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Differential expression of the virulence-associated protein p57 and characterization of its duplicated gene *msa* in virulent and attenuated strains of *Renibacterium salmoninarum*

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ABSTRACT: Virulence mechanisms utilized by the salmonid fish pathogen *Renibacterium salmoninarum* are poorly understood. One potential virulence factor is p57 (also designated MSA for major soluble antigen), an abundant 57 kDa soluble protein that is predominately localized on the bacterial cell surface with significant levels released into the extracellular milieu. Previous studies of an attenuated strain, MT 239, indicated that it differs from virulent strains in the amount of surface-associated p57. In this report, we show overall expression of p57 in *R. salmoninarum* MT 239 is considerably reduced as compared to a virulent strain, ATCC 33209. The amount of cell-associated p57 is decreased while the level of p57 in the culture supernatant is nearly equivalent between the strains. To determine if the lowered amount of cell-associated p57 was due to a sequence defect in p57, a genetic comparison was performed. Two copies of the gene encoding p57 (*msa1* and *msa2*) were found in 33209 and MT 239, as well as in several other virulent isolates. Both copies from 33209 and MT 239 were cloned and sequenced and found to be identical to each other, and identical between the 2 strains. A comparison of *msa1* and *msa2* within each strain showed that their sequences diverge 40 base pairs 5' to the open reading frame, while sequences 3' to the open reading frame are essentially identical for at least 225 base pairs. Northern blot analysis showed no difference in steady state levels of *msa* mRNA between the 2 strains. These data suggest that while cell-surface localization of p57 may be important for *R. salmoninarum* virulence, the differences in localization and total p57 expression between 33209 and MT 239 are not due to differences in *msa* sequence or differences in steady state transcript levels.

KEY WORDS: *Renibacterium salmoninarum* · p57 · *msa* · Gene duplication · Pathogenesis

INTRODUCTION

Bacterial kidney disease (BKD) is one of the most important diseases affecting both hatchery-reared and wild salmonids worldwide. The disease is a chronic, granulomatous infection characterized by grey-white necrotic abscesses in the kidney. In advanced stages, the entire kidney may become enlarged and necrotic (Fryer & Sanders 1981). The disease is found in freshwater salmonids and in anadromous salmonids during both freshwater and saltwater life stages (Fryer & Sanders 1981, Evelyn 1993). The causative agent of BKD, *Renibacterium salmoninarum*, is a slow-growing, Gram-positive diplobacillus that is aerobic and nonmotile (Fryer & Sanders 1981, Fryer & Lannan 1993).

Despite numerous studies, little is known about the pathogen's main portal(s) of entry, and few potential virulence factors and their functions have been identified (Evenden et al. 1993). *Renibacterium salmoninarum* is believed to infect the skin, eye, and other unidentified sites and then localize in the kidney

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(Munro & Bruno 1988). The bacteria are found intracellularly in mononuclear phagocytes, which may be an important characteristic of pathogenesis (Gutenberger et al. 1997). However, the mechanisms utilized by the organism to invade and survive within macrophages are poorly understood. A cloned DNA fragment from *R. salmoninarum* reportedly conveys invasiveness to non-invasive *Escherichia coli*, but searches of available databases with the DNA sequence of this fragment did not show homology to any known bacterial invasion factors (Maulén et al. 1996). Results of one study by electron microscopy suggest that *R. salmoninarum* is encapsulated (Dubreuil et al. 1990b). Many other studies have focused on the role of a 57 kDa cell surface protein (p57). This protein is the immunodominant antigen on the bacterial cell surface (Dubreuil et al. 1990a). It is extremely soluble and easily extracted from the bacterium with water or saline, hence its alternate designation as the major soluble antigen or MSA. Of the total proteins on the *R. salmoninarum* surface, reportedly 70% is p57 (Wood & Kaattari 1996). The presence of p57 has been suggested to correlate with the cell surface hydrophobicity and autoagglutination of *R. salmoninarum*, with hydrophobicity being associated with the virulence of the pathogen (Bruno 1988, 1990). The p57 protein causes haemagglutination of rabbit erythrocytes but does not agglutinate erythrocytes from salmon (Daly & Stevenson 1987). It also suppresses the host immune response, as measured by decreased antibody responses *in vitro* (Turaga et al. 1987, Fredriksen et al. 1997) and lowered haematocrit levels *in vivo* (Turaga et al. 1987). Removal of p57 increased the immunogenicity of the bacteria, resulting in increased antibody titers against other *R. salmoninarum* antigens in a chinook salmon *Oncorhynchus tshawytscha* challenge (Wood & Kaattari 1996). The gene encoding p57, *msa*, was cloned and sequenced (Chien et al. 1992), and investigators have produced recombinant p57 in an *E. coli* expression system (Grayson et al. 1995). Characterization of *msa* revealed an open reading frame encoding a protein of 557 amino acids bearing a 26 amino acid amino-terminal signal peptide that is cleaved off the mature protein (Chien et al. 1992).

Some strains with phenotypic differences in their surface components and lowered virulence have been reported. In an injection challenge of rainbow trout *Oncorhynchus mykiss*, 3 non-agglutinating strains of *Renibacterium salmoninarum* caused dramatically lower mortality (as low as 8%) than agglutinating, hydrophobic *R. salmoninarum* strains (Bruno 1988). One of these non-agglutinating strains, MT 239, displays little or no surface-extractable p57 (Bruno 1990, Daly & Stevenson 1990), and anti-p57 monoclonal antibodies reportedly fail to react with whole cell lysates of MT 239 (Wood et al. 1995). These results suggest that differences in expression and localization of p57 may be partially responsible for the attenuated phenotype of MT 239. To better understand the basis of these differences, we compared total p57 expression between a virulent strain (ATCC 33209) and the attenuated strain MT 239. In addition, we compared the *msa* sequences and evaluated the levels of *msa* transcription between the 2 strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used in this study are shown in Table 1 and were obtained from D. Elliott of the Western Fisheries Research Center, Seattle, WA. *Renibacterium salmoninarum* strain D6 was originally obtained from Oregon State University, Corvallis, OR, and it was cultured from a coho salmon *Oncorhynchus kisutch* that was held in salt water in Oregon. The ATCC strain was isolated from a chinook salmon *Oncorhynchus tshawytscha* in Oregon. The Sawtooth strain was isolated from a chinook salmon *O. tshawytscha* at the Sawtooth National Fish Hatchery in Idaho. The MT 239 strain was originally isolated from a farmed Atlantic salmon *Salmo salar* (Bruno 1988). It was identified as a non-agglutinating strain that may have arisen through routine subculture. Cultures of *R. salmoninarum* for chromosomal DNA extraction and protein expression analyses were grown at 15°C in KDM2 broth following methods previously described (Evelyn 1977) and modified to include 0.05% cysteine-HCl and 10% fetal bovine serum (FBS). For the transcription studies, cultures were grown in KDM2 broth (Evelyn 1977) with 0.05% cysteine-HCl. Cell counts of *R. salmoninarum* in the broth cultures used in expression analyses were determined by using 4',6-diamidino-2-phenylindole (DAPI) staining (100 µl at 1 µg ml^{-1}) (Sigma, St. Louis, MO). *Escherichia coli* TOP 10 F' was grown at 37°C in Luria-Bertani (LB) broth with kanamycin (100 µg ml^{-1}).

DNA manipulations. DNA was extracted from *Renibacterium salmoninarum* following methods described for *Listeria monocytogenes* (Flamm et al. 1984) with the exception that incubation in lysis buffer was extended from 30 min to overnight at 37°C. Standard techniques were used for enzymatic manipulations, ligations, transformations, and DNA electrophoresis (Sambrook et al. 1989). Small-scale plasmid preparations followed a standard alkaline-lysis method (Birnboim & Doly 1979); plasmid preparations for DNA sequencing utilized Qiagen midi-prep columns (Qiagen, Santa Clarita, CA) or the Wizard™ Plus Minipreps DNA Purification System (Promega, Madison, WI). Probes used in northern and Southern blot

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Host, source
Renibacterium salmoninarum		
D6	Virulent strain	*Oncorhynchus kisutch*, Oregon
ATCC 33209-1	Virulent strain	*O. tshawytscha*, Oregon (ATCC)
ATCC 33209-2	Fewer passages than ATCC 33209-1	
Sawtooth	Virulent strain	*O. tshawytscha*, Idaho
MT 239	Attenuated strain	*Salmo salar*, Scotland
Escherichia coli		
TOP 10 F'	F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697*gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
Cloning vector		
pZErO™-2.1	Kan^r Zero background cloning vector	Invitrogen
Recombinant plasmids		
pCO2	5 kb *Bam*H I fragment cloned into pZErO from ATCC 33209, contains *msa1*	This study
pCO4	7 kb *Bam*H I fragment cloned into pZErO from ATCC 33209, contains *msa2*	This study
pCO5	5 kb *Bam*H I fragment cloned into pZErO from MT 239, contains *msa1*	This study
pCO7	7 kb *Bam*H I fragment cloned into pZErO from MT 239, contains *msa2*	This study
pET-30b+*msa*	Expression vector containing 1.6 kb *Bam*H I- *Hin*d III fragment with *msa* (encodes amino acids #22-556)	J. Winton

hybridizations were prepared using the random priming method with digoxygenin (DIG)-labeled dUTP followed by chemiluminescence detection with anti-DIG alkaline phosphatase conjugate and disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5-chloro)tricyclo[3,3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (Genius™ System, nonradioactive detection kit version 2.0, Boehringer Mannheim, Indianapolis, IN).

Analysis of p57 expression. *Renibacterium salmoninarum* strains 33209-1 and MT 239 were grown in KDM2 broth at 15°C for 12 d and equilibrated to an OD_{525} of 0.46 to 0.52. Two 150 ml aliquots of each isolate were transferred to pre-weighed 200 ml conical centrifuge tubes and centrifuged at 10000 × *g* for 15 min. The supernatant was decanted and stored at −80°C. The pellets were washed 1 time in tris-buffered saline solution (TBS, 20 mM Tris, 0.5 M NaCl, pH 7.5), resuspended in TBS at a concentration of 100 mg ml^{-1}, and stored at −80°C. For preparation of supernatant-localized p57, the supernatants were thawed and centrifuged at 10000 × *g* for 15 min, transferred to lyophilization vials, flash-frozen in liquid nitrogen, and lyophilized for 72 h in a Genesis 25 lyophilizer (VirTis Company, Gardiner, NY). The lyophilized material was resuspended to one-tenth the original volume in TBS and then transferred to 4 Slide-A-Lyzer 10K Dialysis Cassettes (10000 molecular weight, MW, cutoff) (Pierce, Rockford, IL) and dialyzed against 1.5 l TBS at 4°C for 72 h (with 3 changes of TBS). Following dialysis, the samples were stored at −80°C. A Bradford protein analysis (Bio-Rad, Hercules, CA) was performed with an aliquot of the dialyzed sample to determine total protein concentration of the supernatant protein preparation.

Total p57 in supernatant and whole cell fractions was compared by western immunoblots. Supernatant fractions were mixed with an equal volume of 2× sample buffer containing 62.5 mM Tris-HCl, pH 6.8; 10% glycerol (v/v); 2% sodium dodecyl sulfate (SDS) (w/v); 5% 2-mercaptoethanol (v/v); 0.005% bromophenol blue (w/v), and heated for 4 min at 95°C. Whole cell fractions were also mixed with an equal volume of the 2× sample buffer and heated for 4 min at 95°C, which completely lysed the bacteria. Samples were then electrophoresed on SDS-12% polyacrylamide gels (Laemmli 1970), and the proteins transferred to nitrocellulose (Towbin et al. 1979). Identification of p57 was carried out by reacting the blot with mouse anti-p57 monoclonal antibody 3H1 (Wiens & Kaattari 1991; generously provided by R. Pascho), and then developed with goat anti-mouse IgG horseradish peroxidase conjugate (Wiens et al. 1990) (Bio-Rad, Richmond, CA).

Nucleotide sequence determination. DNA sequencing was performed by the dideoxy-chain termination method (Sanger et al. 1977) using either the Dye Terminator Cycle Sequencing Ready Reaction Kit on the

ABI PRISM 310 Genetic Analyzer or the dRhodomine dye terminator sequencing protocol on the ABI 377 (all from Applied Biosystems Inc., Perkin-Elmer Corp., Foster City, CA). Sequencing primers were synthesized with a Gene Assembler Special (Pharmacia, Piscataway, NJ) or obtained commercially (Great American Gene Company, Ramona, CA).

RNA preparation. Total RNA was isolated from log phase broth cultures of both 33209-1 and MT 239 (OD_{525} = 0.5 to 0.7) using a modification of a method developed for *Streptococcus agalactiae* (Yim & Rubens 1997). Cultures were kept on ice throughout the procedure except during the hot phenol extraction steps. Growing cultures (30 ml) were transferred to chilled oakridge tubes and centrifuged at 10000 × *g* for 15 min at 4°C. Following centrifugation, the supernatant was decanted, and the pellet was resuspended in 600 µl cold diethyl pyrocarbonate (DEPC)-treated H_2O and transferred to pre-chilled 1.5 ml screw-capped tubes containing 0.3 g glass beads (150 to 212 µm, Sigma, St. Louis, MO), 250 µl of phenol saturated with DEPC-treated H_2O, 50 µl of 2 M sodium acetate (NaOAc, pH 4.0), 25 µl of 20% SDS, and 25 µl DEPC-treated H_2O. The samples were kept on ice and vortexed at maximum speed for 20 s, chilled for at least 1 min, and vortexed again for a total of 5 times in order to break open the cells. Then, 150 µl of chloroform were added, and the mixture was vortexed for 20 s, incubated on ice for 15 s, and centrifuged for 10 min at 13000 × *g* at 4°C. The supernatant was transferred to a 1.5 ml microcentrifuge tube and the aqueous phase extracted a total of 2 times with 500 µl of DEPC-H_2O-saturated phenol. Each extraction included heating the mixture (phenol and aqueous phase) to 65°C for 10 min with vortexing at maximum speed every 2 min. The mixture was then centrifuged at 13000 × *g* for 10 min at 4°C with a microcentrifuge. Following the second phenol extraction, the aqueous phase was transferred to a new 1.5 ml microcentrifuge tube which contained 800 µl of chloroform. The mixture was vortexed briefly at maximum speed and centrifuged at 13000 × *g* for 5 min at 4°C. The upper aqueous phase was transferred to a new 1.5 ml microcentrifuge tube containing 130 µl 3 M sodium acetate (pH 5.2) and 800 µl absolute isopropanol. The samples were placed at −20°C for at least 30 min to precipitate the RNA followed by centrifugation at 10000 × *g* for 30 min at 4°C. The isopropanol mixture was decanted, and 1 ml of 80% ethanol was added to wash the pellet. The samples were centrifuged at 10000 × *g* for 5 min at 4°C. The ethanol was decanted and the pellet allowed to air dry. The dried pellet was resuspended in 50 µl DEPC-treated H_2O and stored at −80°C. Total RNA concentrations were determined by measuring the absorbance at 260 nm of an aliquot diluted in H_2O.

Northern blots. Transcription of *msa* from each strain was determined by northern blot hybridization of total RNA probed with a labeled *msa* DNA probe. The probe used was an internal 1089 base pair *Bgl* II fragment excised from pCO4 (see Fig. 3, Table 1) and labeled with DIG as described above. Equal concentrations of RNA were loaded onto a 1% agarose gel containing 1.1% formaldehyde and 20% 3-(N-morpholino)propanesulfonic acid (MOPS) (Sambrook et al. 1989). For the sample preparation, 4.5 µl of RNA, 2.0 µl 10× MOPS gel-running buffer (200 mM MOPS pH 7.0, 80 mM sodium acetate, 10 mM EDTA pH 8.0) , 3.5 µl of 37% formaldehyde, and 10.0 µl of formamide were added to a 0.5 ml microcentrifuge tube (Sambrook et al. 1989). The samples were heated to 68°C for 15 min and then placed on ice until ready to load onto the gel. RNA loading buffer (Sambrook et al. 1989) was added while samples were on ice. The samples were electrophoresed at 5 V per each centimeter of gel length at constant voltage with 1× MOPS gel-running buffer. The RNA was transferred to a nylon membrane (Boehringer Mannheim, Indianapolis, IN) overnight at room temperature by capillary transfer following established procedures (Sambrook et al. 1989) and probed with DIG-labeled *msa* as described above.

Nucleotide sequence accession numbers. The nucleotide sequences of both *msa1* and *msa2* in 33209-1 and MT 239 have been submitted to the GenBank/EMBL/DDBJ data libraries. For the *msa* genes from 33209, the GenBank accession number for *msa1* is AF123888 and for *msa2* is AF123889. For MT 239, the accession number for *msa1* is AF123890 and for *msa2* is AF123891. The original GenBank accession number for *msa* is Z12174 (Chien et al. 1992).

RESULTS

Surface localization and overall expression of p57

Western blot analysis of whole cell lysates from growing cultures using a monoclonal antibody against p57 showed much higher levels of p57 in 33209-1 when compared to equivalent cell numbers from MT 239 (Fig. 1). However, a direct comparison of culture supernatants showed the presence of comparable levels of p57 (Fig. 1). Western blot analysis with equal weights of whole cell lysates from both strains and with equal concentrations of proteins from culture supernatants also showed the same results as observed with blots of equal cell numbers (data not shown). In addition, comparison of water-extractable p57 (Daly & Stevenson 1990, Griffiths & Lynch 1991) from the surface of both strains showed results identical to those obtained with whole cells (data not shown). Expression

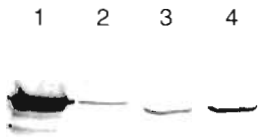

Fig. 1. Western blot hybridization of cell lysates and culture supernatants from *Renibacterium salmoninarum* 33209-1 and MT 239 with a monoclonal anti-p57 antibody, Mab 3H1 Lanes: 1, 33209 whole cell lysates; 2, MT 239 whole cell lysates, both from 8.1×10^4 cells; 3, 33209 supernatant; 4, MT 239 supernatant, both from 1.1×10^7 cells

of other proteins was not different between the 2 strains, as seen in Coomassie-blue stained SDS-PAGE gels of whole cell extracts and supernatants (data not shown). Together these results indicate that the total level of p57 expression is lower in MT 239 than in 33209-1.

Gene duplication of *msa* in *Renibacterium salmoninarum*

An initial Southern blot hybridization analysis of DNA from *Renibacterium salmoninarum* 33209-1 digested with *Bam*H I and *Sal* I showed 2 fragments hybridizing to the *msa* probe (data not shown), a 1.6 kb *Hin*d III-*Bam*H I fragment from pET-30b+*msa* (Table 1). The original published sequence of *msa* (GenBank accession number Z12174) has neither an internal *Bam*H I nor *Sal* I site (Chien et al. 1992). This result indicates that 2 *msa* gene copies are present on the *R. salmoninarum* chromosome. The *Bam*H I fragments were approximately 5 and 7 kb in length (Fig. 2), and the *Sal* I fragments were approximately 14.2 and 16 kb in length (data not shown). A subsequent Southern blot of *Bam*H I-digested chromosomal DNA from 33209-1, 33209-2, D6, Sawtooth, and MT 239 showed that all of the strains contained both 5 and 7 kb fragments that hybridized to the *msa* probe (Fig. 2).

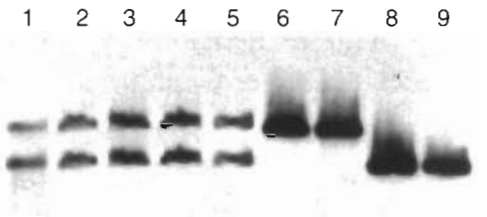

Fig. 2. Southern blot comparison of chromosomal DNA from various strains of *Renibacterium salmoninarum* and plasmid constructs that contain *msa* cloned from 33209-1 and MT 239, digested with *Bam*H I, and probed with *msa*. Lanes: 1, 33209-1; 2, 33209-2; 3, D6; 4, Sawtooth; 5, MT 239; 6, pCO4; 7, pCO7; 8, pCO2; 9, pCO5

Sequence analysis of *msa* in ATCC 33209 and MT 239

To confirm that there are 2 copies of *msa* on the *Renibacterium salmoninarum* chromosome, and to compare the 2 copies with each other and between corresponding copies of the 2 strains, chromosomal DNA containing the 5 and 7 kb *Bam*H I fragments from both strains was directly cloned into pZErO-2.1. The presence of *msa* in the 4 plasmid constructs was confirmed by Southern blot hybridization to the *msa* probe. The constructs containing the cloned fragments were designated pCO2 and pCO4 (5 and 7 kb *Bam*H I fragments from 33209-1, respectively), and pCO5 and pCO7 (5 and 7 kb *Bam*H I fragments from MT 239, respectively). The cloned genes were compared by restriction fragment endonuclease digestion to verify that they matched the original published sequence and to assess whether any restriction site polymorphisms existed between copies. The same size *Bam*H I fragments from 33209-1 and MT 239 (from pCO2 and pCO5, pCO4 and pCO7, respectively) had the same restriction pattern (Fig. 3). However, when comparing the 2 cloned fragments within each isolate (from pCO2 and pCO4, pCO5 and pCO7), an additional *Hin*d III site was observed downstream of *msa* in the 7 kb fragments (Fig. 3).

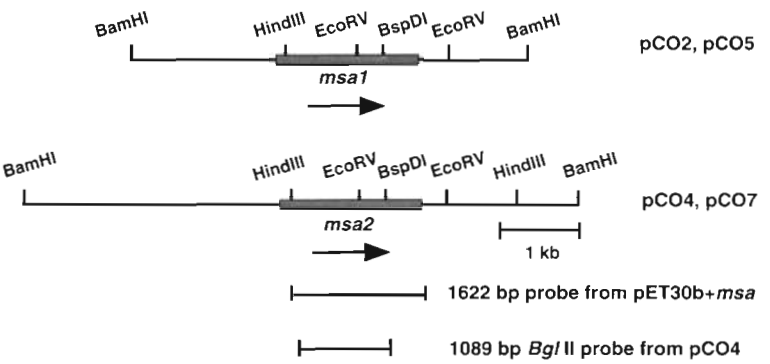

Fig. 3. Restriction map of *Bam*H I fragments containing *msa* cloned from 33209-1 and MT 239. pCO2 is the 5 kb fragment from 33209-1, and pCO5 is the 5 kb fragment from MT 239. pCO4 is the 7 kb fragment from 33209-1, and pCO7 is the 7 kb fragment from MT 239. The genes *msa1* and *msa2* are denoted by the thick bars, and arrows indicate the direction of transcription. The 1622 base pair *Bam*H I - *Hin*d III fragment from pET30b+*msa* (Table 1), and the 1089 base pair *Bgl* II fragment from pCO4, used as probes in Southern and northern blots, respectively, are shown below the maps

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          -112                                                                -53
pCO4      GGCAGCAA-T CCC-G-GCCA CCTCG-GCCA CAAACGA-GC CG-ATTT-GT -TCCCGGTTT
pCO7      GGCAGCAA-T CCC-G-GCCA CCTCG-GCCA CAAACGA-GC CG-ATTT-GT -TCCCGGTTT
pCO2      TCC-GCTCGT TGCAGAGC-A GCA-GTG--A -AAACCATGC CGGAACGCGG GTCC-G-TT-
pCO5      TCC-GCTCGT TGCAGAGC-A GCA-GTG--A -AAACCATGC CGGAACGCGG GTCC-G-TT-
                                                                    +1
pCO4      TCGA-CAA-C GG-GATGGC- TTTCGCTGGT GCGCTGTGGT AAAATAAAAG CTATGAAAAT
pCO7      TCGA-CAA-C GG-GATGGC- TTTCGCTGGT GCGCTGTGGT AAAATAAAAG CTATGAAAAT
pCO2      --GAACGAGC GGAGATGGC- TTTCGCTGGT GCGCTGTGGT AAAATAAAAG CTATGAAAAT
pCO5      --GAACGAGC GGAGATGGC- TTTCGCTGGT GCGCTGTGGT AAAATAAAAG CTATGAAAAT
Z12174       ACGAGC GGAGATGGCC TTTCGCTGGT GCGCTGTGGT AAAATAAAAG CTATGAAAAT
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Fig. 4. Nucleic acid comparison of sequence upstream of *msa1* and *msa2* from 33209-1 (pCO2 and pCO4) and MT 239 (pCO5 and pCO7). The sequence shown as Z12174 is the upstream sequence of *msa* previously published as GenBank accession number Z12174 (Chien et al. 1992). Shaded nucleotides represent areas of differences. Numerical annotation represents the number of nucleotides upstream of the *msa* start codon, which is indicated by +1 and is boxed

Results from the DNA sequence analysis showed that all 4 cloned fragments had identical nucleotide sequences within the coding region of *msa*. Since both copies of *msa* within each strain were identical, they are designated as *msa1* (from pCO2 and pCO5) and *msa2* (from pCO4 and pCO7). All matched the published sequence of Chien et al. (1992) except for an additional codon (GCA) located in all 4 copies 720 nucleotides from the first nucleotide of the start codon. The sequence upstream of *msa1* was identical to the published sequence of *msa* with one exception: the original sequence showed an additional cytosine (C) 33 nucleotides 5′ to the start codon that was not found in any of the 4 copies of *msa* sequenced in this study (Fig. 4). Comparison of the regions immediately surrounding *msa1* and *msa2* show that they begin to diverge 40 nucleotides upstream of the start codon with numerous differences found within 100 nucleotides (Fig. 4). The limited upstream sequence available from the originally published *msa* sequence (GenBank accession number Z12174) most closely matches the upstream sequence of *msa1*. Comparison of the DNA sequence downstream of the *msa* open reading frame showed that all were essentially identical with the exception of a single base substitution 39 nucleotides 3′ to the stop codon. At this position, a C downstream of *msa2* and in Z12174 is a guanosine (G) in *msa1*. With the exception of this difference, the sequence downstream of both *msa* copies is identical for at least the first 225 nucleotides. As shown previously (Chien et al. 1992), database searches with *msa1*, *msa2*, and their flanking regions did not reveal strong homologies to any known genes or proteins.

Transcriptional analysis of *msa* in 33209 and MT 239

Equal concentrations of total RNA from 33209-1 and MT 239 were analyzed by northern blot hybridization to a *msa* probe to determine whether there are any detectable differences in the transcription of *msa*. The probe hybridized to an approximately 1600 nucleotide fragment in both strains, corresponding to the 1671 nucleotide length of *msa* (data not shown). Repeating the northern hybridization with varying concentrations (1, 5, 10, and 15 µg) of RNA (Fig. 5) showed that band intensity was essentially equivalent. Identical results were obtained in 3 separate experiments.

DISCUSSION

The role of p57 in *Renibacterium salmoninarum* virulence has not been clearly established. As standard molecular genetic methods are not yet available for study of this bacteria, we have used other molecular techniques to compare a virulent *R. salmoninarum* strain (ATCC 33209) to a significantly less virulent strain (MT 239) in which the only other reported difference was a lack of p57 on the outer cell surface of MT 239. We report here that the cell-surface localization and overall expression levels of p57 are different between the 2 strains, with the virulent 33209 strain expressing much higher levels of cell-associated p57 than the attenuated strain. There was little difference between the 2 strains in the level of p57 released into

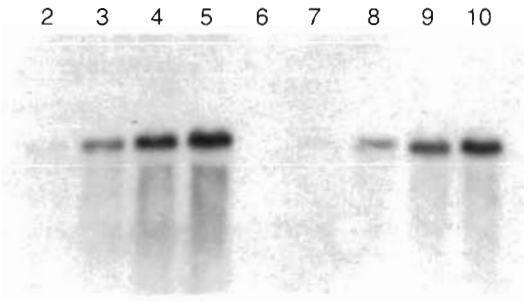

Fig. 5. Northern blot hybridization of total RNA isolated from 33209-1 and MT 239, probed with an internal fragment of *msa*. Lanes 1–5, from 33209-1. 1, 0 µg; 2, 1 µg; 3, 5 µg; 4, 10 µg; 5, 15 µg. Lanes 6–10, from MT 239: 6, 0 µg; 7, 1 µg; 8, 5 µg; 9, 10 µg; 10, 15 µg

culture supernatants. In contrast to Wood et al. (1995), we were able to detect p57 in the MT 239 cell lysates by western blot analysis when using the same p57 monoclonal antibody (3H1). Our observations are based on analyses with both whole cell lysates from equal numbers of bacteria (Fig. 1), and in other trials, equal amounts of total protein by weight. Wood's study does not describe whether equal amounts of total protein were loaded between strains, and this may account for the differences in results between the 2 studies. In addition, the studies differ in growth media and cell preparation, including the use of 10% FBS in our study. However, we obtained the same results as shown in Fig. 1 when strain 33209 and MT 239 were grown in the absence of serum (data not shown). The p57 detected in the culture supernatants appeared to have a slightly lower MW than the p57 detected from whole cell lysates, which may be due to autolytic degradation and cleavage resulting in the extracellular protein fraction containing fragments less than 57 kDa (Griffiths & Lynch 1991, Rockey et al. 1991, Barton et al. 1997). Our monoclonal antibody, however, did not detect p57 degradation products below approximately 43 kDa, and none were detected in the supernatant fractions (Fig. 1). Alternatively, the slightly lower MW of p57 from the culture supernatant could be an artifact of the presence of serum proteins (from the 10% FBS) in the culture medium.

To determine if the phenotypic differences in p57 cell-surface localization and total p57 expression were due to a genetic defect in *msa* from MT 239, we cloned and sequenced the gene from both strains. This led to the interesting discovery that the gene has an identical chromosomal duplicate in 33209 and MT 239, and moreover, this duplication appears to be highly conserved among other strains tested. The sequence data also suggests that the original cloning and subsequent sequencing of *msa* used in the database submission for GenBank accession number Z12174 possibly combined the 5' end of one copy with the 3' end of the other, a distinct possibility as this complete *msa* sequence was obtained from 2 separate DNA fragments (Chien et al. 1992). Analysis of *msa1* and *msa2* from MT 239, however, showed that aberrant expression of p57 in this strain is not due to an alteration of the basic amino acid sequence of the protein resulting in an unstable polypeptide that is more susceptible to degradation or fails to anchor to the cell surface. Interestingly, p57 lacks the surface sorting and anchoring motifs that have been identified in more than 50 different surface proteins of various Gram-positive bacteria (Schneewind et al. 1993, Braun et al. 1997).

Lower expression of p57 in MT 239 could be the result of lower levels of transcripts of *msa* mRNA. However, northern blot hybridization analysis of total RNA showed that there were closely similar if not equal steady-state levels of *msa* transcripts from both 33209 and MT 239 that hybridized to the *msa* probe, so this explanation also appears unlikely as the cause of the differences. Examination of the sequences upstream of *msa* do not reveal obvious promoter regions and the limits of northern blot resolution do not allow us to determine whether transcription occurs from one or both gene copies. The sequence divergence 40 nucleotides upstream of the start codon will allow the use of alternate methods such as RT-PCR or primer extension for promoter identification if transcription is initiated in the variable upstream region. The p57 phenotype of the MT 239 strain may be due to more than a single cause. Our results indicate that a post-transcriptional defect is involved, such as inappropriate protein processing, protein instability, or defective cell-surface anchoring. Lesions in other proteins responsible for post-transcriptional expression of p57, such as regulatory factors for protein modifications, are likely since the respective coding regions of *msa1* and *msa2* are identical between the 2 strains. Although we observed a reduction in total p57 protein levels in the attenuated MT 239 strain, it is possible that MT 239 may actually synthesize wild-type levels of p57, but fails to properly anchor the protein to the cell surface, resulting in extracellular release of p57. Since released p57 is more susceptible to degradation by a serine proteinase activity produced by *Renibacterium salmoninarum* (Griffiths & Lynch 1991, Rockey et al. 1991), it is possible that most of the p57 synthesized by MT 239 is degraded in the extracellular milieu. The slightly elevated levels of supernatant-derived p57 from MT 239 over 33209 shown in Fig. 1 would support this hypothesis. Although supernatant protein levels of p57 in Fig. 1 (lanes 3 and 4) appear to be different, additional experiments carried out in our laboratory reveal that the differences may not be of sufficient magnitude to be considered biologically relevant. While these results suggest that this difference (less than 2-fold) may be reproducible, further experimentation is required to confirm this observation. In contrast, the considerable difference observed between the 2 strains in the amount of cell-associated p57 (Fig. 1, lanes 1 and 2) represents 2 orders of magnitude fewer cells and is clearly biologically important. In addition, since total cellular protein levels were not different between the 2 strains and growth of MT 239 in culture is not hampered (our observations), it is unlikely that MT 239 suffers from a general protein synthesis defect.

The discovery of identical duplicate genes encoding p57 in *Renibacterium salmoninarum* is intriguing. There are numerous examples of gene duplication in prokaryotes. The evolution of a number of cell surface proteins, known to be important for pathogenesis of

Gram-positive bacteria by aiding colonization and/or invasion, has involved gene duplication (Goward et al. 1993). This phenomenon is a potentially important mechanism in the development of successful virulence mechanisms. Examples include cell surface proteins in Gram-positive bacteria such as Group A and Group G *Streptococcus* and *Staphylococcus aureus* that have evolved by the process of internal gene duplication (Goward et al. 1993). In *Neisseria meningitidis*, duplication following horizontal transfer occurs within part or all of the *opa* virulence genes from the same and from other meningococcal strains into other *opa* loci to alter the repertoire of Opa proteins for increasing surface antigen variability (Hobbs et al. 1994). Gene duplication has also been observed with antibiotic resistance genes such as *tet* in Gram-negative bacteria (Rubin et al. 1990) and is believed to be involved in the evolution of some heat shock proteins (Gupta & Singh 1992). Genes that have duplicated may be located in near proximity to one another, as reported with the *mer* genes which encode regulation of mercuric ion resistance in *Thiobacillus ferrooxidans* (Inoue et al. 1991). With the *msa* duplication, it is not known how close in proximity the 2 genes are in the *R. salmoninarum* genome.

It is tempting to speculate that duplication of *msa* has some important implications for virulence of *Renibacterium salmoninarum*. A similar example has been recently reported, where it was suggested that a tandem gene duplication of the *pstS1* genes encoding phosphate binding proteins in *Mycobacterium intracellulare* was the result of evolutionary pressure for intracellular pathogenesis (Thangaraj et al. 1996). However, in this case as well as every other instance of gene duplication cited in the examples above, the duplicated genes were not identical as was observed in this study with *msa*. It would be expected that if this phenomenon is as widespread as our survey of strains indicates, then there must be some physiological pressure to maintain the identical sequence. For example, high levels of p57 may be required to maintain the virulence of *R. salmoninarum* by aiding colonization, lowering host immune responses, or increasing intracellular survival. Alternatively, the duplication may have been a relatively recent evolutionary event which may explain the absence of any silent point mutations in either copy of *msa*. One would expect, however, the presence of some silent point mutations in *msa* when comparing strains isolated from different locations and from different host species; it is interesting to note this is not the case when comparing *msa* from ATCC 33209 and MT 239. Furthermore, we have recently discovered the existence of *IS3*-like insertion sequences flanking the *msa* copies at a distance of 2 to 3 kb, leading us to speculate that intragenic recombination may be the mechanism of *msa* gene duplication (L. Rhodes & M. Strom, Northwest Fisheries Science Center, National Marine Fisheries Service, NOAA, Seattle, WA, unpubl.).

In summary, we have shown that differences exist in p57 cell-surface localization and in overall expression between the 33209 and the MT 239 strains of *Renibacterium salmoninarum in vitro*. These differences may explain the attenuated virulence observed with MT 239 *in vivo*. Our studies also revealed the presence of a widespread *msa* gene duplication in *R. salmoninarum*. While the significance of this finding is unknown, it is hoped that continued studies will aid in understanding the role of the p57 protein in the virulence of *R. salmoninarum*.

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Erratum

Differential expression of the virulence-associated protein p57 and characterization of its duplicated gene *msa* in virulent and attenuated strains of *Renibacterium salmoninarum*

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Dis Aquat Org 38(2): 115–123, 1999

- On pages 119 and 120, the molecular weight and DNA size markers were omitted from Figs. 1, 2 & 5. The corrected figures are reproduced here with their legends.

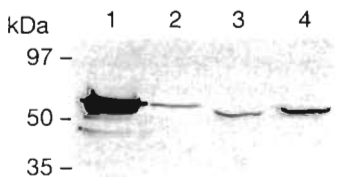

Fig. 1. Western blot hybridization of cell lysates and culture supernatants from *Renibacterium salmoninarum* 33209-1 and MT 239 with a monoclonal anti-p57 antibody, Mab 3H1. Lanes: 1, 33209 whole cell lysates; 2, MT 239 whole cell lysates, both from 8.1×10^4 cells; 3, 33209 supernatant; 4, MT 239 supernatant, both from 1.1×10^7 cells

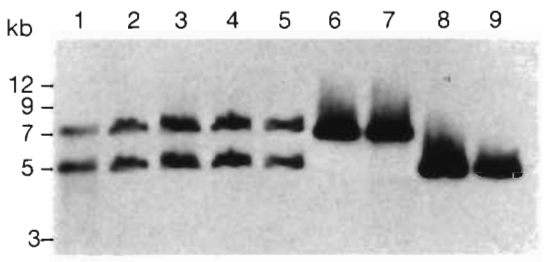

Fig. 2. Southern blot comparison of chromosomal DNA from various strains of *Renibacterium salmoninarum* and plasmid constructs that contain *msa* cloned from 33209-1 and MT 239, digested with *Bam*H I, and probed with *msa*. Lanes: 1, 33209-1; 2, 33209-2; 3, D6; 4, Sawtooth; 5, MT 239; 6, pCO4; 7, pCO7; 8, pCO2; 9, pCO5

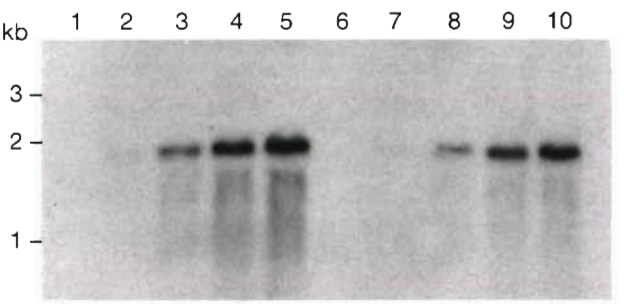

Fig. 5. Northern blot hybridization of total RNA isolated from 33209-1 and MT 239, probed with an internal fragment of *msa*. Lanes 1–5, from 33209-1: 1, 0 µg; 2, 1 µg; 3, 5 µg; 4, 10 µg; 5, 15 µg. Lanes 6–10, from MT 239: 6, 0 µg; 7, 1 µg; 8, 5 µg; 9, 10 µg; 10, 15 µg