Both *msa* Genes in *Renibacterium salmoninarum* Are Needed for Full Virulence in Bacterial Kidney Disease

Alison M. Coady,¹ Anthony L. Murray,² Diane G. Elliott,² and Linda D. Rhodes^{1*}

Northwest Fisheries Science Center, 2725 Montlake Boulevard East, Seattle, Washington 98112,¹ and Western Fisheries Research Center, U.S. Geological Survey, 6505 Northeast 65th Street, Seattle, Washington 98115²

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***Renibacterium salmoninarum*, a gram-positive diplococcobacillus that causes bacterial kidney disease among salmon and trout, has two chromosomal loci encoding the major soluble antigen (*msa*) gene. Because the MSA protein is widely suspected to be an important virulence factor, we used insertion-duplication mutagenesis to generate disruptions of either the *msa1* or *msa2* gene. Surprisingly, expression of MSA protein in broth cultures appeared unaffected. However, the virulence of either mutant in juvenile chinook salmon (*Oncorhynchus tshawytscha*) by intraperitoneal challenge was severely attenuated, suggesting that disruption of the *msa1* or *msa2* gene affected in vivo expression.**

Bacterial kidney disease (BKD) has been reported in most areas of the world where salmonid species are found (4, 11, 13, 20, 27, 28, 35, 37, 42, 46), and it is a principal source of morbidity and mortality among cultured salmon and trout. Acute BKD is characterized by ascites, swollen kidneys, exophthalmia, melanosis, and granulomatous lesions of internal organs such as the kidney, whereas asymptomatic carriers can complete an entire life cycle and successfully spawn (16). The infectious agent of BKD, *Renibacterium salmoninarum*, can be transmitted both horizontally among cohorts and vertically by intraovum inclusion (2, 14). Current disease controls include broodstock segregation or culling, antibiotic treatment, and vaccination, but these methods have only moderate efficacy in preventing or treating the disease (1, 30, 32).

R. salmoninarum is a gram-positive diplococcobacillus that grows very slowly and has fastidious nutritional requirements (8, 12, 39, 40). *R. salmoninarum* produces abundant quantities of an extracellular, 57-kDa protein called p57 or major soluble antigen (MSA) (7, 17, 41). MSA is associated with the bacterial surface and is released into surrounding tissues, and it has been associated with leukocyte agglutination (7, 43, 44) and immunomodulation (3, 15, 41, 45). A variant strain of *R. salmoninarum* displays both reduced MSA expression and lowered mortality upon infection (5, 24, 38), whereas *R. salmoninarum* isolates possessing an additional copy of the gene encoding MSA are more virulent than isolates lacking the additional copy (32). MSA is encoded by duplicate chromosomal loci (25), and both loci are expressed (33). These observations suggest that MSA is a significant virulence determinant, and we hypothesized that disruption of either chromosomal locus would result in a less virulent strain of *R. salmoninarum*. The goals of the present study were to generate and characterize *R. salmoninarum* clones bearing disruptions of either *msa1* or *msa2*, to compare the production of MSA protein in the mutants and the parental *R. salmoninarum* strain, and to evaluate

the virulence of the mutant and parental *R. salmoninarum* strains for chinook salmon, *Oncorhynchus tshawytscha*.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The parental *R. salmoninarum* strain used for transformation was the ATCC type strain 33209 (American Type Culture Collection, Manassas, VA). Broth and agar plate cultures were grown in modified KDM2 broth and modified KDM2 agar, respectively, as previously described (32). Culture purity was determined by Gram stain, and identity was confirmed by direct fluorescent antibody staining with an anti-*R. salmoninarum* polyclonal antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Kanamycin selection was at 50 $\mu\text{g mL}^{-1}$ for both liquid and plate cultures. The *R. salmoninarum* recombinant strains AMC1 and AMC2 are stable transformants of ATCC 33209 with the insertion-duplication mutagenesis (IDM) construct pACP41, described below.

Plasmids. Plasmid constructions were performed in *Escherichia coli* Top10 F' {F' [*lacI*^q Tn10 (Tet^r)] *mcrA*Δ(*mrr-hsdRMS-mcrBC*) F80 *lacZ*Δ*M15* Δ*lacX74* *recA1* *araD139*Δ(*ara-leu*)7697 *galU* *galK* *rpsL* (Str^r) *endA1* *nupG*}. A 600-bp fragment of the *msa* open reading frame (ORF) was obtained by PCR amplification with primers p57forwardp4 and CO-4R (Table 1) of pCO2, which contains the entire ORF of *msa1* (25). This *msa* amplicon was ligated into pCRII according to the manufacturer's protocol (Invitrogen), creating pACP4. A 656-bp BamHI-EcoRV fragment was excised from pACP4 and cloned into BamHI-EcoRV-digested pZER02.1 (Invitrogen), creating pACP41. Orientation of the *msa* insert in pACP41 was confirmed by restriction analysis and PCR, and sequencing was performed by cycle sequencing with BigDye terminator mix v3.1 and analysis with an ABI 3100 (ABI, Foster City, CA).

Transformation of *R. salmoninarum*. Transformation of *R. salmoninarum* was accomplished by electroporation as previously described (33). After electroporation, cells were allowed to recover at 15°C with agitation for 22 to 24 h. Cells were then selected on modified KDM2 plates containing 50 $\mu\text{g mL}^{-1}$ kanamycin at 15°C. Plates were examined weekly for colony growth.

Chromosomal DNA analysis. Chromosomal DNA was prepared as previously described (33) and analyzed by standard Southern blotting (36). Probes were random prime labeled with digoxigenin-11-dUTP, and hybridized probes were detected by chemiluminescence according to the manufacturer's instructions (Roche, Indianapolis, IN). The *msa* probe was synthesized from a 1.06-kb BglII fragment within the *msa* ORF of pCO2. The pZER02.1-specific probe was synthesized from the plasmid without an insert. Analysis of the integration junction was performed by PCR amplification of chromosomal DNA with an *msa1*-specific primer (CO-22F), an *msa2*-specific primer (CO-9F), or an *msa* ORF primer (p57forwardp4, p57reverseB6, or CO-4R) in combination with vector-specific primers (*lacZ*forward, *lacZ*reverse, M13reverse, M13-40, or Sp6pro) (Table 1). Amplicons were purified (MoBio, Carlsbad, CA) and subjected to cycle sequencing as described above for the plasmid pACP41.

Protein analysis. Equivalent numbers of *R. salmoninarum* cells in mid-logarithmic growth (optical density at 525 nm [OD₅₂₅] between 0.7 and 0.9) were

* Corresponding author. Mailing address: Northwest Fisheries Science Center, 2725 Montlake Blvd. East, Seattle, WA 98112. Phone: (206) 860-3279. Fax: (206) 860-3467. E-mail: linda.rhodes@noaa.gov.

TABLE 1. Primers used

Primer name	Binding site	Sequence (5' to 3')
p57forwardp4	<i>msa</i> ORF	AATTCTTCCACTTCAACAGTAC AAGG
p57reverseB6	<i>msa</i> ORF	ACCAATCGATGTTTTACC
CO-4R	<i>msa</i> ORF	GGAAACTCTCGTACGTTA
CO-9F	<i>msa2</i> specific (5' to ORF)	TTTCTGCCCGCATCCAACC
CO-22F	<i>msa1</i> specific (5' to ORF)	ATCATCGGATCCACGGGCTAAC CCCGCCCT
lacZforward	Vector lacZ	CCTCTTCGCTATTACGCC
placreverse	Vector plac	CGCAACGCAATTAATGTGAG
M13reverse	Vector	CAGGAAACAGCTATGAC
M13(-40)	Vector	GTTTTCCTCCAGTCACGAC
SP6pro	Vector	AGCTATTTAGGTGACACTATAG

harvested by centrifugation for 15 min at $10,000 \times g$. Phenylmethylsulfonyl fluoride was added to decanted, filter-sterilized supernatants to a final concentration of 1.4 mM, and equivalent volumes of supernatants were concentrated 50-fold using Centricon-30 filters (Millipore, Billerica, MA). The cell pellet was resuspended in 1.4 mM phenylmethylsulfonyl fluoride and incubated at room temperature with gentle shaking for 1 hour. This suspension was centrifuged for 15 min at $10,000 \times g$, and the supernatant was removed. This supernatant contained the cell surface-associated protein extract. The remaining cell pellet was resuspended in 0.5% sodium dodecyl sulfate, disrupted in a Branson sonicator at 60°C for 15 min, passaged through a 26-gauge needle 10 times, and centrifuged at $10,000 \times g$ for 10 min. The supernatant from this preparation contained cell-associated protein extract. All preparations (supernatant, cell surface-associated extract, and cell-associated extract) were stored at -20°C until analysis. Samples were heat denatured and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), and probed with monoclonal antibody 3H1 (44) with standard alkaline phosphatase visualization (19).

Pathogenicity in salmon. Fish used in the study were a cohort of chinook salmon from the stock at the Soos Creek Hatchery (Washington Department of Fish and Wildlife, Auburn, Wash.). Adult fish were screened at spawning for the presence of *R. salmoninarum* antigens in kidney tissue by an enzyme-linked immunosorbent assay (ELISA) (30). Eggs from 21 females with no detectable *R. salmoninarum* antigen were fertilized with milt from 21 males with no detectable *R. salmoninarum* antigen and selected for the study. Gametes were transported on ice to the Western Fisheries Research Center, Seattle, Wash., for fertilization and hatching and were reared for about 6 months after hatching. Fish were maintained in sand-filtered, UV-treated Lake Washington water at 12°C throughout the study and were fed ad libitum with a pelleted, semimoist commercial salmon diet (Bio-Oregon, Warrenton, OR).

R. salmoninarum strains used for the challenges included the parental ATCC type strain 33209, a clone with an *msa1* disruption (AMC1), and a clone with an *msa2* disruption (AMC2). Challenge cultures were grown in modified KDM2 broth as previously described (32), except that the culture medium for AMC1 and AMC2 contained kanamycin at $50 \mu\text{g ml}^{-1}$. The cultures were grown for 5 to 7 days at 15°C with stirring to an OD_{525} of ≥ 0.6 and then were harvested by centrifugation at $5,000 \times g$ for 20 min and resuspended in 0.01 M phosphate-buffered saline, pH 7.4 (PBS), supplemented with 0.1% peptone (PBS-peptone). Bacteria were enumerated by the membrane filtration fluorescent antibody technique (10) and diluted to the appropriate concentration in PBS-peptone. Bacterial viability was confirmed by culture plate counts. Because differences in growth rates among the *R. salmoninarum* strains created difficulties in achieving synchronous bacterial growth of all strains on a given date, separate injection and immersion challenges were conducted comparing AMC1 to 33209 and AMC2 to 33209.

For each injection challenge, fish (mean weight, 3.6 g) were anesthetized with tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA) and injected intraperitoneally with $50 \mu\text{l}$ of PBS-peptone (vehicle control) or an equal volume of one of the *R. salmoninarum* isolates at 1×10^6 bacteria fish $^{-1}$. One hundred fish were included in each injection group. Fish were held in 122-liter circular, flowthrough tanks at 12°C and monitored daily for 115 days. Kidneys from mortalities were tested for the presence of *R. salmoninarum* by a direct fluorescent antibody test (30) and by plate culture to confirm cause of death.

For each treatment group in the immersion challenge, 100 fish (4.6 g mean weight) were placed in 8 liters of water containing 40 ml of one of the *R. salmoninarum* strains or an equal volume of PBS-peptone (vehicle control). Each treatment was performed in duplicate at a final challenge concentration of 1×10^7 bacteria ml $^{-1}$ in a static system for 24 h. The challenge water temperature was maintained at 12°C , and oxygen was continuously supplied to each bucket during challenge. At the end of the challenge, fish were placed in 122-liter circular, flowthrough tanks at 12°C and monitored daily for 150 days. Kidneys from mortalities were tested for the presence of *R. salmoninarum* by direct fluorescent antibody test and plate culture. Kidneys from fish surviving at the end of the postchallenge monitoring period were randomly subsampled (30 per tank, 60 per treatment) and analyzed individually for *R. salmoninarum* antigen levels by ELISA (30). The genotypes of all challenge strains and the bacteria recovered from mortalities were confirmed by PCR and Southern blot hybridization.

Survival curves were estimated by the Kaplan-Meier algorithm and compared by the log rank test. For comparison of *R. salmoninarum* antigen levels in fish testing positive by the ELISA within a given challenge group, a log transformation was first applied to ELISA absorbance values to achieve common variances of means among various data groups (11). Student's *t* test was then used to compare *R. salmoninarum* antigen levels between the two groups of fish exposed to *R. salmoninarum* in a challenge.

RESULTS

Transformation of *R. salmoninarum*. Because previous work demonstrated *R. salmoninarum* could be stably transformed by single-crossover homologous recombination at a very low frequency (33), we chose to use an IDM approach. Sequence analysis of the *msa* fragment in the IDM construct pACP41 identified a single base insertion positioned 90 bp from the upstream primer site, creating an in-frame stop codon with the remainder of the *msa* ORF. Because both copies of *msa* have identical ORFs, homologous recombination could occur with either *msa1* or *msa2*. Parallel aliquots of type strain ATCC 33209 were electroporated with no DNA, a plasmid lacking an insert (pZErO2.1), a plasmid previously used in homologous recombination experiments with *R. salmoninarum* (pLDR109), or pACP41. Ten weeks after growth under selection, colonies were visible for the electroporation with pLDR109 or pACP41, but not for the electroporation with pZErO2.1 or with no DNA. The cells from these colonies were gram-positive diplococci bacilli that were labeled with anti-*R. salmoninarum* polyclonal antibody. The integration frequency of pACP41 was $108 \text{ CFU } \mu\text{g}^{-1} \text{ DNA}$.

Genotype of transformed *R. salmoninarum*. The candidate recombinant clones were screened by PCR with either an *msa1*-specific or *msa2*-specific primer with a vector primer for evidence of homologous recombination. Amplification by *msa1*-specific PCR generated a 1.35-kb product from some clones, whereas amplification by *msa2*-specific PCR generated a 1.04-kb amplicon from other clones. Amplification with the primer pairs was mutually exclusive, and neither pair generated a product from DNA from the parental 33209 strain (Fig. 1). The observed 1.35-kb and 1.04-kb amplicons were consistent with predicted patterns resulting from homologous recombination at the *msa1* or *msa2* locus, respectively, and clones with potential integrations were further analyzed by Southern blot hybridization and sequence analysis.

Southern blot hybridization with an *msa* ORF probe revealed two patterns, and both patterns differed from that for the parental 33209 strain. For a BamHI digestion, one pattern lacked the 5.1-kb band specific for *msa1* (32) and displayed novel 2.4-kb and 6.4-kb bands, while the 7.0-kb band that is specific for *msa2* was unaltered (Fig. 2). This pattern is consis-

FIG. 1. PCR amplifications specific for the *msa1* (left image) or *msa2* (right image) locus. The upstream primer for each reaction was specific for one *msa* locus, and the downstream primer was specific for vector pZeRO2.1. Expected product sizes were 1.35 kb for *msa1* integration and 1.05 kb for *msa2* integration. Templates used were the parental ATCC type strain 33209 (lane 1), clones with *msa2* integrations (lanes 2 and 3), and clones with *msa1* integrations (lanes 4, 5, and 6). The molecular mass marker (lane 7) is a 1-kb ladder (New England BioLabs), and marker sizes (in kilobases) are shown to the right of each image.

FIG. 2. Southern blot of 1 µg of chromosomal DNA from the parental strain and selected integration clones hybridized with an *msa* ORF probe. Chromosomal DNAs, digested with the indicated enzyme, were from the parental ATCC type strain 33209 (lanes 1), one clone bearing *msa1* integrations (lanes 2), and one clone bearing *msa2* integrations (lanes 3). Lane s1 contains molecular mass marker II (Boehringer Mannheim), and relative masses are labeled to the left. Lane s2 contains molecular mass marker VII (Boehringer Mannheim), and relative masses are labeled to the right. Sizes are in kilobases. Blot images were captured with an ImageStation 440CF using 1D Image Analysis software, v. 3.5.3 (Kodak).

tent with integration of pACP41 into the *msa1* locus (Fig. 3A). The second pattern lacked the *msa2*-specific 7.0-kb BamHI band and displayed novel 4.0-kb and 6.7-kb bands, while the *msa1*-specific 5.1-kb band was unaltered (Fig. 2). This pattern is consistent with integration of pACP41 into the *msa2* locus (Fig. 3B). Hybridization with chromosomal DNA digested with HindIII or XhoI also yielded dual pattern results (Fig. 2), which corroborated the predictions of integration into *msa1* or *msa2* (Fig. 3). Hybridization with a probe generated from an empty pZeRO2.1 vector confirmed the presence of vector sequences of the expected restriction fragment size (data not shown).

The 5' and 3' integration sites of selected clones were amplified with combinations of vector primers and primers specific to *msa1*, *msa2*, or the *msa* ORF, and the amplicons were sequenced. The integrations were precise to the nucleotide (data not shown). Furthermore, the point mutation introduced in pACP41 permitted determination of the position of the *msa* fragment in pACP41 within the chromosomal integration. For each *msa* integration, the cloned *msa* fragment was located 3' to the plasmid sequences and the homologous chromosomal sequences (Fig. 3).

Expression of MSA in transformed *R. salmoninarum*. The IDM construct pACP41 was designed to disrupt expression from either *msa* gene upon integration. Because both *msa1* and *msa2* are transcriptionally active, a decrease in MSA expression was expected if one locus was inactivated. MSA protein expressed in broth cultures can be fractionated into three compartments: MSA released from the cell (supernatant), MSA weakly associated with the cell surface (cell surface associated), and MSA strongly associated with the cell (cell associated). MSA expression of the parental ATCC type strain 33209 was compared with expression from clones with disruption of *msa1* (AMC1) or *msa2* (AMC2). Equivalent numbers of cells were harvested from late-logarithmic-phase broth cul-

FIG. 3. Schematic representation of the organization of the chromosomal integration sites at the *msa1* locus (A) or *msa2* locus (B). Chromosomal sequences are represented by thick lines, and plasmid sequences are represented by thin lines. The plasmid gene conferring resistance to kanamycin is labeled "kan." The *msa* ORF fragment in pACP41 is a shaded box. The approximate position of an in-frame stop codon is indicated by a short, vertical arrow. The position of the *msa* ORF probe used in Southern hybridizations is shown by a diagonally hatched box beneath the *msa1* figure. Restriction sites are indicated beneath each figure as follows: B, BamHI; H, HindIII; X, XhoI.

tures (OD₅₂₅ of 0.9 to 1.2), and the three protein fractions were isolated and analyzed by Western blot analysis with a monoclonal antibody against MSA. Surprisingly, the levels of MSA from all three fractions were not significantly different among the parental strain, AMC1, and AMC2 (Fig. 4).

Virulence of integration clones in chinook salmon. In separate injection challenges, we compared the parental type strain, 33209, to either AMC1 (challenge 2) or AMC2 (challenge 1), and both challenges were terminated at 115 days postinjection. The parental type strain caused significantly greater mortality throughout the study period ($P < 0.001$; log rank test) than either of the two mutants (Fig. 5a and b). At the conclusion of the challenges, survival among fish exposed to AMC1 was 74%, and among fish exposed to AMC2 it was 85%, whereas survival among fish exposed to the parental type strain 33209 was 21% and 16%, respectively. In comparison, survival in the PBS-peptone-injected control groups exceeded 98%. Median survival among fish exposed to the parental wild-type strain 33209 was 65 days in each challenge.

At the conclusion of the immersion challenges (150 days after exposure), survival was 94% to 95% in groups of fish exposed to any of the *R. salmoninarum* isolates, with no significant difference in survival among the *R. salmoninarum*-challenged groups ($P > 0.05$). Survival in the PBS-peptone

control groups was 99%. A subsample of surviving fish in each treatment group was analyzed for *R. salmoninarum* antigens in the kidney tissue by ELISA, and $\geq 98\%$ of fish exposed to either AMC1 or AMC2 or to the parental type strain 33209

FIG. 4. Western blot of cell-associated extract (CA), cell surface-associated extract (CSE), and supernatant (SUP) from broth cultures of the parental strain (lanes 1), a clone bearing an *msa1* disruption (lanes 2), and a clone bearing an *msa2* disruption (lanes 3). Cell-equivalent amounts of protein for each strain were analyzed, and MSA protein was identified with monoclonal antibody. Lane m contains molecular mass standards, and indicated sizes are in kilodaltons. The blot image was captured with an ImageStation 440CF using 1D Image Analysis software, v. 3.5.3 (Kodak).

FIG. 5. Cumulative percent survival in groups of chinook salmon challenged by intraperitoneal injection with PBS-peptone (vehicle control) or one of the *R. salmoninarum* strains. Fish were held in 12°C water and monitored daily for 115 days after challenge; data markers are shown at 10-day intervals. (a) Survival of fish injected with the parental type strain ATCC 33209 or with a clone bearing an *msa2* disruption (AMC2). (b) Survival of fish injected with the parental type strain 33209 or with a clone bearing an *msa1* disruption (AMC1).

tested positive for *R. salmoninarum* antigens (data not shown). However, Student's *t* comparison of transformed ELISA ODs from tissues of infected fish showed that bacterial antigen levels in fish exposed to either AMC1 or AMC2 were significantly lower ($P < 0.0001$ and $P = 0.012$, respectively) than levels in fish exposed to the parental wild-type strain.

Isolates were cultured from challenge mortalities, and for challenges with the integration clones isolates were recovered both with and without kanamycin selection. The *msa* genotype of isolates recovered from mortalities was characterized by PCR and by Southern blot analysis. In all cases, the *msa* genotype of the recovered isolates matched that of the challenge strain (data not shown).

DISCUSSION

Although the MSA protein has been previously implicated as an important virulence factor in BKD, this study provides the first empirical evidence that each *msa* gene is necessary for full pathogenicity by *R. salmoninarum*. The MSA protein has been implicated as a significant factor in pathogenicity of *R. salmoninarum*, in part because of its abundance on the bacterial surface and its high immunogenicity. MSA mediates agglutination of eukaryotic cells (salmon leukocytes, trout spermatozoa, and rabbit erythrocytes) and bacterial autoagglutination (5, 7, 15, 43, 44), and greatly reduced levels of MSA are associated with loss of agglutination and lowered virulence (5, 24, 38, 45). The role of MSA in BKD pathogenicity is unknown, although its putative activities include immunosuppression (3, 15, 41), proteolytic activity (34), and modulation of the macrophage respiratory burst (9).

The injection challenge experiments were monitored far beyond the median survival of the parental wild-type strain and demonstrated that each mutant had significantly attenuated virulence. Isolates recovered from mortalities retained the *msa* genotype of the respective challenge strain, indicating that the integrations are stable through disease progression and subsequent culture without selection. Because chromosomal regions known to be benign for *R. salmoninarum* function have not yet been identified, our design did not include plasmid integrations into other sites for comparative virulence testing. The growth

of both *msa* mutants in broth culture is indistinguishable from the parental strain (data not shown), suggesting that the plasmid sequences are not inhibitory. However, the effects of the integrated plasmid sequences during infection and disease are unknown and may contribute to the attenuated phenotype.

In contrast to the injection challenges, the immersion challenges resulted in low mortality during the 150-day monitoring period, even for the parental wild-type strain. This is not surprising, considering the chronic nature of BKD; the mean time to death may exceed 200 days for fish challenged by immersion with virulent *R. salmoninarum* at an average water temperature of 12.6°C (23). Significantly lower levels of bacterial antigens detected in kidneys from surviving fish challenged with AMC1 or AMC2 in comparison to 33209 could be indicative of a reduced bacterial ability to establish infection, effective clearance by the host, or slower proliferation by the bacterium. The finding that $\geq 89\%$ of surviving fish challenged with any of the *R. salmoninarum* strains showed medium to high *R. salmoninarum* antigen levels ($OD_{405} \geq 0.200$) (11, 30) at the termination of the monitoring period suggested that all strains of the bacterium proliferated to some extent in the majority of fish after immersion exposure. Nevertheless, because ELISA cannot distinguish between antigens of live and dead *R. salmoninarum* organisms and antigens from the bacterium can persist for at least 110 days in the absence of live bacteria (31), the ELISA analysis could not determine whether reduced antigen levels in fish challenged with AMC1 or AMC2 resulted from slower proliferation of the mutant strains, from more effective clearance of these strains by the host after initial infection, or from a combination of factors. Further investigation of the ability of AMC1 or AMC2 to establish infections through horizontal transmission may require more rapidly efficacious exposure conditions, such as cohabitation with severely infected fish (21, 23), and the use of a method such as culture for enumeration of viable *R. salmoninarum* in fish.

Disruption of the *msa* locus was accomplished by insertion-duplication mutagenesis with a plasmid containing 600 bp of cloned *msa* sequence and a selectable kanamycin marker. There is physical evidence that homologous recombination and integration of this plasmid disrupted the targeted gene loci. The integrations into the chromosomal loci were precise, re-

sulting in a drastically truncated *msa* ORF (694 bp out of 1,677 bp) adjacent to the endogenous promoter and a partial *msa* ORF lacking both the endogenous promoter and the first 94 bp positioned 3' to the integrated plasmid sequences (Fig. 2). Although it is possible that a promoter(s) within the plasmid sequence could have initiated transcription into the downstream, partial *msa* ORF, the in-frame stop codon introduced by PCR within the *msa* fragment in the IDM construct would halt translation near the beginning of the ORF (Fig. 2). Furthermore, evidence of truncated MSA products unique to either IDM mutant was not observed in Western blot analysis of protein fractions.

Our previous attempts to employ transposon mutagenesis (e.g., Tn10 and Tn5) for gene disruption were unsuccessful, suggesting that common transposases may not be active in *R. salmoninarum*. We chose to exploit IDM, a method successfully used for over 20 years (22, 26), to disrupt bacterial gene expression because we had previously observed homologous recombination in *R. salmoninarum* (33). Our IDM plasmid was not specifically designed to permit transcription through the cloned inserts to ensure nonpolar mutations (18). However, there is a 97-bp hairpin structure starting 6 bp upstream of the *msa* ORF stop codon in each chromosomal *msa* locus that presents a strong transcriptional termination motif (6), suggesting that the IDM was unlikely to have a polar effect in the direction of *msa* transcription at either locus. There is no strong termination motif in the 5'-flanking regions of either locus, and it is formally possible that the observed attenuated phenotypes are due to polar effects on genes positioned 5' to both *msa* genes. To date, no plasmids have been identified in *R. salmoninarum*, eliminating the opportunity to test for polar effects through complementation.

The absence of an effect upon *in vitro* expression of MSA by the disruption of either locus could be a result of posttranscriptional, translational, or posttranslational regulation, because previous analysis showed that both loci are transcribed *in vitro* (33). Both *msa* loci have identical sequences extending from 40 bp upstream of the ORF through 225 bp downstream of the ORF, and our efforts to identify unique transcribed sequences for each *msa* locus have been unsuccessful (unpublished observations), suggesting that transcription initiates within common sequences. The requirement for both genes for full virulence may be due to differential regulation of gene expression or the protein level *in vivo*.

In summary, we have used IDM to disrupt expression from each chromosomal locus of *msa* and provided evidence that each locus is required for full virulence of *R. salmoninarum*. If MSA interferes with an effective host response to infection, ablation of MSA is predicted to result in a highly attenuated strain that may be a vaccine candidate against BKD. Therefore, targeting a double mutant at both *msa* loci is a priority.

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