Expression of Duplicate msa Genes in the Salmonid Pathogen

Renibacterium salmoninarum

Linda D. Rhodes,* Alison M. Coady, and Mark S. Strom

Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, United States Department of Commerce, Seattle, Washington 98112

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Renibacterium salmoninarum is a gram-positive bacterium responsible for bacterial kidney disease of salmon and trout. R. salmoninarum has two identical copies of the gene encoding major soluble antigen (MSA), an immunodominant, extracellular protein. To determine whether one or both copies of msa are expressed, reporter plasmids encoding a fusion of MSA and green fluorescent protein controlled by 0.6 kb of promoter region from msa1 or msa2 were constructed and introduced into R. salmoninarum. Single copies of the reporter plasmids integrated into the chromosome by homologous recombination. Expression of mRNA and protein from the integrated plasmids was detected, and transformed cells were fluorescent, demonstrating that both msa1 and msa2 are expressed under in vitro conditions. This is the first report of successful transformation and homologous recombination in R. salmoninarum.

Bacterial kidney disease (BKD) in salmonid fish is a debilitating, systemic condition that is characterized by granulomatous lesions, primarily of the kidney and other internal organs. The disease is widely reported throughout North America, the British Isles, northern continental Europe, and Japan (14), with infection prevalences among some hatchery populations in the western United States approaching 100% (8). BKD may be endemic among feral salmon, where the prevalence of infection can exceed 30% (20, 32). The listing of multiple stocks of wild salmon in the United States under the Endangered Species Act has resulted in substantial recovery and restoration efforts, including the rearing of captive broodstock. BKD poses a significant threat to those efforts.

The causative agent of BKD is Renibacterium salmoninarum, a fastidious and slowly growing gram-positive diplococcobacillus (6, 9, 44). The bacterium is transmitted horizontally, probably by a fecal-oral route (1), and vertically in the egg (11, 24). It produces an abundant, 57-kDa protein associated with the extracellular surface (13, 16, 46). This heat-stable protein, called major soluble antigen (MSA), is released by bacteria in situ (45), and it has been associated elsewhere with agglutinating activity (5, 7), immunomodulation (48), and virulence (2). Cloning of the msa gene was reported in 1992 (4), and subsequently it was recognized that two identical copies existed (30), a rare occurrence among prokaryotes. Because of the abundance of the MSA protein, we hypothesized that both copies of the msa gene are expressed.

To test this hypothesis, the ability to transform R. salmoninarum was required. Until now, there has been no report of genetic manipulation of R. salmoninarum. Here, we report the first successful transformation of this bacterium and the chromosomal integration of two reporter plasmids by single-cross-over (SCO) homologous recombination. Based on expression of the integrated plasmids, both msa1 and msa2 are expressed in R. salmoninarum under in vitro culture conditions.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The type strain R. salmoninarum ATCC 33209 (American Type Culture Collection), which expresses abundant levels of MSA (5, 16, 47), was used as the parental strain for transformation. R. salmoninarum isolate MT239 (2, 5, 41) was used in the fluorescence analysis. Liquid cultures were grown in modified KDM2 broth (1% Bacto Peptone, 0.05% yeast extract, 0.05% L-cysteine, 10% newborn calf serum, 5% sucrose) 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% sucrose) at 15°C. Culture purity was determined by Gram staining, and identity was confirmed by direct fluorescent antibody staining with an anti-R. salmoninarum polyclonal antibody (Kirkegaard and Perry Laboratories). Kana-mycin selection was at 50 μg/ml for both liquid and plate cultures. Recombinant strains 42-8.5, 42-8.6, 43-6.1, and 43-6.2 were transformed with pLDR109, and recombinant strains 43-7.1, 43-7.2, 43-8.1, and 43-8.2 were transformed with pLDR111.

Plasmid constructions were performed in Escherichia coli Top10 F’ (F" [lacI 807 (Tn10) [F'),'proAB(mrB-lacBM15 lacZAM15 ΔlacX74 recA1 araD139 endA1]) and transformed into R. salmoninarum. Materials and methods are listed in Table 1. A 722-bp XbaI fragment from pEGFP (Clontech) containing the open reading frame (ORF) of enhanced green fluorescent protein (GFP) was ligated into the XbaI site of plasmid pZErO2.1 (Invitrogen), creating pLDR109. The presumptive promoter regions of msa1 and msa2 were obtained by PCR amplification of pCO2 and pCO4, respectively, which each contain the entire msa ORF and more than 1 kb of flanking sequence (30). The upstream primer for the msa1 PCR was 5’-ATCA TCGGATCCACGGGCTAACCCCGCCCT-3’ and the upstream primer for the msa2 PCR was 5’-ATCAGATGCAGTACGTGACTGCCGCTC-3’. The downstream primer for both reactions was 5’-AAAGGTACCATGGAAGAAT-3’ and the primers included BamHI or NcoI restriction sites near the 3’ end to facilitate cloning. PCR products were ligated into pCRII (Invitrogen) and plasmids were excised and cloned into pLDR104 to create the reporter plasmids. Plasmid pLDR109 contains 558 bp of 5’ sequence flanking the msa1 ORF, 102 bp of the msa ORF, and the entire gfp ORF. Plasmid pLDR111 is identical to pLDR109 except that it contains 580 bp of 5’ sequence flanking the msa2 ORF in place of the msa1 sequences. Plasmids were cycle sequenced with BigDye Terminator reactions (PE Biosystems) to confirm that the plasmids encoded the desired constructs.
Protein analysis. Equivalent numbers of *R. salmoninarum* cells in late logarithmic growth (OD_{623}, between 1.0 and 1.2) were harvested by centrifugation for 10 min at 10,000 × g, and the cell pellets were resuspended and lysed at 99°C in 1× sample buffer (0.3 M Tris, pH 6.8, 2.5% glycerol, 0.5% SDS, 1.25% β-mercaptoethanol). Heat-denatured samples were analyzed by SDS-polyacrylamide gel electrophoresis and the standard immunoblot method with alkaline phosphatase visualization (18). GFP was detected with monoclonal antibody JL-1 (Clontech), and MSA was detected with monoclonal antibody 3H1 (47).

Analysis of fluorescence in transformants. Liquid cultures were grown to an OD_{623} between 0.5 and 0.8, and 0.5 ml of cells was harvested by centrifugation for 10 min at 10,000 × g, washed with 10 mM Tris (pH 7.5), and resuspended in 200 μl of 10 mM Tris (pH 7.5). Serial halving dilutions with 10 mM Tris were made in 96-well immunosassay plates (Costar). Cell density was read at 595 nm with an ELX808I U microplate reader (Bio-Tek), and fluorescence was read (excitation at 485 nm; excitation at 530 nm) with an ELX800 fluorescence microplate reader (Bio-Tek). For each clone, fluorescence units were regressed on optical densities by a simple linear model, and regression slopes were compared by Student’s t test.

RESULTS

**Transformation of *R. salmoninarum*.** Our previous efforts to transform *R. salmoninarum* with a plasmid containing the ColE1 origin of replication and a kanamycin resistance gene suggested that transient transformation was possible (data not shown). The regions immediately upstream of the *msa1* and *msa2* genes (558 and 580 bp, respectively) and 102 bp of the MSA ORF were amplified by PCR and cloned directly adjacent to the GFP ORF, which had been inserted into the commercially available vector pZErO2.1. The fusion protein from this construct would contain the first 34 amino acids of MSA preceding the entire GFP. Plasmid pLDR109 contains the putative promoter sequence from *msa1* and pLDR111 contains the promoter sequence from *msa2*. A control plasmid, pLDR104, contains the GFP reporter without *R. salmoninarum* sequences.

Plasmid DNA was introduced into *R. salmoninarum* ATCC 33209 by electroporation. In addition to pLDR109 and pLDR111, parallel aliquots of bacteria were electroporated with no DNA, pZErO2.1, or pLDR104 as controls. All electroporated aliquots were incubated on KDM2 plates containing kanamycin. By 16 weeks of incubation, no colonies developed from the aliquots that received no DNA. Four weeks after electroporation, colonies were visible at a 20× magnification for all remaining aliquots. However, microscopic colonies from aliquots receiving pZErO2.1 and pLDR104 failed to increase in size with continued incubation up to 16 weeks. After 11 weeks of incubation, larger fluorescent colonies appeared only on plates inoculated with aliquots receiving pLDR109 and pLDR111. The cells from these fluorescent colonies were gram-positive diplococcobacilli and were labeled by the anti-MSA antibody 3H1. This morphology and phenotype are consistent with those of *R. salmoninarum*. Integration frequencies varied between 5.8 × 10⁻⁶ and 8.8 × 10⁻⁶/μg of DNA for six independent transformations.

**Genetic analysis of transformed *R. salmoninarum*.** From the larger fluorescent colonies, eight clones (four from transformation by pLDR109 and four from transformation by pLDR111) were subjected to extraction procedures for chromosomal and plasmid DNA. The plasmid preparations were analyzed by gel electrophoresis and were used to transform *E. coli* for selection by kanamycin resistance. No plasmids were observed by gel electrophoresis, and no kanamycin-resistant *E.

### TABLE 1. Plasmids used

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td>pCO2</td>
<td>Km&lt;sup&gt;′&lt;/sup&gt;; contains a 5-kb BamHI fragment carrying the <em>msa1</em> ORF and at least 1 kb of flanking sequence</td>
<td>30</td>
</tr>
<tr>
<td>pCO4</td>
<td>Km&lt;sup&gt;′&lt;/sup&gt;; contains a 7-kb BamHI fragment carrying the <em>msa2</em> ORF and at least 1 kb of flanking sequence</td>
<td>30</td>
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<tr>
<td>pEGFP</td>
<td>Ap&lt;sup&gt;′&lt;/sup&gt;; enhanced GFP</td>
<td>Clontech</td>
</tr>
<tr>
<td>pZErO2.1</td>
<td>Km&lt;sup&gt;′&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCR1II</td>
<td>Km&lt;sup&gt;′&lt;/sup&gt; Ap&lt;sup&gt;′&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<tr>
<td>pLDR104</td>
<td>Km&lt;sup&gt;′&lt;/sup&gt;; contains an XbaI fragment carrying enhanced gfp ORF as a BamHI-NcoI fragment in pLDR104</td>
<td>This study</td>
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<tr>
<td>pLDR109</td>
<td>Km&lt;sup&gt;′&lt;/sup&gt;; contains 555 bp from <em>msa1</em> promoter and 102 bp of <em>msa ORF</em> as a BamHI-NcoI fragment in pLDR104</td>
<td>This study</td>
</tr>
<tr>
<td>pLDR111</td>
<td>Km&lt;sup&gt;′&lt;/sup&gt;; contains 580 bp from <em>msa2</em> promoter and 102 bp of <em>msa ORF</em> as a BamHI-NcoI fragment in pLDR104</td>
<td>This study</td>
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Transformation of *R. salmoninarum*. Plasmid DNA was prepared with column filters for plasmid purification (Qiagen) and was resuspended in water to a concentration of 100 ng/μl. *R. salmoninarum* liquid cultures grown for 5 to 7 days to an optical density at 525 nm (OD<sub>525</sub>) of 1.0 to 1.5 were harvested by centrifugation for 20 min at 10,000 × g. Pellets were resuspended in an equal volume of glycerol medium (modified KDM2 broth supplemented with 0.5 M sucrose, 20 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, and 1% glycerine), and the cells were incubated with agitation at 15°C for 45 to 48 h. On the day of electroporation, 1.5-mL aliquots of the glycerinated cells were centrifuged and washed once with electroporation solution (0.5 M sucrose with 10% glycerol). Washed cell pellets were resuspended in 50 μl of electroporation solution and equilibrated at 15°C for at least 15 min. Plasmid DNA (500 ng in 5 μl) was added, and the mixture was transferred to a 1-mm cuvette (Invitrogen) which had been equilibrated to 15°C. A 33209 by electroporation. In addition to pLDR109 and pLDR111, parallel aliquots of bacteria were electroporated with no DNA, pZErO2.1, or pLDR104 as controls. All electroporated aliquots were incubated on KDM2 plates containing kanamycin. By 16 weeks of incubation, no colonies developed from the aliquots that received no DNA. Four weeks after electroporation, colonies were visible at a 20× magnification for all remaining aliquots. However, microscopic colonies from aliquots receiving pZErO2.1 and pLDR104 failed to increase in size with continued incubation up to 16 weeks. After 11 weeks of incubation, larger fluorescent colonies appeared only on plates inoculated with aliquots receiving pLDR109 and pLDR111. The cells from these fluorescent colonies were gram-positive diplococcobacilli and were labeled by the anti-MSA antibody 3H1. This morphology and phenotype are consistent with those of *R. salmoninarum*. Integration frequencies varied between 5.8 × 10⁻⁶ and 8.8 × 10⁻⁶/μg of DNA for six independent transformations.
coli colonies were isolated. These results suggested that the plasmids may have integrated, in whole or in part, into the R. salmoninarum chromosome. To test this possibility, chromosomal DNA from the eight clones was analyzed by Southern blotting. Among the eight clones, the hybridization pattern of BamHI-digested DNA was identical with that of either the msa1 or gfp probe. The pattern observed was consistent with integration into the corresponding msa promoter region by homologous recombination (Fig. 1C).

If the integration occurred by precise homologous integration, the junction between the cloned promoter and the chromosomal promoter sequences could be assessed. One method of assessment that we performed was by PCR with one primer positioned within the gfp ORF and the second primer posi-
tioned in the chromosomal region flanking the cloned msa promoter (see primer positions in Fig. 1C). Only an integration into the homologous region would result in a PCR product, and the sizes of the expected PCR products would be 813 bp (for msa1) and 874 bp (for msa2). Integrations that varied 50 or more bp from the homologous site would result in a detectably larger PCR product or no product. PCR of chromosomal DNA from all eight clones generated products of the predicted sizes, whereas the parental strain and the two reporter plasmids failed to produce specific PCR products (Fig. 2). From these results, we conclude that the regions from the msa genes that are cloned into the gfp reporter plasmids are sufficient to mediate homologous recombination by SCO in R. salmoninarum.

The stability of the integrated plasmids was tested by successive passage in broth culture without kanamycin selection. After 2 weeks in the absence of selection (approximately 14 doublings), chromosomal DNA was analyzed by Southern blotting as previously described. The hybridization patterns with the msa and gfp probes were identical to those in Fig. 1A and B (data not shown), suggesting that the integrated plasmids do not require selection for maintenance.

Expression of GFP in transformed R. salmoninarum. The purpose of the reporter plasmids was to determine whether one or both of the msa genes were expressed. Northern blot analysis of total RNA with a gfp probe showed steady-state transcripts from all eight of the transformed strains (Fig. 3). The size of these transcripts was approximately 800 bp, which is consistent with the transcription start site occurring within the putative promoter regions of the msa genes.

To determine whether protein from the reporter plasmid was expressed in R. salmoninarum, mid-logarithmic-phase broth cultures of each of the eight clones were harvested, and equivalent numbers of cells were lysed and examined for GFP and MSA expression by immunoblot analysis. All eight of the clones displayed a cell-associated, 27- to 29-kDa protein that reacted with anti-GFP monoclonal antibody JL-1, while the parental strain 33209 lacked this protein (Fig. 4A). In contrast, all eight clones and the parental strain expressed abundant quantities of MSA protein (Fig. 4B). There was no evidence that the cell-associated levels of MSA protein were significantly affected by the integration of either reporter plasmid.

To determine whether the transformed R. salmoninarum clones displayed increased fluorescence as a result of GFP expression, serial dilutions of broth cultures of the transformed clones, the parental strain 33209, and another untransformed isolate of R. salmoninarum (MT239) were measured for fluo-
rescence (excitation at 485 nm, emission at 530 nm) and for cell density (OD 595). For each strain, a regression of fluorescence on cell density was determined, and the regression slopes and y intercepts were compared. Although untransformed \textit{R. salmoninarum} exhibits a slight autofluorescence, the transformed clones displayed significantly greater fluorescence ($P < 0.01$) than did the untransformed isolates of \textit{R. salmoninarum} (Fig. 5). There were no significant differences in regression slopes or y intercepts among the eight transformed clones ($P > 0.05$). Epifluorescence microscopy of the transformed clones revealed that all eight clones were visibly fluorescent either as colonies on plates or in wet mounts of resuspended cells (data not shown). Based on these results, we reach several conclusions. First, GFP is expressed from each of the integrated reporter constructs. Second, the expressed GFP is properly folding because increases in fluorescence can be observed. Finally, the levels of expression of GFP from the two \textit{msa} promoters are similar.

**DISCUSSION**

Until now, there have been no reports of genetic manipulation of \textit{R. salmoninarum}. The ability to introduce DNA into this problematic, gram-positive pathogen would open a new arena of research. This is the first report of both successful transformation and site-specific integration of exogenous DNA into the \textit{R. salmoninarum} chromosome. Chromosomal analyses show that the reporter plasmids integrated by an SCO event, perhaps through a Campbell insertion mechanism (3).

Based on the integrated GFP reporter plasmids, both \textit{msa1} and \textit{msa2} are expressed under in vitro culture conditions. This finding was not unexpected, because the ORFs of \textit{msa1} and \textit{msa2} are identical (30), suggesting that there may be selective pressure on both copies. While gene duplication is widely observed among bacterial genomes (23, 42), the occurrence of identical copies of genes is unusual except for ribosomal DNA and tDNA (34). Among genes with high (>95%) nucleotide identity, all copies are typically expressed. Duplicate methane monooxygenase gene clusters in \textit{Methylococcus capsulatus} Bath are nearly identical (99.996%), and disruption of any of the genes within either cluster reduced the growth rate, indicating that each copy was expressed (40, 43). Similarly, insertional inactivation of nearly identical (99.879%) \textit{amoA} genes within duplicated ammonia monooxygenase gene clusters in \textit{Nitrosomonas europaea} showed that both copies are expressed (19, 28). Duplicate genes \textit{tufA} and \textit{tufB} both appear to contribute to the synthesis of the encoded gene product, elongation factor EF-Tu, a highly abundant cytoplasmic protein in \textit{E. coli} (12, 15, 39, 50). Gene duplication is a way to amplify production of a gene product or to ensure the synthesis of an essential, multifunctional protein. Expression of both \textit{msa1} and \textit{msa2} may be
required for high levels of MSA. In vivo, MSA occurs in such high abundance that it is the principal antigen recognized by a polyclonal immunosorbent diagnostic assay for *R. salmoninarum* in salmon kidney tissue (31). Gene duplication also provides a way to differentially regulate expression of the same protein. For example, lysyl-tRNA synthetase genes in *E. coli* are differentially expressed, with *lysS* displaying constitutive behavior and *lysU* exhibiting inducible behavior (21, 37). While both copies of *msa* have identical sequences up to 40 bp upstream of the ORF, the sequences beyond are quite divergent. If promoters or other regulatory sequences occur in the divergent region, *msa1* and *msa2* may be differentially expressed under in vivo conditions.

The differences in the amounts of GFP from the reporter genes and MSA from the endogenous *msa* genes are striking. Because MSA is a high-abundance protein both in culture and in infected fish (35, 45), we expected GFP expression to be substantial. Although the GFP encoded by the integrated genes has a reported in vivo half-life of >24 h (27), it is possible that GFP stability was reduced by the presence of the MSA sequences at the amino terminus of the fusion protein. Alternatively, the fusion protein may lack sequences that confer stability on the MSA protein. Although MSA can undergo proteolysis at elevated temperatures, it exhibits no degradation after 10 h at 15°C (17, 36), the optimal culture temperature for *R. salmoninarum*. The high levels of MSA observed in tissues of infected fish (up to 200 μg/g) and its stability at ambient environmental temperatures suggest that MSA is persistent under a range of conditions.

Each of the reporter plasmids integrated into the targeted *msa* gene copy. As a result, the endogenous 5’-flanking sequence was preserved, and the *msa-gfp* ORF fusion was subject to the full promoter context for each *msa* gene. In this respect, an integrated reporter is superior to an extrachromosomal reporter gene because no assumptions about the size or context of the promoter need to be made. While SCO integration is more frequently used for gene inactivation or heterologous gene expression, this and other studies (29) demonstrate its utility for monitoring promoter activity.

The vector plasmid pZErO2.1 and the promoterless GFP reporter construct pLDR104 failed to produce integrants, suggesting that the plasmids lacked sequences that ensured sustained extrachromosomal replication. However, we found evidence that the ColE1-based plasmid could persist in *R. salmoninarum* for a transient period. The appearance of microscopic kanamycin-resistant colonies of *R. salmoninarum* after 4 weeks of incubation in all electroporation groups, except the group receiving no DNA, indicated that the plasmids had not been rapidly destroyed by an endogenous restriction system and that the kanamycin gene was expressed. These microscopic colonies failed to enlarge with prolonged (>16 weeks) incubation, leading us to speculate that the initial transformants may have received multiple plasmids which were subsequently segregated to daughter cells. As a result, the ColE1-based plasmid behaved as a suicide vector, favoring integrations under selective pressure.

The frequency of recombinants obtained was extremely low. This may be a limitation of the length of the homologous regions in the reporter plasmids, a site-dependent effect on recombination frequency, or a result of suboptimal electroporation conditions. The transformation efficiency by plasmid insertion among gram-positive bacteria demonstrates a strong dependence on the length of the homologous site (25). Because the 600- to 700-bp homologous regions used in our GFP reporter plasmids are well above the shortest lengths of homology capable of recombination observed for *Lactococcus sake* (300 bp [26]), *Streptococcus pneumoniae* (96 bp [25]), and *Bacillus subtilis* (70 bp [22]), we conclude that low recombination efficiency was not due to this factor. In order to determine whether there is a site-dependent effect on recombination by the *msa* promoter regions, comparisons with similarly sized inserts from other parts of the *R. salmoninarum* chromosome would be needed. When this comparison was made for *S. pneumoniae*, there was a very small site-dependent effect on recombination frequency among nondisrupted or nonessential, disrupted gene targets (25). Because only one of the *msa* gene copies was targeted by each reporter plasmid and MSA protein expression was not reduced, it is not likely that the integrated
reporter plasmids disrupted an essential gene function. This suggests that any site-dependent effect on recombination frequency was not strong. On the other hand, suboptimal electrotransformation conditions would exert a strong influence on the frequency of recombinants recovered. While insertional mutagenesis of targeted genes is now possible by the method described here, generating random mutagenic libraries with a high degree of saturation will require substantial improvements in transformation efficiency.

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