

# Analysis of *p51*, *groESL*, and the Major Antigen P51 in Various Species of *Neorickettsia*, an Obligatory Intracellular Bacterium That Infects Trematodes and Mammals

Yasuko Rikihisa,<sup>1\*</sup> Chunbin Zhang,<sup>1</sup> Manuel Kanter,<sup>1</sup> Zhihui Cheng,<sup>1</sup>  
Norio Ohashi,<sup>1†</sup> and Takeo Fukuda<sup>2</sup>

Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio,<sup>1</sup>  
and Miyazaki Prefecture Institute for Public Health and Environment, Miyazaki, Japan<sup>2</sup>

Received 18 February 2004/Returned for modification 11 April 2004/Accepted 22 April 2004

**The *p51* gene that encodes the major antigenic 51-kDa protein in *Neorickettsia risticii* was identified in strains of *Neorickettsia sennetsu* and the *Stellantchasmus falcatus* agent but not in *Neorickettsia helminthoeca*, suggesting that *p51*-based diagnosis would be useful to distinguish among them. *groESL* sequencing results delineated the phylogenetic relationships among *Neorickettsia* spp.**

*Neorickettsia* spp. are obligatory intracellular bacteria and belong to the family *Anaplasmataceae*, in the order *Rickettsiales*. Currently, three species are recognized in the genus *Neorickettsia*, namely, *N. risticii*, *N. sennetsu*, and *N. helminthoeca* (4). The ecology and transmission of *Neorickettsia* spp. are unique among bacteria, in that this agent parasitizes both trematodes and mammals (17, 18). In mammals, these bacteria reside within cytoplasmic vacuoles, primarily in monocytes in the blood and in macrophages of lymphoid or other tissues, and they can cause systemic diseases. *N. helminthoeca* causes salmon poisoning disease, an acute and highly fatal disease of domestic and wild canidae (17). *N. risticii* causes Potomac horse fever, an acute diarrheal disease of horses (18). *N. sennetsu* (6, 10) causes human sennetsu rickettsiosis. In addition, the SF agent isolated directly from the metacercaria of *Stellantchasmus falcatus* trematodes that encyst within gray mullet fish (7, 21, 22) belongs to the genus *Neorickettsia*. The adult stage of *S. falcatus* can parasitize the human intestine (8). Despite the wide environmental distribution of *Neorickettsia* spp. and their importance to public health and veterinary medicine, few molecular and antigenic markers have been identified for this group of bacteria.

*N. risticii*, *N. sennetsu*, SF agent, and *N. helminthoeca* are antigenically cross-reactive, and inoculation with *N. sennetsu* protects horses from Potomac horse fever (15, 21). However, other than approximate molecular sizes, the nature of these cross-reacting antigens is unknown. A 51-kDa protein (P51) is the major antigen recognized in horses with Potomac horse fever (19). P51 is encoded by the *p51* gene, which is not found in any other bacteria based on a search of the GenBank database, and has been found in all *N. risticii* strains identified to date (2, 5, 9, 11). P51 is predicted to be an outer membrane protein by PSORT analysis (<http://psort.nibb.ac.jp/>). There-

fore, *p51* may be a diagnostically important gene for *N. risticii*. However, several critical issues have never been addressed. These include the following: (i) whether the *p51* gene exists in other *Neorickettsia* species, (ii) whether the P51 protein is expressed by the organisms isolated from cultures that can be used as diagnostic antigens, and (iii) whether P51 is antigenically cross-reactive among *Neorickettsia* spp. Therefore, the present study was designed to address these questions and to delineate the phylogenetic relationships among *Neorickettsia* spp. *Neorickettsia* strains used in this study are shown in Table 1.

***p51* and *murE*.** We obtained a total of seven new DNA sequences comprising *p51*, the intergenic space, and an upstream open reading frame. These include the following: a 2,463-bp DNA fragment (relative positions 75 to 2,534 of *N. risticii* 90-12) of *N. risticii* Illinois<sup>T</sup>; 2,438-bp fragments (relative positions 22 to 2,443 of *N. risticii* 90-12) of the *N. sennetsu* Miyayama<sup>T</sup>, Nakazaki, and 11098 strains; a 1,481-bp fragment (relative positions 899 to 2,376 of *N. risticii* 90-12) of the Kawano strain of *N. sennetsu*; and two 2,417-bp fragments (relative positions 22 to 2,444 of *N. risticii* 90-12) from the SF agent Hirose in DH82 cells that were frozen in 1994 (21) and the Oregon strain isolated from the frozen spleen of a dog, which was fed with trout caught in Oregon in 1990.

TABLE 1. *Neorickettsia* species used in this study

Species	Strain	Origin	Source or reference
<i>N. risticii</i>	Illinois <sup>T</sup>	Horse blood, Md.	ATCC
<i>N. sennetsu</i>	Miyayama <sup>T</sup>	Human blood, Japan	G. Dasch <sup>a</sup>
<i>N. sennetsu</i>	11098	Human blood, Malaysia	G. Dasch
<i>N. sennetsu</i>	Nakazaki	Human blood, Japan	S. Yamamoto <sup>b</sup>
<i>N. sennetsu</i>	Kawano	Human blood, Japan	S. Yamamoto
SF agent	Hirose	Metacercaria, Japan	21
SF agent	Oregon	Trout, Oreg.	This study
<i>N. helminthoeca</i>	Oregon <sup>T</sup>	Salmon, Oreg.	16
<i>N. helminthoeca</i>	California 1	Dog blood, Calif.	This study
<i>N. helminthoeca</i>	California 2	Dog blood, Calif.	This study

<sup>a</sup> Centers for Disease Control and Prevention, Atlanta, Ga.

<sup>b</sup> Miyazaki Prefecture Institute for Public Health and Environment.

\* Corresponding author. Mailing address: Department of Veterinary Biosciences, The Ohio State University, 1925 Coffey Rd., Columbus, OH 43210. Phone: (614) 292-5661. Fax: (614) 292-6473. E-mail: rikihisa.1@osu.edu.

† Present address: Laboratory of Environmental Microbiology, Institute of Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan.

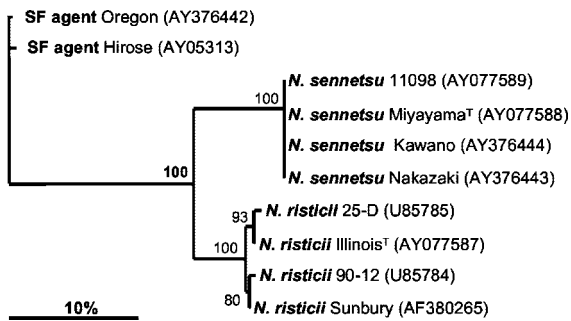


FIG. 1. Phylogram of *Neorickettsia* spp. based on *p51* sequence comparisons. GenBank accession numbers are shown in parentheses. Numbers beside the internal nodes indicate the percentage of 1,000 bootstrap replicates that supported the branch. Bar, the percentage of divergence.

Unexpectedly, the *p51* DNA sequences among four *N. sennetsu* strains, including three strains from Japan (Miyayama, Nakazaki, and Kawano), and one strain from Malaysia (11098) shared 100% identity. The *p51* sequences of an SF strain from Japan and an SF agent strain from the state of Oregon shared 99.3% identity. The 1,461 bp (including gaps) of almost-complete *p51*s were compared among four *N. risticii* strains, four *N. sennetsu* strains, and two SF agent strains, showing clustering within each species (Fig. 1). Although more-divergent *p51* sequences from other *N. risticii* strains are available (2, 9, 11, 14), they are too short to be included in this comparison.

The partial open reading frame was located 408 bp upstream of *p51* in the *N. risticii* Illinois<sup>T</sup> and 90-12 strains, 425 bp upstream of *p51* in the *N. sennetsu* Miyayama<sup>T</sup>, 11098, and Nakazaki strains, and 418 bp upstream of *p51* in the Hirose and Oregon SF agents. This element was identified as the *murE* gene (BlastP E values of 9e-07 to *murE* of *Brucella melitensis*), which encodes UDP-*N*-acetylmuramoylalanyl-D-glutamate-2,6-diamino-pimelate ligase. Comparison of a 418-bp fragment of the *murE* sequence (relative positions 10011 to 10428 of *Rickettsia rickettsii murE*; BlastP E values of 4e-06; GenBank accession number AJ293314) in these seven *Neorickettsia* spp. showed identity levels ranging from 84.0 to 100.0% (Fig. 2).

The *p51* gene in *N. helminthoeca* Oregon<sup>T</sup> as well as in the

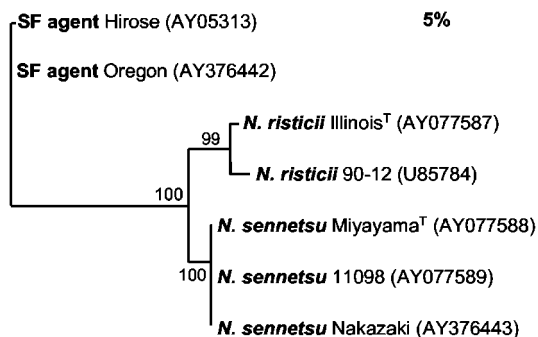
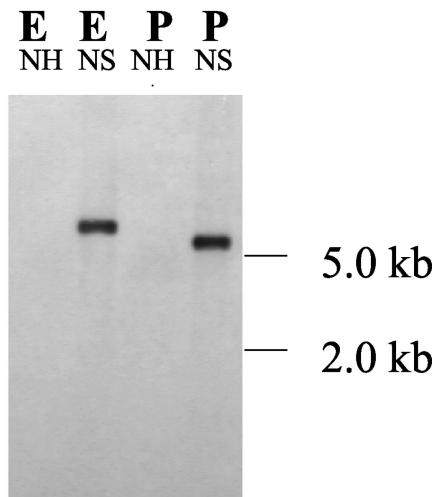


FIG. 2. Phylogram of *Neorickettsia* spp. based on *murE* sequence comparison. GenBank accession numbers are shown in parentheses. Numbers beside the internal nodes indicate the percentage of 1,000 bootstrap replicates that supported the branch. Bar, the percentage of divergence.



P: *Pst* I  
E: *EcoR* I  
NH: *N. helminthoeca*  
NS: *N. sennetsu*

FIG. 3. Southern blot analysis. Samples of genomic DNA from *N. sennetsu* (NS) and *N. helminthoeca* (NH) were digested with either *EcoRI* (E) or *PstI* (P). The DNA probe used to detect the *p51* gene was amplified from *N. sennetsu* genomic DNA and was labeled by digoxigenin, as described in the text. No *p51* band was detected in *N. helminthoeca* Oregon<sup>T</sup>, whereas the *N. sennetsu* Miyayama<sup>T</sup> positive control showed a clear band upon digestion with either *EcoRI* or *PstI*.

two new strains from blood specimens of two dogs that were naturally infected in California in 2003 was not detectable by PCR using primers specific to the conserved regions of *p51*. 16S rRNA sequences from these two strains (443 bp; GenBank accession numbers AY510029 and AY510030) were 99% identical to that of *N. helminthoeca* Oregon<sup>T</sup>. To confirm this result, Southern blot analysis was performed using genomic DNA extracted by the phenol-chloroform method from organisms purified by Percoll gradient centrifugation (12). The blot was hybridized with digoxigenin-labeled *N. risticii* Illinois<sup>T</sup> *p51* (1,153 bp, corresponding to nucleotides 894 to 2046 of the *N. sennetsu* Miyayama *p51*). As positive control, genomic DNA from *N. sennetsu* was digested with either *PstI* or *EcoRI*, whereupon a single clear band was observed. In contrast, when genomic DNA from *N. helminthoeca* Oregon<sup>T</sup> was digested with either of these two restriction enzymes, no band was detected (Fig. 3). This result supports the fact that there is no ortholog of *p51* in *N. helminthoeca* or that *p51* in this organism is highly divergent from that of *N. sennetsu* or *N. risticii*.

**Western immunoblot analysis.** To determine whether P51 is expressed by *Neorickettsia* spp. isolated from cell cultures and whether it is antigenically cross-reactive, we cloned and expressed the entire *p51* from *N. risticii* Illinois<sup>T</sup>, which encodes a mature protein with a molecular mass of 52.8 kDa (after cleavage of a 20-amino-acid signal peptide), by using a pET30a expression vector (Novagen, Inc., Madison, Wis.) as described elsewhere (12).

Histidine-tagged recombinant *N. risticii* Illinois<sup>T</sup> (rP51; *M<sub>r</sub>*

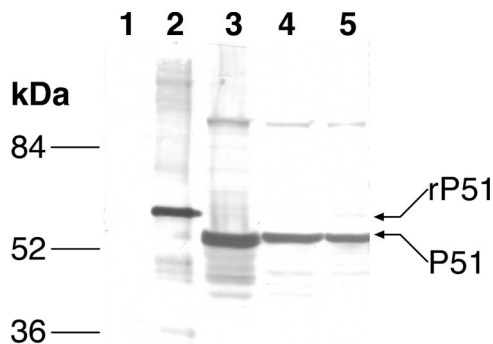


FIG. 4. Detection of P51 levels by Western blot analysis using rabbit anti-*N. risticii* Illinois<sup>T</sup> rP51 serum. Lane 1, P388D<sub>1</sub> cells (negative control); lane 2, *N. risticii* Illinois<sup>T</sup> rP51; lane 3, native *N. risticii* Illinois<sup>T</sup>; lane 4, *N. sennetsu* 11908; and lane 5, *N. sennetsu* Miyayama<sup>T</sup> antigens.

57,128.57 fusion protein) was purified, and an antibody specific to rP51 was prepared in rabbits as described elsewhere (12). Western blot analysis revealed that rabbit anti-rP51 serum strongly reacted with both purified rP51 and with 51-kDa native proteins of *N. risticii* Illinois<sup>T</sup>, *N. sennetsu* Miyayama<sup>T</sup>, and *N. sennetsu* 11908 cultured in P388D<sub>1</sub> cells (Fig. 4). Thus, P51 protein is expressed by the *N. risticii* Illinois<sup>T</sup> and *N. sennetsu* strains in cell culture and is a major cross-reacting antigen between *N. risticii* and *N. sennetsu*.

**groESL sequences.** New sequences totaling 1,914 bp of *N. helminthoeca* Oregon<sup>T</sup> *groESL* (relative positions 191 to 2,104 bp of *N. sennetsu* Miyayama<sup>T</sup> *groESL*) and 2,112 bp of SF agent Hirose *groESL* (relative positions 30 to 2,142 bp of *N. sennetsu* Miyayama<sup>T</sup> *groESL*) were obtained. The intergenic spaces between *groES* and *groEL* of *N. helminthoeca* Oregon<sup>T</sup>, SF agent Hirose, *N. risticii* Illinois<sup>T</sup>, and *N. sennetsu* Miyayama<sup>T</sup> were all -1 bp (i.e., they overlapped). Comparison of 1,296 bp of *groESL* (relative positions 436 to 1,731 bp of *N. sennetsu* Miyayama<sup>T</sup> *groESL*, including 70 bp of *groES* and 1,227 bp of *groEL* with 1-bp overlaps) revealed that a cluster of SF agent Hirose, *N. risticii* Illinois<sup>T</sup>, and *N. sennetsu* Miyayama<sup>T</sup> (identity, 91.2 to 95.4%) is separated from *N. helminthoeca* Oregon<sup>T</sup> (identity, 77.8 to 78.5%) (Fig. 5). We also compared the citrate synthase (*gltA*) DNA sequences (relative positions 1 to 1,323 of *N. risticii* Illinois<sup>T</sup>) available from GenBank. *gltA* DNA sequences from *N. sennetsu* Miyayama<sup>T</sup> (GenBank no. AF304148) and *N. risticii* Illinois<sup>T</sup> (GenBank no. AF304147) shared 94% identity, but there was only 54.3 to 56.2% identity between *N. sennetsu* Miyayama<sup>T</sup> or *N. risticii* Illinois<sup>T</sup> and *N. helminthoeca* Oregon<sup>T</sup> (GenBank no. AF304149).

The present study revealed that *N. helminthoeca* is distinct from the other known *Neorickettsia* species and that *p51* can serve as a molecular and antigenic marker to distinguish this species. This is consistent with newly obtained *groESL* sequences, *gltA* sequences, and the previous observation showing that 16S rRNA gene sequences among *N. risticii*, *N. sennetsu*, and the SF agent share close similarity and are divergent from the 16S rRNA gene sequence of *N. helminthoeca* (13, 21). Significant differences in levels of genetic and antigenic divergence were found among *N. risticii* and *N. sennetsu* strains. It has been previously shown that *N. risticii* strains isolated from

horses are both genetically and antigenically diverse (2, 3, 9, 11). The greatest differences in the nucleotide sequence of 16S rRNA are found between *N. risticii* Illinois<sup>T</sup> and *N. risticii* Bunn (14 different bases), followed by *N. risticii* 081 (10 different bases). Even within the same geographic region, there are diverse strains. For example, comparison of the 16S rRNA sequence in the 081 strain isolated in the state of Ohio is significantly different from that of the other known Ohio strains, and the protein compositions also differ, as determined by Western blot analysis with monoclonal and polyclonal antibodies (3, 20). Sequences of *p51* segments among *N. risticii* strains in the United States are up to ~20% divergent (2, 9, 11, 14). Compared to *N. risticii*, much less is known about the genetic and antigenic diversity of *N. sennetsu* strains. The 16S rRNA gene sequences of *N. sennetsu* Miyayama<sup>T</sup> and *N. sennetsu* 11908 are identical (1), and the present study shows that the *p51* sequences of *N. sennetsu* Miyayama<sup>T</sup> (Japanese 1950 isolate), 11908, Nakazaki, and Kawano were identical, suggesting that *N. sennetsu* human isolates are nearly identical clones. Culturing and sequencing of *p51* sequences derived from *N. sennetsu* Miyayama<sup>T</sup>, *N. sennetsu* 11908, and Nakazaki strains were repeated independently 1 year later from different stock sources with a different laboratory member verifying the results. *N. sennetsu* 11908 was originally isolated in culture (Malaysian 1970 isolate) in the laboratory of M. Ristic at the University of Illinois and was later transferred to the laboratory of E. Weiss at the Naval Medical Research Center (Bethesda, Md.), and stocks frozen on 28 February 1989 were used in this study. The *N. sennetsu*, Nakazaki, and Kawano strains had been kept only at the Miyazaki Prefecture Institute for Public Health and Environment, Miyazaki, Japan, where the Miyayama or 11908 strains were never received. This suggests that only a single strain of *N. sennetsu* identified so far is pathogenic to humans, whereas diverse strains of *N. risticii* can infect and cause disease in horses. The *N. sennetsu* Miyayama strain is not pathogenic to horses and protects the horses from *N. risticii* challenge (15). Thus, the present study contributes to understanding these closely related *Neorickettsia* species of different mammalian host pathogenicity.

**Nucleotide sequence accession numbers.** GenBank accession numbers of sequences obtained in this study are shown in the text and the figures.

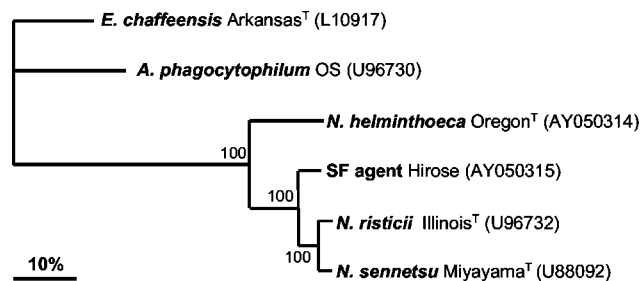


FIG. 5. Phylogram of *groEL* of *Neorickettsia* spp. and two other members of the family *Anaplasmataceae*. GenBank accession numbers are shown in parentheses. Numbers beside the internal nodes indicate the percentage of 1,000 bootstrap replicates that supported the branch. Bar, the percentage of divergence.

This work was supported by grant R01AI47885 from the National Institutes of Health and grant 99-35204-8531 from the United States Department of Agriculture.

We appreciate Shogo Yamamoto for providing *N. sennetsu* Nakazaki and Kawano strains and Gregory Dasch for providing *N. sennetsu* Miyayama and 11098 strains. We also appreciate Ahmet Unver for his help in immunizing the rabbit.

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