicate

```
                Primers 6 and 5                                                  Primers 1 and 3
33209 msa1  agtctccgctcgttgcagagcagcagtgaaaaccatgccggaacgcgggtccgttgaacgagcggagatgg
33209 msa2  -tc-gg-agcaa-cc-g-ccacctc-gcc-c-aacgag-c--tttgttcc-g---ttcgacaac-g-----
684 msa1                                                  ----------------------------------
684 msa2                                        -aacgag-c--tttgttcc-g---ttcgacaac-g-----

                                                     1
p57                                                  M   K   I   K   K   I   L   A   L
33209 1&2   ctttcgctggtgcgctgtggtaaaataaaagct        atg aaa ata aaa aaa att tta gcg ctg
684 msa1    ---------------------------------        --- --- --- --- --- --- --- --- ---
684 msa2    ---------------------------------        --- --- --- --- --- --- --- --- ---
            10                                                   20
p57          T   A   V   T   A   V   T   F   F   G   V   A   P   L   A   Q   A   S
33209 1&2   act gca gtc act gca gtc act ttt ttt ggg gtg gct cct ctt gcg caa gct tcg
684 msa1    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
684 msa2    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                    30                                                   40
p57          Q   G   E   G   N   S   S   T   S   T   V   Q   G   F   S   S   V   N
33209 1&2   caa ggt gaa ggg aat tct tcc act tca aca gta caa ggc ttc agc agt gtt aac
684 msa1    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
684 msa2    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                        50                                                   60
p57          I   F   Q   Q   G   G   Y   S   A   F   F   H   E   L   R   P   D   G
33209 1&2   att ttt caa cag ggt ggt tat tct gct ttc ttt cac gag tta agg cct gac ggg
684 msa1    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
684 msa2    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                            70                                                   80
p57          T   S   S   A   A   G   G   P   V   A   A   G   G   S   Y   S   S   G
33209 1&2   acc tcc agc gcc gca gga gga cca gtt gca gct ggt ggc tct tat agt tct ggt
684 msa1    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
684 msa2    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                90
p57          D   L   G   K   K   G   F   Q   D   G   D   L   V   V   P   V   V   N
33209 1&2   gat tta ggt aaa aag ggc ttc gaa gac gga gat ctt gtt gtt ccc cta gta aac
684 msa1    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
684 msa2    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
            100                                                  110
p57          A   V   F   G   N   E   Q   K   G   S   S   F   V   Y   N   K   D   G
33209 1&2   gcg gtt ttt gga aat gag caa aaa gga agc tcc ttt gtt tat aac aaa gat ggt
684 msa1    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
684 msa2    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                    120                                                  130
p57          P   A   K   E   L   K   V   W   G   T   T   L   N   V   G   F   G   C
33209 1&2   cct gcc aag gaa ctg aaa gtc tgg ggg acg act cta aac gta ggt ttc ggg tgt
684 msa1    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
684 msa2    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                        140                                                  150
p57           N   D   N   A   P   S   P   M   G   C   G   P   A   N   K   P   V   A
33209 1&2   aac gat aat gcg cca tcc cca atg gga tgt ggc ccg gca aat aaa cct gtt gca
                        E
684 msa1    --- --- --- -a- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
684 msa2    --- --- --- -a- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
```

FIG. 5. DNA sequences and predicted amino acids of p57 encoded by *msa1* and *msa2* from *R. salmoninarum* strains ATCC 33209 and 684. The 5′ primers used to amplify *msa1* or *msa2* are underlined. Dashes indicate identity with ATCC 33209 *msa1*. Only one sequence for ATCC 33209 *msa1* and *msa2* is shown as the sequences are identical starting 38 bp 5′ of the ATG start site through the entire coding region.

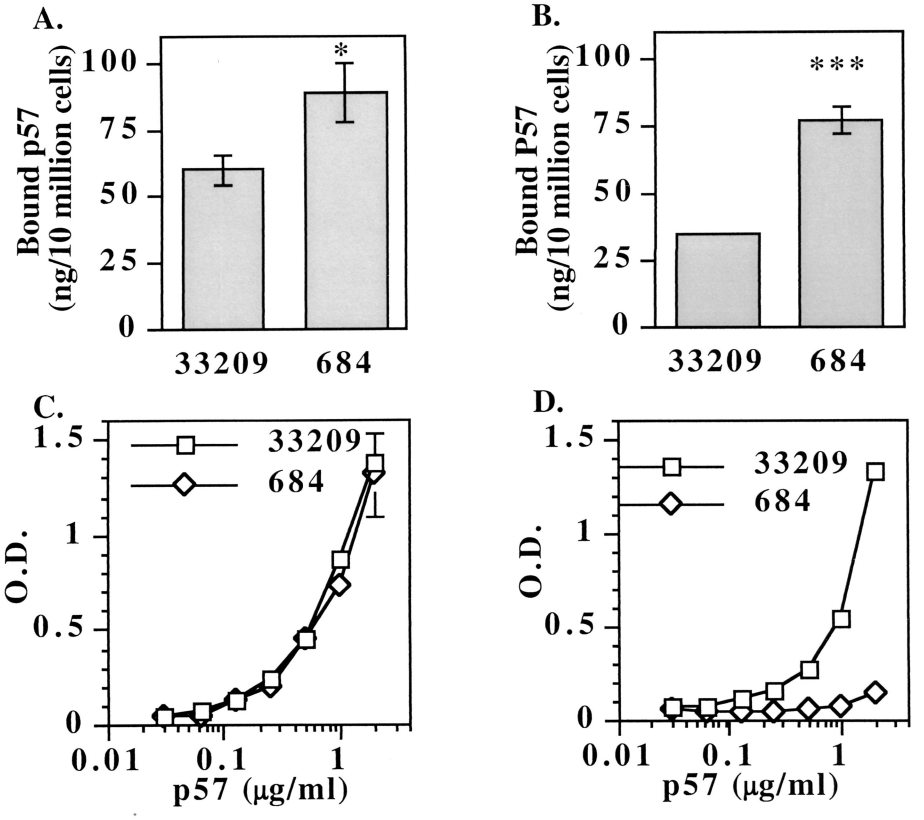


FIG. 6. (A) p57 produced by *R. salmoninarum* strain 684 has 50% increased binding activity for chinook salmon anterior kidney leukocytes compared to the binding activity of an equivalent amount of p57 isolated from *R. salmoninarum* strain ATCC 33209. The data are averages ± standard errors of the means for binding of p57 to leukocytes from three salmon. The asterisk indicates that the results were significantly different at a *P* value of <0.05 (Student's *t* test). ECP was purified from culture supernatants of bacteria grown at the same time under identical conditions. The purification procedure and binding assay are described in Materials and Methods. (B) p57 from strain 684 exhibits a twofold increase in binding to rabbit red blood cells. The three asterisks indicate that the results were significantly different at a *P* value of <0.001 (Student's *t* test). (C) Identical amounts of immunoreactive p57 are present, as determined by a capture ELISA. MAb 3H1 was coated onto a plate, followed by dilutions of the ECP containing p57 from either strain ATCC 33209 or strain 684. Bacterial protein concentrations were determined by the Bradford protein assay. Biotinylated MAb 4D3 was used as a capture antibody, and this was followed by detection with a strepavadin-alkaline phosphatase conjugate. O.D., optical density. (D) Secreted p57 produced by strain 684 lacks the 4C11 epitope. The ELISA was identical to the ELISA whose results are shown in panel C, except that biotinylated MAb 4C11 was used instead of biotinylated MAb 4D3. The data are representative of the data from two experiments performed with the two preparations of ATCC 33209 and 684 ECP.

transfer of *R. salmoninarum* to eastern Canada via infected eggs or fish. It will be interesting to examine additional *R. salmoninarum* isolates from the eastern United States and Canada and Scandinavia for nucleotide substitutions in the Ala^{139} codon to determine the prevalence of the Glu substitution and to investigate whether these strains share other genetic loci indicative of a common origin.

A surprising finding was that the two 684 *msa* genes had a common mutation, while the remaining coding sequences of 684 *msa1* and *msa2* were indistinguishable. With the exception of a single nucleotide change, the 684 *msa1* and *msa2* coding sequences were also identical to the reported coding sequences of ATCC 33209 *msa1* and *msa2* (36). O'Farrell and Strom (36) have proposed that the *msa* genes arose via duplication of an ancestral *msa* gene and that the presence of the two genes provides a selective advantage to *R. salmoninarum*. Here, we confirmed and extended the findings of these authors by demonstrating the presence of *msa1* and *msa2* in both ATCC 33209 and a Norwegian *R. salmoninarum* isolate. Examination of the genomic organization

TABLE 2. MAb 4C11 inhibits ATCC 33209 p57-mediated agglutination of leukocytes but not 684 p57-mediated agglutination of leukocytes[a]

Inhibitor	Concn (μg ml^{-1})	Agglutination			
		Preparation 1		Preparation 2	
		ATCC 33209	684	ATCC 33209	684
Control		+[b]	+	+	+
4C11	80	−	+	−	+
	40	−	+	−	+
	20	±	+	±	+
	10	+	+	±	+
	5	+	+	+	+
PCG1-1	80	+	+	+	+

[a] ECP (50 μg ml^{-1}) was preincubated with dilutions of protein A-purified MAb 4C11, PCG1-1 (antibody of the same isotype that does not bind p57), or tissue culture medium alone (control).

[b] +, agglutination; −, no agglutination; ±, weak agglutination.

of the *msa* genes by *Bam*HI, *Hin*dIII, and *Eco*RI digestion followed by Southern blotting identified hybridizing DNA fragments that were the same size in both 684 and ATCC 33209. These data suggest that the genomic organization of *msa1* and *msa2* is similar in the two strains and that two *msa* genes are present in *R. salmoninarum* isolates from diverse geographic areas. However, there are also differences, as *Xho*I digestion produced a 6.2-kb band in ATCC 33209 and a 6.9-kb hybridizing band in 684. Perhaps this difference and the presence of the 4C11 epitope may be useful for further molecular classification of *R. salmoninarum* isolates. It should be noted that only five isolates, including isolate 684, have been examined to date for the presence of *msa1* and *msa2* and that a more extensive survey is required to determine how prevalent these genes are in other *R. salmoninarum* isolates. The primers that we have identified which selectively amplify either *msa1* and *msa2* may facilitate further analysis of these genes.

The mechanism by which the identical changes were introduced into *msa1* and *msa2* of strain 684 is unclear. One possibility is that a spontaneous mutation occurred in one of the *msa* genes, which was followed by nonreciprocal recombination with the other *msa* gene. In support of this mechanism, the single nucleotide difference 3′ of the stop codon in ATCC 33209 (Fig. 1) is absent in isolate 684. In ATCC 33209, *msa1* contains a G while *msa2* contains a C at this position. Like the ATCC 33209 *msa2* gene, the 684 *msa1* and *msa2* genes both contain a C located 37 bp 3′ of the stop codon. If the nonreciprocal recombination mechanism hypothesis is correct, then in the simplest scenario the mutation may have been first introduced into *msa2* and then introduced by recombination into the *msa1* locus, thus resulting in identical coding sequences as well as identical 3′ sequences. It should be noted that the 5′ sequences, at least as far as the −69 (*msa1*) and −73 (*msa2*) nucleotides 5′ of the translational initiation codon, resemble those determined for ATCC 33209 *msa1* and *msa2* and thus may not be involved in recombination. It is notable that IS*3*-like insertion sequences have been reported to flank the *msa* copies at a distance of 2 to 3 kb and thus may have been involved in *msa* gene duplication, potentially though recombination (36, 40). Recombination and unidirectional transfer of DNA between a pseudogene(s) and a functional gene(s) or between complete gene copies appear to be a common mechanism of antigenic variation in a number of pathogenic microorganisms (5, 30, 31, 35, 38, 46). A well-studied example is *Neisseria gonorrhoeae*, in which pilin antigenic variation can occur by unidirectional transfer of DNA sequences from a silent pilin locus to the expressed pilin gene through high-frequency recombination events (28, 29, 33, 44). At present we do not know if both *msa* genes are expressed or the frequency of the putative recombination process in the *R. salmoninarum* *msa* genes. The divergent 5′ sequences of *msa1* and *msa2* suggest that the *msa* genes may be differentially regulated. In addition to recombination as a mechanism of antigenic variation, we do not exclude the possibility that the identical mutations may be due to a bias in the mutational machinery or repair process targeting this region of the *msa* genes.

A surprising finding was that p57 isolated from isolate 684 culture supernatant displayed increased agglutinating and binding activity for salmonid leukocytes and rabbit erythrocytes. We envisage several possible mechanisms by which the mutation enhances binding activity. One possibility is that the mutation may increase the stability of p57. We have previously demonstrated that p57 is highly susceptible to digestion by an *R. salmoninarum*-produced serine protease (39, 42, 48). In addition, proteolytic activity may reside within the p57 protein itself (3). Alternatively, the mutation may increase the binding affinity for the putative receptor(s) on fish leukocytes and rabbit red blood cells. Enhanced binding could be due to an altered conformation of p57 or a change in the actual binding site. It is of interest that the mutation occurs close to the A1 repeat in p57 (48). The A1 and A2 direct repeats are 81 amino acids long, and each repeat contains a transcription factor-immunoglobulin (TIG)-like domain (2), also called an IPT domain (immunoglobulin-like fold shared by plexins and transcription factors [4]). The TIG/IPT domain is predicted to form an unusual type E immunoglobulin fold and is found in the extracellular regions of members of the plexin family of adhesion-repulsion molecules. If the A1 and A2 repeats are involved in binding to fish leukocytes, it is intriguing to speculate that the mutation at position 139 may alter these domains or their function. A final possibility is that the mutation may alter the binding of a cofactor involved in the binding of p57 to leukocytes. Investigations are under way to distinguish among these possibilities and fully map the binding site(s) on p57.

Although the role of p57 in pathogenesis is unclear, this protein is a well-accepted marker of infection by *R. salmoninarum* (6, 11, 13, 34, 37, 41). The correlation of cell-associated p57 with isolate virulence, the high levels of synthesis by *R. salmoninarum*, the in vitro binding activity with fish leukocytes, and the maintenance of duplicated *msa* genes support the hypothesis that p57 plays an important role in the pathogenesis of bacterial kidney disease. In this study we identified a p57 antigenic variant that has lost the 4C11 epitope and has increased in vitro binding and agglutinating activities. If this binding activity is relevant in pathogenesis, then loss of a neutralizing epitope may help *R. salmoninarum* escape a salmonid immune response. Alternatively, the mutation may be involved in tropism among tissues or among different salmonid species. A novel finding of this work was the demonstration of identical mutations in the coding regions of both the *msa1* and *msa2* genes. The development of isogenic strains and in vivo challenge experiments are required to determine the contribution of the p57 Ala^{139}-to-Glu mutation to *R. salmoninarum* virulence.

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