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Identification of a Third *msa* Gene in *Renibacterium salmoninarum* and the Associated Virulence Phenotype

Linda D. Rhodes,* Alison M. Coady,† and Rebecca K. Deinhard‡

Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, United States Department of Commerce, Seattle, Washington

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***Renibacterium salmoninarum*, a gram-positive diplococcobacillus, causes bacterial kidney disease, a condition that can result in extensive morbidity and mortality among stocks of fish. An immunodominant extracellular protein, called major soluble antigen (MSA), is encoded by two identical genes, *msa1* and *msa2*. We found evidence for a third *msa* gene, *msa3*, which appears to be a duplication of *msa1*. Unlike *msa1* and *msa2*, *msa3* is not present in all isolates of *R. salmoninarum*. The presence of the *msa3* locus does not affect total MSA production in culture conditions. In a challenge study, isolates possessing the *msa3* locus reduced median survival in juvenile chinook salmon (*Oncorhynchus tshawytscha*) by an average of 34% at doses of $\leq 10^5$ cells per fish compared to isolates lacking the *msa3* locus. In contrast, no difference in survival was observed at the highest dose, 10^6 cells per fish. The phenotype associated with the *msa3* locus and its nonuniform distribution may contribute to observed differences in virulence among *R. salmoninarum* isolates.**

Bacterial kidney disease (BKD) is a persistent, debilitating disease in salmonid fishes. *Renibacterium salmoninarum*, the etiological agent of BKD, accumulates primarily in the kidney, causing edema, granuloma development, and membranous glomerulonephritis (36) and resulting in morbidity and mortality (17). The prevalence of chinook salmon (*Oncorhynchus tshawytscha*) infected with *R. salmoninarum* can be as high as 90% in hatcheries (41) and 91% in outmigrating river populations (27). BKD is a persistent infectious disease problem in salmon culture (26), affecting wildlife conservation and restoration efforts. Twenty-six stocks of Pacific salmon and steelhead have been designated threatened or endangered by the United States federal government (23), and restoration activities such as captive rearing are hampered by recurrent outbreaks of BKD.

Fish infected with *R. salmoninarum* mount an antibody response that is directed principally against a 57-kDa extracellular protein (5) called major soluble antigen (MSA). MSA has been implicated as a major pathogenicity determinant that is involved in host immunosuppression (6, 15, 19, 40, 45), leukocyte agglutination (43, 44), and virulence (7, 24, 37). MSA is encoded by two identical genes, *msa1* and *msa2* (25), and both genes are transcriptionally active (31). During a survey of *R. salmoninarum* isolates for mutations in the two *msa* genes, we discovered evidence of a third *msa* gene that was not present in all isolates. In this study we characterized the third *msa* gene and identified an associated virulence phenotype.

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

† Present address: Peace Corps/Zambia, Kasama, Zambia.

‡ Present address: Pacific States Marine Fisheries Commission, Portland, OR 97202.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 26 isolates of *R. salmoninarum* were used in this study. The geographic origin and source of each isolate are shown in Table 1. Liquid cultures were grown in modified KDM2 broth (1.0% Bacto Peptone [Difco], 0.05% yeast extract, 0.05% L-cysteine; adjusted to pH 6.5) (12) at 15°C. Plate cultures were grown on modified KDM2 agar (1% Bacto Peptone, 0.05% yeast extract, 0.05% L-cysteine, 10% newborn calf serum, 5% *R. salmoninarum*-conditioned medium [13]; adjusted to pH 7.5) at 15°C. Culture purity was determined by Gram staining, and identities were confirmed by direct fluorescent antibody staining with an anti-*R. salmoninarum* polyclonal antibody (Kirkegaard and Perry). Cultures recovered from the pathogenicity challenges (see below) for species confirmation and genotyping were inoculated onto selective KDM2 (SKDM2) agar, which contained modified KDM2 agar as described above supplemented with cycloheximide (final concentration, 50 μg ml^{-1}), D-cycloserine (final concentration, 12.5 μg ml^{-1}), polymyxin B (final concentration, 25 μg ml^{-1}), and oxolinic acid (final concentration, 2.5 μg ml^{-1}) (1).

Chromosomal DNA analysis. Chromosomal DNA was prepared as previously described (32) and was analyzed by standard Southern blotting (35). Probes were labeled with digoxigenin-11-dUTP, and hybridized probes were detected by chemiluminescence according to the manufacturer's instructions (Boehringer Mannheim). The *msa* open reading frame (ORF) probe was synthesized by random prime labeling of a 1.06-kb BglII fragment from *msa1* cloned into pCO2 (25). Probes specific for sequences flanking the *msa1* ORF were synthesized by PCR with pCO2 and the following primers pairs: for the 5′ flanking sequences, 5′-GAAGCTGAACTGACTGA-3′ and 5′-AACAGGGCCACCAGATC-3′; and for the 3′ flanking sequences, 5′-GAAAGCCACAAACCGTCTGCTG-3′ and 5′-AACCAACTACAGAGACAGCGGTA-3′. Sequence analysis was performed by cycle sequencing with a Big Dye terminator kit (version 3.1; Applied Biosystems) and analysis with an ABI 3100 sequencer. The 5′ portion of *msa3* was derived by excising and eluting the 8.4-kb XhoI fragment from isolate MMMvir and amplifying a 1.0-kb product with primers 5′-GTTTCTGAGGGACGGGCCGC-3′ and 5′-GTCTTAGGCTCGAGAGTTGCTGCTGT-3′; this product was subjected to sequence analysis. The 3′ portion of *msa3* was derived by excising and eluting the 4.5-kb XhoI fragment from isolate MMMvir and cloning it into vector pCR2.1 (Invitrogen) for subsequent sequence analysis.

Protein analysis. Equivalent numbers of *R. salmoninarum* cells in broth cultures from early to late logarithmic growth (optical densities at 525 nm between 0.12 and 1.35) were harvested by centrifugation for 20 min at 10,000 × *g*, and dry pellets were stored at −20°C until analysis. The volume of the harvested culture supernatant was adjusted on the basis of the optical density at 525 nm of the culture. Phenylmethylsulfonyl fluoride (Sigma) was added to a final concentration of 1.4 mM, and samples were stored at −20°C until analysis. Cells pellets were prepared for analysis by resuspending cells in 1× sample buffer (0.31 M Tris[pH 6.8], 2.5% glycerol, 0.5% sodium dodecyl sulfate,1.25% β-mercapto-

TABLE 1. Geographic origins, years of isolation, and sources of *R. salmoninarum* isolates used in this study

Isolate	Geographic origin	Year of isolation	Source[a]
ATCC 33209^T	Leaburg Hatchery, Oregon	1974	D. G. Elliott (WFRC)
Bonneville-1	Bonneville Hatchery, Oregon	1993	C. R. Banner (OSU)
Bonneville-5	Bonneville Hatchery, Oregon	1998	C. R. Banner (OSU)
BPA2001-6031	Hood Canal, Washington	2001	S. C. Corbett (NWFSC)
BPA2001-6050	Hood Canal, Washington	2001	S. C. Corbett (NWFSC)
Carson5b	Carson Hatchery, Washington	1993	C. R. Banner (OSU)
Cow-ChS-94	Cowlitz Hatchery, Washington	1994	C. R. Banner (OSU)
D6	Oregon State University	1982	C. R. Banner (OSU)
DWK90	Dworshak Hatchery, Idaho	1990	D. G. Elliott (WFRC)
DWK91	Dworshak Hatchery, Idaho	1991	D. G. Elliott (WFRC)
GL64	Lake Michigan	1990	D. G. Elliott (WFRC)
91-127 Idaho	Redfish Lake captive broodstock, Oregon	1991	C. R. Banner (OSU)
Lake Billy Chinook	Lake Billy Chinook, Oregon	1998	C. R. Banner (OSU)
Little Goose	Little Goose Dam, Washington	1984	C. R. Banner (OSU)
Lookingglass	Lookingglass Hatchery, Oregon	1993	C. R. Banner (OSU)
Lost18	Lostine River captive broodstock, Oregon	1999	F. T. Poysky (NWFSC)
LR95	Lostine River captive broodstock, Oregon	1999	C. R. Banner (OSU)
Marion Forks	Marion Forks Hatchery, Oregon	1994	C. R. Banner (OSU)
MK91	MacKenzie Hatchery, Oregon	1991	C. R. Banner (OSU)
MMMvir	Seattle, Wash.	1999	M. M. Moore (NWFSC)
MT239	Aberdeen, Scotland	1988	D. G. Elliott (WFRC)
Round Butte	Round Butte Hatchery, Oregon	1991	C. R. Banner (OSU)
SAW91	Sawtooth Hatchery, Idaho	1991	D. G. Elliott (WFRC)
ss-ChS-94-1	South Santiam Hatchery, Oregon	1994	C. R. Banner (OSU)
W88B2	McNary Dam (water), Oregon/Washington	1989	D. G. Elliott (WFRC)
Willamette	Willamette Hatchery, Oregon	1993	C. R. Banner (OSU)

[a] Abbreviations: WFRC, Western Fisheries Research Center, Seattle, Wash.; OSU, Oregon State University, Corvallis; NWFSC, Northwest Fisheries Science Center, Seattle, Wash.

ethanol). Supernatants were concentrated by using Centriprep-30 or Centricon-30 spin concentrators (Amicon) according to the manufacturer's instructions; in this procedure we made sure that samples from a given sampling time received equivalent concentrations. Sample buffer was added to the supernatants to obtain preparations containing 1× (final concentration) sample buffer. Samples were heat denatured at 99°C for 10 min and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the standard immunoblot method with alkaline phosphatase visualization (20). MSA was detected with monoclonal antibody 3H1 (43).

BKD ELISA. The level of *R. salmoninarum* antigens in the kidney was determined by an enzyme-linked immunosorbent assay (ELISA) (28, 29) by using a goat antibody directed against whole *R. salmoninarum* (Kirkegaard and Perry). For detection we used horseradish peroxidase activity measured at 405 nm. The threshold value for negative controls was 0.0896.

Pathogenicity in salmon. A cohort of juvenile fall chinook salmon (*O. tshawytscha*) was reared to smoltification and transferred to seawater approximately 6 months after hatching. The fish were reared for an additional 8 months in seawater to a mean weight of 15.3 g and to a mean length of 108.4 mm without significant morbidity or mortality. Prior to challenge, 10 fish were examined for the presence of *R. salmoninarum* in kidney tissue by a nested PCR assay (8), and all fish were negative. For the challenge, fish were inoculated intraperitoneally with 0.1 ml of peptone-saline (0.1% Bacto Peptone, 0.85% NaCl) containing no bacteria (vehicle control) or peptone-saline containing *R. salmoninarum* at a concentration of 10^4, 10^5, 10^6, or 10^7 cells ml^{-1}. Twenty fish were used in the vehicle control groups. For each isolate and dose, 15 to 19 fish were inoculated. Cell concentrations were determined by a membrane fluorescent antibody technique (11), and viability was confirmed by culture on modified KDM2 agar. The fish were held in 3-foot-diameter, circular, flowthrough tanks receiving ambient, UV-treated seawater, and the temperature ranged from 8 to 11.5°C from the beginning to the end of the experimental period. The fish were fed 2-mm grower diet (Ewos) at a rate of 2% of body weight on alternate days. Dead fish were removed daily from the tanks, stored on ice, and necropsied within 48 h of removal. Bacteria were recovered from the kidney by plating on SKDM2 agar, and then the entire kidney was removed and stored at −20°C for subsequent BKD ELISA analysis. The identity of bacteria on SKDM2 agar was confirmed by fluorescent antibody microscopy with a polyclonal antibody directed against *R. salmoninarum* (Kirkegaard and Perry). Mortality was attributed to BKD if viable *R. salmoninarum* was recovered on SKDM2 agar or the BKD ELISA value was greater than or equal to 0.5. No BKD mortality was observed among fish inoculated with peptone-saline without bacteria. The duration of the experiment for each treatment group was as follows: 10^6 cells per fish, 85 days; 10^5 cells per fish, 92 days; 10^4 cells per fish, 102 days; and 10^3 cells per fish, 106 days. Kaplan-Meier survival analysis was used to estimate the median length of survival (in days) and the proportion of susceptible fish surviving at the end of the experimental period. Survival curves were compared by the log rank test.

RESULTS

Identification of a third *msa* locus. The occurrence of two copies of the *msa* gene with identical ORFs has been known for several years (25), and these two copies can be distinguished by Southern analysis of genomic DNA digested with BamHI (Fig. 1A). The *msa* ORF contains a single XhoI restriction site, located near the middle of the ORF (nucleotides 727 to 732 in the 1,677-nucleotide ORF), and Southern analysis of XhoI-digested DNA from type strain ATCC 33209^T with an *msa* ORF probe revealed hybridizing bands at 2.7, 6.4, and >17 kb (Fig. 1A). Sequence analysis of the regions flanking the *msa1* and *msa2* ORFs and Southern analysis with copy-specific probes identified the 2.7-kb band as the 5′ portion of *msa2* (data not shown) and the 6.4-kb band as the 5′ portion of *msa1* (Fig. 1A). The hybridizing fragment larger than 17 kb actually consists of two bands (G. Wiens, personal communication), representing the 3′ portions of both *msa1* and *msa2*.

Surprisingly, some isolates of *R. salmoninarum* displayed two additional hybridizing bands at 4.5 and 8.4 kb after XhoI digestion. Because Southern analysis of BamHI-digested DNA from these isolates revealed only two *msa* loci, we predicted that the third copy was a duplication of either *msa1* or *msa2*.

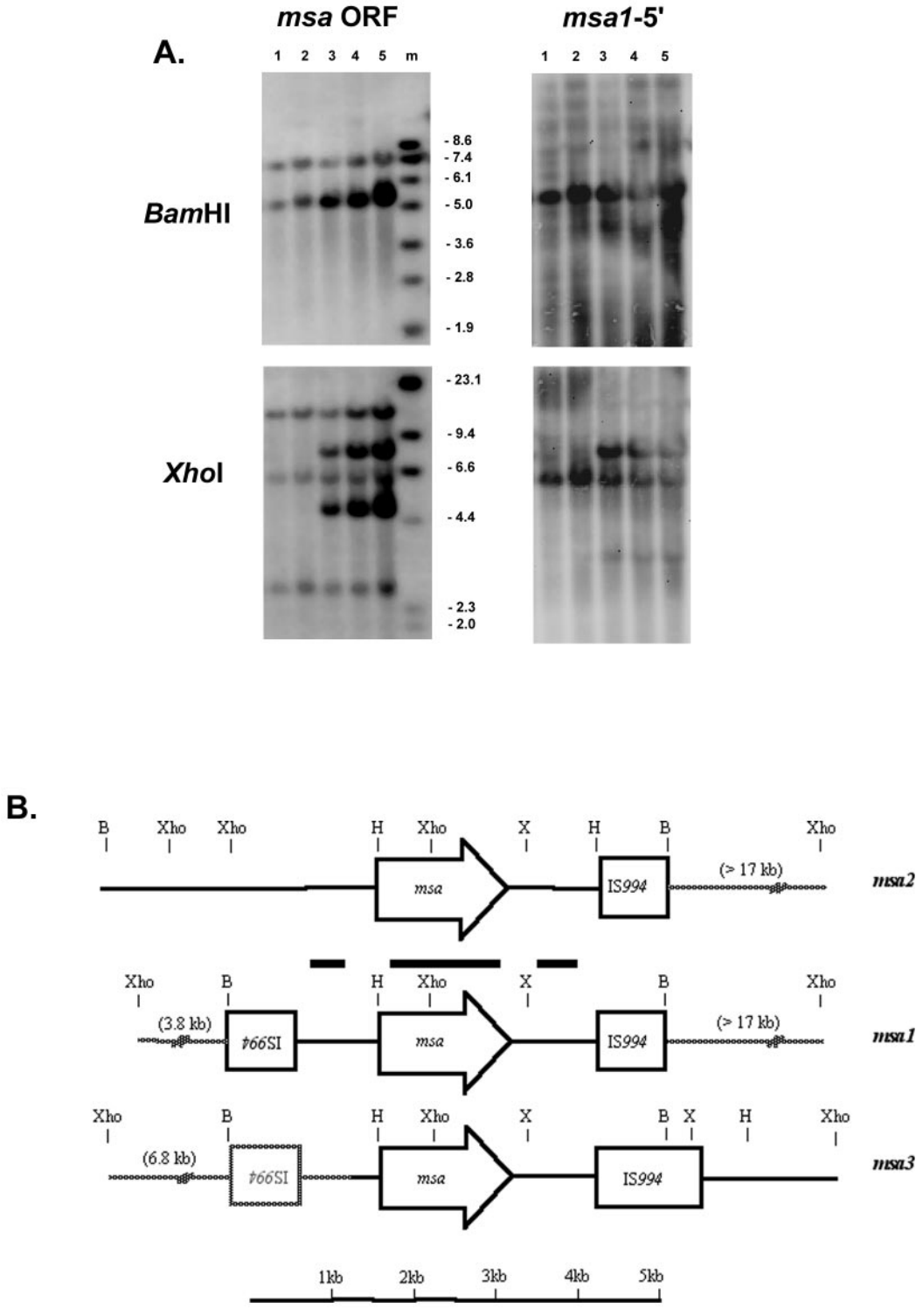

FIG. 1. Demonstration of a third *msa* locus in some isolates of *R. salmoninarum*. Blot images were captured with an ImageStation 440CF by using the 1D Image Analysis software, version 3.5.3 (Kodak). (A) Southern blot analysis of 1 μg of genomic DNA from five isolates of *R. salmoninarum*. The blots on the left were hybridized to an *msa* ORF probe and then stripped and hybridized to a probe specific for sequences located 5′ to the *msa1* ORF (blots on the right). The DNA in the upper blots was digested with BamHI, and the DNA in the lower blots was digested with XhoI. Lane 1, ATCC 33209^T; lane 2, Marion Forks; lane 3, MMMvir; lane 4, Bonneville-1; lane 5, Carson 5b. The restriction digestion used is indicated at the left. Lane m contained molecular mass marker VII (Boehringer Mannheim) in the upper blot and molecular mass marker II (Boehringer Mannheim) in the lower blot. Sizes (in kilobases) are indicated next to lane m. (B) Schematic diagram comparing the three *msa* loci based on sequence analysis (solid black lines) or Southern analysis (gray lines). Restriction sites are indicated as follows: Xho, XhoI; B, BamHI; H, HindIII; X, XbaI. The solid bars above *msa1* show the positions (from left to right) of the *msa1* 5′-specific probe, the *msa* ORF probe, and the *msa1* 3′-specific probe.

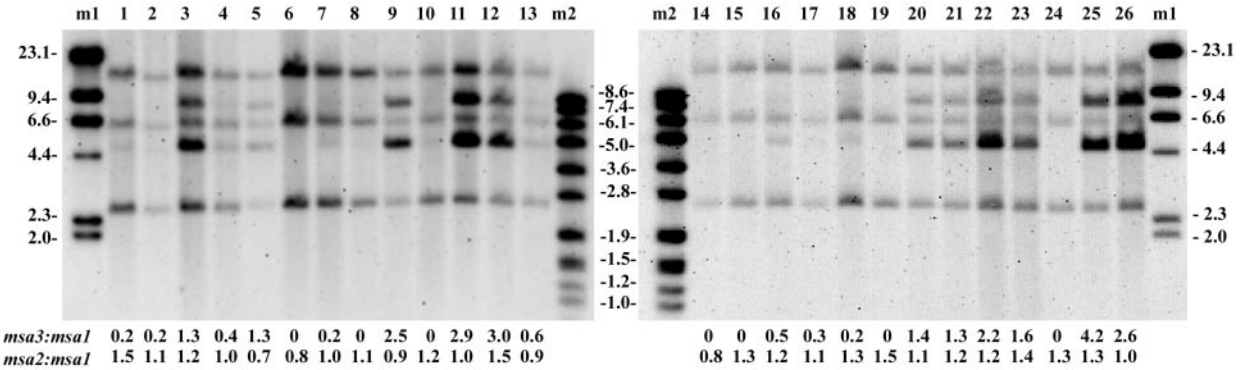

FIG. 2. Distribution of the *msa3* locus in 26 isolates of *R. salmoninarum*: Southern blot analysis of 500 ng of XhoI-digested genomic DNA with an *msa* ORF probe. Lane 1, Little Goose; lane 2, D6; lane 3, 91-127; lane 4, Bonneville-1; lane 5, Lookingglass; lane 6, Round Butte; lane 7, LR-95; lane 8, Marion Forks; lane 9, Lake Billy Chinook; lane 10, Bonneville-5; lane 11, MK91; lane 12, ss-ChS-94-1; lane 13, Cow-ChS-94; lane 14, W88B2; lane 15, GL64; lane 16, Sawtooth91; lane 17, DWK91; lane 18, DWK90; lane 19, MT239; lane 20, BPA2001-6031; lane 21, BPA2001-6050; lane 22, Lost18; lane 23, MMMvir; lane 24, ATCC 33209^T; lane 25, Carson5b; lane 26, Willamette. Lanes m1 contained molecular mass marker II (Boehringer Mannheim), and lanes m2 contained molecular mass marker VII (Boehringer Mannheim). Sizes (in kilobases) are indicated next to the gels. Blot images were captured with an ImageStation 440CF by using the 1D Image Analysis software (version 3.5.3; Kodak). The ratios of densitometric measurements for the 8.4-kb band to densitometric measurements for the 6.4-kb band (*msa3*:*msa1*) and the ratios of densitometric measurements for the 2.7-kb band to densitometric measurements for the 6.4-kb band (*msa2*:*msa1*) are shown beneath the lanes.

For isolates displaying the two additional XhoI bands, the *msa* ORF probe exhibited increased hybridization signal intensity for the 5.0-kb BamHI band corresponding to *msa1*, suggesting that there were more copies of *msa1* than of *msa2* (Fig. 1A). Region-specific probes for *msa1* showed that the 4.5-kb band hybridized to the 3′ portion of *msa1* (data not shown) and the 8.4-kb band hybridized to the 5′ fragment of *msa1* (Fig. 1A). The 4.5-kb XhoI band was directly cloned and sequenced, which revealed that this fragment indeed contained 943 nucleotides of the *msa* ORF and the 3′ flanking sequences unique to *msa1*. The isolated 8.4-kb XhoI fragment was used to generate a 1.0-kb PCR product encompassing 301 bp of the 5′ flank and the first 729 bp of the *msa* ORF, including the XhoI site that was in the downstream primer. This PCR product was sequenced and was found to be identical to the corresponding region of *msa1*, confirming the Southern analysis observations. From these results, we concluded that the third copy of *msa* is a duplication of *msa1*. Because the context of this third copy differs from that of *msa1*, we provisionally designated the third copy *msa3*. A schematic diagram comparing the three loci is shown in Fig. 1B.

To assess the distribution of *msa3* in *R. salmoninarum*, we examined 26 isolates obtained from a variety of geographic locations and at different times (Table 1) by Southern analysis. The majority (19 of 26) of the isolates examined exhibited the three-locus genotype (Fig. 2). The origins of the isolates displaying the two-locus genotype varied and included locations in Oregon (Round Butte, Marion Forks, ATCC 33209^T), on the Columbia River (Bonneville-5, W88B2), on Lake Michigan (GL64), and in Scotland (MT239). Because the probe used in the Southern analysis whose results are shown in Fig. 2 was derived from the *msa* ORF, which is identical at the three *msa* loci, the hybridization signal intensity was expected to be proportional to the number of copies for each locus. For isolates with the *msa3* locus, the densities of the hybridization signal intensity for the 8.4-kb band (*msa3*), the 2.7-kb band (*msa2*), and the 6.4-kb band (*msa1*) were determined, and the density ratios of the *msa3* and *msa1* bands and of the *msa2* and *msa1* bands were calculated. For the 19 isolates, the average *msa3*/*msa1* ratio was 1.42 with a coefficient of variation of 83.9%, while the average *msa2*/*msa1* ratio for hybridization signal intensity was 1.14 with a coefficient of variation of 19.3%. Thus, while the average *msa3*/*msa1* and *msa2*/*msa1* ratios of signal intensities were similar, the coefficient of variation for the *msa3*/*msa1* ratio was four times greater than that for the *msa2*/*msa1* ratio. These results suggest that the *msa3* copy number is not constant among the isolates.

In vitro phenotype of isolates with *msa3*. The presence of a third copy of *msa* may affect in vitro characteristics of *R. salmoninarum*, such as replication. The growth of two isolates with two *msa* loci (type strain ATCC 33209^T and Marion Forks) and four isolates with three *msa* loci (MMMvir, Bonneville-1, Carson 5b, and 91-127 Idaho) was compared daily for up to 4 days by visible light spectrophotometry. No significant differences in the growth rate were observed for the two the genotypes (data not shown).

The protein encoded by *msa*, MSA, has been implicated as a virulence factor in the pathogenesis of BKD, and previous work has shown that *msa1* and *msa2* are expressed in vitro (31). If a protein is expressed from *msa3*, the levels of MSA may be greater in isolates bearing *msa3* than in isolates bearing only *msa1* and *msa2*. We compared two isolates with the two-locus genotype (ATCC 33209^T and Marion Forks) with four isolates having the three-locus genotype (MMMvir, 91-127 Idaho, Bonneville-1, and Carson 5b). Broth cultures were harvested as the culture density increased, and total protein was isolated from the culture supernatants and the cell pellets. The MSA levels were assessed by Western blot analysis of equivalent cell amounts from each fraction (Fig. 3). No consistent, detectable differences were observed in any of the fractions at any time during culture growth for any of the isolates, demonstrating that the total in vitro MSA levels were not significantly affected by the presence of *msa3*.

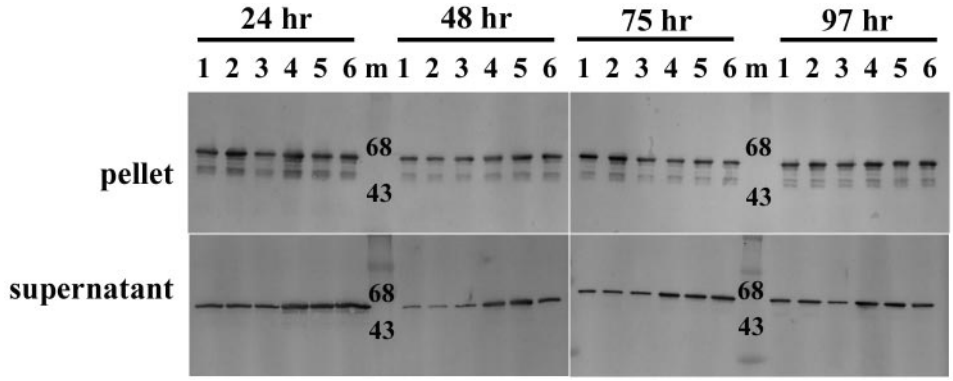

FIG. 3. Western blot analysis for MSA from six isolates of *R. salmoninarum*. Bacterial pellets and supernatants from broth cultures were harvested at the times indicated. Total proteins from equivalent numbers of cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blots were probed with monoclonal antibody 3H1. Lane 1, ATCC 33209^T; lane 2, Marion Forks; lane 3, MMMvir; lane 4, 91-127; lane 5, Bonneville-1; lane 6, Carson5b. Lanes m contained protein size markers (Invitrogen), and the bands corresponding to 68 and 43 kDa are labeled. Blot images were captured with an ImageStation 440CF by using the 1D Image Analysis software (version 3.5.3; Kodak).

In vivo phenotype of isolates with *msa3*. Although the level of MSA in vitro was not affected by the presence of *msa3*, it is possible that the *msa3* locus has an in vivo effect. This possibility was tested by challenging juvenile chinook salmon (*O. tshawytscha*) with isolates bearing *msa3* (MMMvir, Bonneville-1, and Carson 5b) or lacking *msa3* (type strain ATCC 33209^T and Marion Forks). In addition to a mock-challenged group, the following four challenge doses for each isolate were used: 10^6, 10^5, 10^4, and 10^3 bacteria per fish. Mortality due to *R. salmoninarum* was confirmed by both bacterial culture and BKD ELISA analysis of the kidney, and no BKD-associated mortality was observed in the mock-challenged fish.

At the highest dose (10^6 bacteria per fish), there was no statistical difference among the survival curves ($P = 0.6470$, as determined by the log rank test; df = 3), and the median survival times ranged from 30.0 to 36.0 days (Table 2). For the three lower doses, significantly lower levels of survival for fish challenged with isolates bearing *msa3* were observed. The median survival time was reduced by at least 16.5 days at a dose of 10^5 bacteria per fish, by 22.5 days at a dose of 10^4 bacteria per fish, and by 25.0 days at a dose of 10^3 bacteria per fish (Table 2). For these three treatments, the median level of survival was reduced by an average of 34.0% (range, 32.1 to 35.7%) for the fish challenged with isolates bearing *msa3* compared to the fish challenged with isolates lacking *msa3*. The proportions of fish surviving at the end of the experiment were also different for the isolates with and without *msa3*. Few or no fish challenged with isolates bearing *msa3* survived to the end of the experimental period, regardless of the challenge dose. In contrast, substantial proportions of the fish challenged with isolates lacking *msa3* survived to the end of the experimental period in the group that received the lowest dose (Table 2). At each of the three lower doses, there was no significant difference among the survival curves for the three *msa3*-bearing isolates ($P \geq 0.0680$, as determined by the log rank test for each comparison; df = 2). However, the survival curves for type strain ATCC 33209^T and Marion Forks differed significantly from those for the *msa3*-bearing isolates for each of the three dose groups ($P \leq 0.0042$, as determined by the log rank test for each comparison; df = 4).

TABLE 2. Median survival times and proportions of susceptible fish surviving at the end of the experimental challenges with five isolates of *R. salmoninarum*[a]

Challenge dose (cells per fish)	Parameter	ATCC 33209^T (*msa3*)[b]	Marion Forks (*msa3*)	MMMvir (*msa3*$^+$)	Bonneville-1 (*msa3*$^+$)	Carson 5b (*msa3*$^+$)
10^6						
	Median survival time (days)	30.5	30.0	NT[c]	32.0	36.0
	Surviving proportion	0.125	0.156	NT	0.000	0.000
10^5						
	Median survival time (days)	69.0	63.5	47.0	44.0	40.0
	Surviving proportion	0.438	0.000	0.000	0.000	0.000
10^4						
	Median survival time (days)	79.5	81.0	57.0	54.0	52.5
	Surviving proportion	0.500	0.202	0.000	0.056	0.000
10^3						
	Median survival time (days)	>106.0	92.0	63.0	67.0	61.0
	Surviving proportion	0.784	0.412	0.064	0.063	0.063

[a] Values were derived from a Kaplan-Meier survival analysis.
[b] *msa3*, lacking *msa3*; *msa3*$^+$, possessing *msa3*.
[c] NT, not tested.

To assess the genotypes of the recovered bacteria, Southern blot analysis of chromosomal DNA from three to five recovery cultures for each isolate was performed. *R. salmoninarum* recovered from fish challenged with type strain ATCC 33209^T or Marion Forks lacked the *msa3* locus, while bacteria recovered from MMMvir, Bonneville-1, and Carson5b possessed *msa3* (data not shown). Thus, the *msa3* genotypes of the challenge bacteria and the bacteria recovered appeared to be identical.

DISCUSSION

The discovery of another *msa* locus in *R. salmoninarum* is an intriguing finding for this salmonid pathogen, because two functional copies of the *msa* gene have been identified previously. Our characterization indicates that *msa3* is within a 5-kb BamHI region that is a copy of the region containing *msa1*. In the *msa1* locus, the BamHI sites are located within inverted, flanking IS*994* elements (32). Because all isolates possess *msa1* but not all isolates possess *msa3*, it is likely that *msa3* was duplicated from *msa1*. The mechanism for this kind of duplication in *R. salmoninarum* is unknown, but the flanking IS*994* elements may be involved (33, 34).

The physical context of the *msa3* locus (i.e., chromosomal or extrachromosomal) was not determined in this study. The apparent stoichiometry of *msa3* to *msa1* varied widely among the isolates examined (Fig. 2), indicating that the *msa3* copy number may vary. The *msa3* locus could be located within an amplified chromosomal region or within mobile DNA, such as a plasmid or a bacteriophage. Chromosomal amplifications can occur spontaneously (34) or can result from selective pressures, such as metabolic demands (22, 30). If MSA is essential for survival, it is possible that amplification producing the *msa3* locus may compensate for defects in *msa1* or *msa2*. Because the genotype of the recovered bacteria was the same as that of the starting challenge bacteria, the *msa3* locus does not appear to be a result of selective pressure during disease progression. Attempts to isolate bacteriophages, repetitive elements, or pathogenicity islands in *R. salmoninarum* have not been reported yet, although there has been a single anecdotal report of a bacteriophage associated with *R. salmoninarum* (16). An effort to isolate plasmid DNA from *R. salmoninarum* was unsuccessful (39), but this attempt involved type strain ATCC 33209^T, which lacks *msa3*. Analysis of sequences flanking the *msa3* locus in the 4.5-kb XhoI fragment resulted in identification of ORFs with significant homology to plasmid partition proteins (our observations), suggesting that in future investigations workers should address the possibility of a plasmid context.

Because *msa1* and *msa2* are transcribed at a range of cell densities under in vitro conditions (31), it was expected that *msa3* would contribute to MSA levels. Although sequence analysis revealed that the promoter region and ORF of *msa3* are identical to those of *msa1*, the total level of MSA protein in cultured cells was not elevated in isolates possessing the *msa3* locus. This unexpected result may have been due to posttranscriptional regulation of total MSA levels, which would mask contributions from *msa3*. The MSA protein can be involved in host immune cell interactions, such as leukocyte adhesion (42, 44), and a role for MSA in pathogenicity has been implicated (7, 24, 37). Close regulation of an important pathogenicity protein could be critical for appropriate function.

While in vitro MSA levels were not affected by the presence of the *msa3* locus, a distinct difference in virulence was observed between isolates possessing *msa3* and isolates lacking *msa3*. Mortality occurred more rapidly in fish challenged with isolates possessing the *msa3* locus, and nearly all of the fish challenged with *msa3*-bearing isolates died before the end of the experimental period (Table 2). Notably, no significant difference in survival was observed among all of the isolates at the highest challenge dose (10^6 cells per fish), indicating that the effect is negligible above a threshold inoculum. If protein encoded by *msa3* contributes to increased virulence, MSA expression must be differentially regulated under in vivo conditions. However, it is possible that the increased virulence is associated with sequences flanking the *msa3* locus rather than with the *msa3* gene itself.

Although the ATCC 33209^T and Marion Forks isolates both lacked *msa3*, ATCC 33209^T exhibited lower virulence than the Marion Forks isolate. The relatively lower pathogenicity of ATCC 33209^T has been observed previously. This isolate has greatly reduced virulence in chinook and coho salmon (*O. tshawytscha* and *Oncorhynchus kisutch*) compared to the virulence of other isolates (38), and it has been reported that ATCC 33209^T does not produce BKD symptoms in rainbow trout (21). Furthermore, ATCC 33209^T is unable to infect the *Epithelioma papillosum* cell line, whereas more virulent isolates of *R. salmoninarum* can invade and multiply in this fish cell line (21). Nonetheless, the ATCC 33209^T strain exhibits biochemical and morphological properties similar to those of more virulent isolates (3, 4, 9, 14, 18), including a capsule (10), and it survives within macrophages as well as more virulent strains (2). The Marion Forks strain was isolated in 1994 and has been subjected to few laboratory passages. In contrast, the ATCC 33209^T strain was isolated in 1974 and has undergone extensive laboratory culture, which may have resulted in a mutation(s) or gene loss(es) that contributes to its relatively reduced virulence. Southern hybridization with a probe located 3′ of the *msa3* locus (i.e., flanking the BamHI site in the 4.5-kb XhoI fragment) hybridized to genomic DNA from the Marion Forks strain, but not to genomic DNA from the ATCC 33209^T strain (data not shown). While the absence of the *msa3* locus is at least partially responsible for the lower virulence of these two isolates, it is likely that there are additional factors not present in ATCC 33209^T that are responsible for its lower-virulence phenotype.

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