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Phylogenetic analysis of *Piscirickettsia salmonis* by 16S, internal transcribed spacer (ITS) and 23S ribosomal DNA sequencing

M. J. Mauel*, S. J. Giovannoni, J. L. Fryer**

Department of Microbiology and The Center for Salmon Disease Research, 220 Nash Hall, Oregon State University, Corvallis, Oregon 97331-3804, USA

ABSTRACT: *Piscirickettsia salmonis*, the etiologic agent of piscirickettsiosis, is a systemic disease of salmonid fish. Variations in virulence and mortality have been observed during epizootics at different geographical regions and in laboratory experiments with isolates from these different locations. This raises the possibility that biogeographical patterns of genetic variation might be a significant factor with this disease. To assess the genetic variability the 16S ribosomal DNA, the internal transcribed spacer (ITS) and the 23S ribosomal DNA of isolates from 3 different hosts and 3 geographic origins were amplified using the polymerase chain reaction (PCR). Results of this analysis confirm that *P. salmonis* is a member of the gamma subgroup of the Proteobacteria and show that the isolates form a tight monophyletic cluster with 16S rDNA similarities ranging from 99.7 to 98.5%. The ITS regions were 309 base pairs (bp), did not contain tRNA genes, and varied between isolates (95.2 to 99.7% similarity). Two-thirds of the 23S rRNA gene was sequenced from 5 of the isolates, yielding similarities ranging from 97.9 to 99.8%. Phylogenetic trees were constructed based on the 16S rDNA, ITS and 23S rDNA sequence data and compared. The trees were topologically similar, suggesting that the 3 types of molecules provided similar phylogenetic information. Five of the isolates are closely related (>99.4% 16S rDNA similarity, 99.1 to 99.7% ITS and 99.3 to 99.8% 23S rDNA similarities). The sequence of one Chilean isolate, EM-90, was unique, with 16S rDNA similarities to the other isolates ranging from 98.5 to 98.9%, the ITS from 95.2 to 96.9% and the 23S rDNA from 97.6 to 98.5%.

KEY WORDS: *Piscirickettsia salmonis* · rDNA · Phylogeny

INTRODUCTION

The bacterium *Piscirickettsia salmonis* is an obligate intracellular parasite and the causative agent of piscirickettsiosis, a systemic disease of salmonids. The pathogen has been reported in Chile (Bravo & Compos 1989, Fryer et al. 1990), Norway (Olsen et al. 1993), Ireland (Rodger & Drinan 1993, Palmer et al. 1997) and the Atlantic and Pacific coasts of Canada (Evelyn 1992, Jones et al. 1998).

The mortality associated with piscirickettsiosis varies with geographic region and host species (Fryer & Lannon 1994). Mortalities between 30 and 90% in netpen reared coho salmon *Oncorhynchus kisutch* have been recorded in Chile (Bravo & Campos 1989), while those in Atlantic salmon *Salmo salar* from the Pacific coast of Canada, Norway and Ireland vary between 0.6 and 15% (Evelyn 1992, Olsen et al. 1993, Rodger & Drinan 1993). Differences in mortality may be due to variation in factor(s) intrinsic to the bacterium, host species resistance, vector species (if present), environmental factors or the mariculture practices that exist between the areas.

At present the molecular phylogenetic placement of *Piscirickettsia salmonis* has been based on the 16S rRNA gene sequence of a single isolate, LF-89^T the

*Present address: Center for Vector-borne Disease, University of Rhode Island, Kingston, Rhode Island 02881, USA
**Addressee for correspondence.
E-mail: fryerj@bcc.orst.edu

type strain (Fryer et al. 1992). This analysis showed that *P. salmonis* is a member of the gamma subdivision of the Proteobacteria, as are the genera *Coxiella* and *Francisella*. The bacteria of the genera *Neorickettsia*, *Rickettsia*, *Cowdria*, *Anaplasma*, and *Ehrlichia* are members of the alpha subdivision of the Proteobacteria. Thus, *Piscirickettsia* strains are expected to share more characteristics with *Coxiella* and *Francisella* than with *Neorickettsia*, *Rickettsia*, *Cowdria*, *Anaplasma*, and *Ehrlichia*.

Comparisons of 16S rDNA sequences have become routine methodology for study of microbial phylogeny and have been used previously to classify members of the Rickettsiaceae (Anderson et al. 1991, Wen et al. 1995a,b). 23S rRNA genes are larger and more variable than 16S rRNA genes and therefore may be useful for phylogenetic comparisons of closely related organisms (Ludwig & Schleifer 1994). The internal transcribed spacer (ITS) regions of rRNA operons also show a large amount of sequence and length variation that is particularly useful for differentiating within species of prokaryotic organisms (Barry et al. 1991, Navarro et al. 1992, Leblond-Bourget et al. 1996, Trevisanato et al. 1996).

To determine if genetic differences occur among isolates from different geographical locations, the 16S rRNA genes and approximately two-thirds of the 23S rRNA genes were sequenced from 5 isolates of *Piscirickettsia salmonis* and the complete ITSs were sequenced from 6 isolates.

MATERIAL AND METHODS

***Piscirickettsia salmonis* isolates.** *Piscirickettsia salmonis* type strain LF-89^T (ATCC VR 1361) was isolated from coho salmon in Chile (Fryer et al. 1990, 1992). Chilean isolate EM-90 was cultured from Atlantic salmon and provided by E. Madrid (Marine Harvest, Puerto Montt, Chile). ATL-4-91 was isolated from Atlantic salmon collected off the east coast of Vancouver Island, British Columbia, Canada (Brocklebank et al. 1992) and was provided by G. Traxler (Pacific Biological Station, Nanaimo, BC). Norwegian isolate NOR-92 was obtained from Atlantic salmon and provided by H. P. Melby (National Veterinary Institute, Oslo, Norway). Chilean isolates SLGO-94 and C1-95 were supplied by P. Smith (University of Chile, Santiago, Chile). SLGO-94 was obtained from rainbow trout *Oncorhynchus mykiss* and C1-95 was isolated from coho salmon.

Culture of bacteria and isolation of DNA. *Piscirickettsia salmonis* was grown at 15°C in the chinook salmon embryo cell line CHSE-214 (Lannan et al. 1984) in antibiotic-free Eagle's minimum essential medium (MEM) with Earle's salts (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) until 90% of the cell sheet was lysed (approximately 14 d). Genomic DNA was isolated using DNA-STAT 60 (Tel-Tex, Inc., Friendswood, TX) following the manufacturer's protocol.

Table 1. Sequences of primers utilized for internal transcribed spacer (ITS) and 23S rRNA gene PCR and sequencing. F: forward; R: reverse

Primer/location	Sequence (5'-3')
PS16SA (1387F)[a]	GCCTTGTACACAACCGCCC
PSI6SH (1516F)[a]	CCTGCGGCTGGATTACCT
PS23SB (507R)[b]	CCTTTCCCTCACGGTCAT
PS23SC (203R)[b]	TAGATGTTTCAGTTCCCC
PS441F[b]	GTGAACTAGTACCGTGAGGG

[a]Numbering corresponds to *Escherichia coli* 16S rRNA gene
[b]Number of bases from the 5' end of the *Piscirickettsia salmonis* 23S rRNA gene

Oligonucleotide design and synthesis. The bacterial 16S primers, EubA and EubB, the 16S sequencing primers, 519F, 519R, 1100F, 1100R, 1406F, 1406R, and the *Piscirickettsia*-specific primers, PS2S and PS2AS, have been described (Giovannoni 1991, Lane 1991, Mauel et al. 1996). The ITS polymerase chain reaction (PCR) primer pair, PS16SA and PS23SB (Table 1), are modifications of previously described primers (East & Collins 1993, Frothingham & Wilson 1993, Gurtler 1993). The ITS sequencing primers PS16SH and PS23SB were designed using 16S and 23S sequence data derived in the course of this study (Table 1). The 23S PCR and sequencing primers, 761F, 1104F, 1623F, 1948F, 2253F, 2512F, 2758F, 716R, 1091R, 1608R, 1930R, 2241R, 2498R, 2747R, have been previously described by Stothard et al. (1994), except primer PS441F (Table 1), which was designed from the *P. salmonis* sequence.

PCR amplification of the 16S rRNA gene. To amplify the 16S gene, 5 µl DNA lysate was added to 45 µl of reaction mixture consisting of PCR buffer (Promega, Madison, WI; 10 mM Tris HCl [pH 8.4], 1.5 mM $MgCl_2$, 50 mM KCl, 100 mg ml^{-1} gelatin, 0.05% NP-40), 200 µM each of dATP, dCTP, dTTP, dGTP, 1 µM EubA primer, 1 µM EubB primer, 2.5 U Taq DNA polymerase (Promega) and covered with 50 µl mineral oil. The mixture was denatured at 94°C for 2 min and amplification was achieved with 35 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 3 min.

Amplification of the ITS gene was accomplished by substituting 2 µM each of the primers PS16SA and PS23SC for the EubA and EubB primers, with 35 cycles of 94°C for 1 min, 57°C for 2 min and 72°C for 3 min. To amplify the 23S gene, 5 µl DNA lysate was added to

45 µl of the PCR reaction mixture described above and covered with 50 µl mineral oil. The first 457 nucleotides of the 5' end of the 23S genes were amplified and sequenced with the ITS region. Another approximately 2000 nucleotides of the 23S gene were amplified in 2 sections with the primer pairs PS441F - 1930R and 1623F - 2498R. The mixture was denatured at 94°C for 2 min and amplified with 35 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 3 min.

All amplifications were followed by an extension period of 72°C for 7 min, and the tubes held at 4°C. Negative controls with no DNA template were included whenever samples were amplified. Aliquots (10 µl) of the PCR reaction mixture were fractionated in a 2% agarose 1× TAE pH 8.0 (40 mM Tris acetate/ 1 mM EDTA) gel containing 10 µg/50 ml ethidium bromide and photographed under UV transillumination to confirm amplification of the correct size of PCR products.

Cloning of the 16S rRNA genes and the ITS regions. The PCR products were purified using the PCRquick Kit (Qiagen, Valencia, CA). The purified PCR products were cloned into the TA cloning vector pCRII® using the manufacturer's protocol (InVitrogen Corporation, San Diego, CA). Vector plasmids were digested with EcoRI (Promega) and separated by gel electrophoresis to confirm insertion of DNA fragments of the expected size.

Sequence determination. The 16S rDNA and ITS sequences were determined by sequencing PCR products inserted into the vector pCRII®. 23S rRNA gene sequences were determined by direct sequencing of the PCR products. Sequencing was carried out on an ABI 373 automated sequencer (Perkin Elmer, Applied Biosystems, Inc., Foster City, CA) using the dye-primer (T7 and SP6 primer sites on the pCRII plasmid) and dye-terminator kits supplied by Applied Biosystems, Inc.

The sequences determined in this investigation have been deposited in the GenBank nucleotide sequence data base and given the following accession numbers: 16S rDNA; LF-89 U36941, EM-90 U36940, ATL-4-91 U36915, NOR-92 U36942, SLGO-94 U55015: ITS and 23S rDNA; LF-89 U36943, EM-90 U36944, ATL-4-91 U36945, NOR-92 U36946, SLGO-94 U62104 C1-95 U62103.

Additional taxa. The 16S gene sequences of Proteobacteria representing diversity within the phylum were compared to the *Piscirickettsia salmonis* isolates. These organisms and the GenBank accession for the nucleotide sequences used in this study are: *Rickettsia rickettsii* M21293, *Ehrlichia risticii* M21290, *Cowdria ruminantium* X61659, *Ehrlichia canis* M73221, *Anaplasma marginale* M60313, *Ehrlichia equi* M73223, *Neorickettsia helminthoeca* U12457, *Afipia clevelandensis* M69186, *Bartonella quintana* M11927, *Brucella abortus* X13695, *Wolbachia persica* M21292, *Francisella tularensis* Z21932, *Coxiella burnetii* M21291, *Legionella pneumophila* M36025, *Pseudomonas aeruginosa* X06684, *Vibrio anguillarum* X16895, and *Escherichia coli* rrnB operon J01695. Since chlamydial infections have been reported in fish (Evelyn 1992), *Chlamydia trachomatis* M59178 was included in the analysis. The *Bacillus subtilis* rrnB operon M10606 was used as an outgroup.

Phylogenetic analysis. Each *Piscirickettsia salmonis* sequence was confirmed from the 3 separate clones, each of which was obtained from a different amplification reaction. The clones were sequenced in both forward and reverse directions. Sequences were aligned manually and phylogenetic trees were constructed from the sequence data using programs in PHYLIP, phylogenetic inference package version 3.5 (Felsenstein 1989). A matrix of evolutionary distances was constructed from the aligned data by the method of Jukes & Cantor (1969), which assumes that independent changes occur at all sites with equal probability. Phylogenetic trees were inferred from distance estimates by the method of De Soete (1983). In addition, a cladistic reconstruction of the phylogenetic relationships of the taxa was performed using the parsimony program PAUP version 3.0r (Swofford 1993). Only data for sequence positions that were known to be present in all isolates used in the analysis were compared. This resulted in the use of 1527 bases from the 16S rRNA genes for comparisons among the *P. salmonis* isolates and 1313 bases for comparison between *P. salmonis* and the selected non-*P. salmonis* sequences. For comparisons between the *P. salmonis* isolates using the ITS region and the 23S rRNA genes, 309 and 1902 bases were utilized, respectively.

RESULTS

The phylogenetic relationships of *Piscirickettsia salmonis* and other bacteria, including Chlamydiae and members of the Rickettsiaceae, are shown in Fig. 1. Phylogenetic comparisons of the *P. salmonis* 16S rRNA to other bacterial genes indicated that *P. salmonis* is a member of the gamma subgroup of the Proteobacteria and is only distantly related to the genera *Coxiella* and *Francisella* (Table 2). Analysis by both distance ane parsimony methods produced the same branching order (Fig. 1).

Comparisons among the 16S gene sequences of 5 *Piscirickettsia salmonis* isolates revealed high levels of similarity (98.5%) within the group (Fig. 2), and showed that they are monophyletic. Isolates LF-89 and SLGO-94 differed by only 2 nucleotides and are 99.8%

Table 2. 16S rDNA sequence similarities between *Piscirickettsia salmonis*, selected rickettsiae and bacteria

Species	% similarity to[a]																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. *Piscirickettsia salmonis*																			
2. *Clamydia trachomitis*	75.1																		
3. *Rickettsia rickettsii*	80.8	71.0																	
4. *Ehrlichia ristic*	75.6	67.9	81.2																
5. *Cowdria ruminanticum*	78.1	70.0	83.3	84.6															
6. *Ehrlichia canis*	76.7	69.4	82.6	84.2	97.8														
7. *Anaplasma marginale*	72.2	71.5	83.0	85.1	93.3	93.4													
8. *Ehrlichia equi*	77.6	70.6	83.2	85.0	93.5	93.0	98.1												
9. *Neorickettsia helminthoeca*	77.1	66.2	82.1	96.4	84.6	84.5	84.2	84.6											
10. *Afipia clevelandensis*	80.2	69.7	83.4	78.3	79.7	78.7	80.5	80.5	78.7										
11. *Bartonella quintana*	81.6	70.8	85.3	79.8	81.2	80.9	82.1	82.1	79.7	89.6									
12. *Brucella abortus*	81.9	71.3	85.5	80.1	80.9	80.1	81.2	81.5	80.5	90.6	95.5								
13. *Wolbachia persica*	86.5	71.4	78.0	74.9	76.5	75.3	76.3	76.2	74.4	78.4	79.6	78.7							
14. *Francisella tularensis*	86.3	71.6	77.8	74.9	76.4	75.3	76.4	76.3	74.7	78.8	79.9	79.2	99.1						
15. *Coxiella burnetii*	89.5	72.8	81.7	78.8	78.8	78.3	79.4	79.2	78.3	82.0	82.1	82.5	84.8	84.7					
16. *Legionella pneumophila*	89.2	73.7	80.7	77.8	78.0	77.1	78.7	78.5	77.4	81.5	80.7	80.9	85.3	85.5	89.7				
17. *Pseudomonas aeruginosa*	86.8	71.9	78.6	75.6	77.2	76.4	78.5	78.4	75.0	78.8	79.8	80.5	84.9	84.7	88.1	88.6			
18. *Vibrio anguillarum*	85.1	71.1	77.4	74.7	76.4	75.7	76.8	76.3	74.5	77.2	78.7	79.2	81.9	81.8	83.8	85.5	84.9		
19. *Escherichia coli*	86.2	71.0	76.8	75.9	76.0	74.7	77.1	76.6	75.6	77.7	78.2	79.7	82.6	83.0	84.4	85.0	86.5	90.9	
20. *Bacillus subtilis*	76.1	72.2	77.7	74.8	75.9	75.7	75.9	75.0	74.8	79.0	78.8	80.1	76.3	76.1	77.5	76.1	77.5	74.2	75.8

[a]Regions of uncertain homology and ambiguity were eliminated, leaving 1313 positions for comparison. Similarities were calculated as described in 'Material and methods'

similar. LF-89 differed from the sequences of ATL-4-91 and NOR-92 by 6 and 7 nucleotides respectively (level of similarity 99.4%). ATL-4-91 and NOR-92 differed by 5 nucleotides (99.7% similarity). EM-90 differed from LF-89 by 22 nucleotides (98.5% similarity) and from ATL-4-91 by 20 nucleotides, NOR-92 by 19 nucleotides, and SLGO-94 by 22 nucleotides (98.7, 98.8 and 98.5% similarity respectively). Nine of the base differences between EM-90 and the other isolates were found between bases 1003 and 1020. They corresponded to paired bases in a stem-loop structure (Fig. 3). Analysis of the 16S rRNA gene data by either distance or parsimony methods produced the same tree (Fig. 2). The bootstrap values for all nodes were 100%.

The complete ITS rDNA sequences of 6 isolates of *Piscirickettsia salmonis* were determined by sequencing cloned amplicons prepared with PCR primers for conserved regions in the 16S and 23S rRNA genes. Only 1 ITS sequence was obtained from each isolate (Fig. 4). ATL-4-91 and NOR-92 have ITS regions of 309 base pairs (bp) and LF-89, EM-90, SLGO-94 and C1-96 have ITS regions of 308 bp in length. The similarity values used in the phylogenetic comparisons of the *P. salmonis* isolates were calculated from 309 nucleotide positions that were unambiguously determined for all 6 isolates. This region included insertions and deletions (Table 3). Comparison of the ITS sequence alignments of the 6 *P. salmonis* isolates revealed high levels of similarity (ranging from 95.2 to 99.4%). Analysis of the ITS data by either distance or parsimony methods produced the same tree (Fig. 2). The bootstrap values for all nodes were 100%.

Approximately 2500 bp of the 23S rRNA gene was amplified and directly sequenced for all of the isolates except NOR-92, from which a 460 bp of the 5' end of the 23S gene was cloned and sequenced along with the ITS region.

The 23S rRNA genes were analyzed in 2 ways. A 460 bp region of the 5' end of the gene was used in an analysis that included all 6 isolates. In addition, the 5' regions of 23S genes of 5 of the isolates were amplified and sequenced to position 2495. Similarity matrices were constructed from both datasets (Table 3) and used to infer phylogenetic trees (Fig. 2). Overall, the phylogenetic rela-

tionships observed in the two 23S trees and the ITS tree were alike. Analysis of the 23S rRNA gene data by either distance or parsimony methods produced the same topology. The bootstrap values for all nodes were 100%. The 23S rDNA sequence of LF-89 was aligned and compared to the 23S rDNA sequences of the Ribosomal Data Base (Maidak et al. 1994). LF-89 was found to be most similar to the 23S rDNA sequences in the gamma subunit of the Proteobacteria, in agreement with the 16S rDNA analysis.

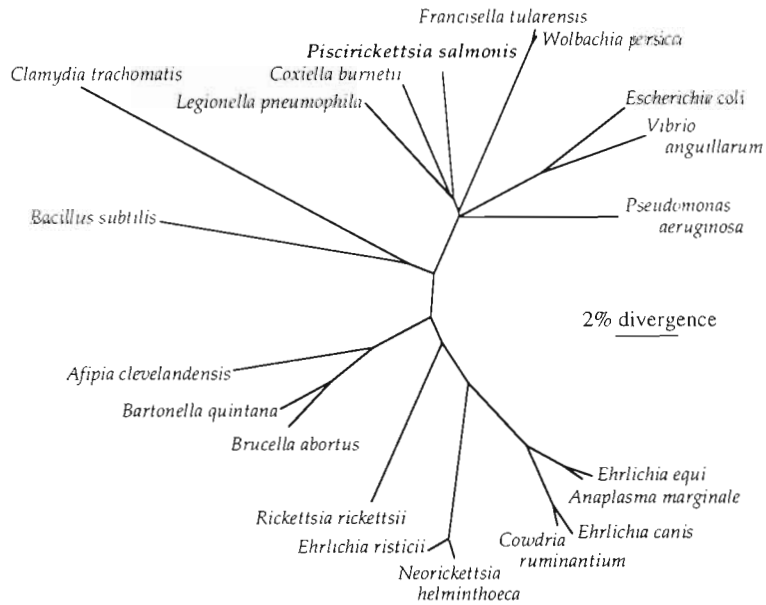

Fig. 1. Phylogenetic relationships among the 16S rRNAs of *Piscirickettsia salmonis*, selected rickettsias and other bacteria. The tree was inferred by the method of De Soete (1983) from evolutionary distances that were calculated by the method of Jukes & Cantor (1969). After eliminating regions of ambiguity and uncertain homology, 1313 positions were compared

DISCUSSION

The isolation of *Piscirickettsia salmonis* from different geographical regions prompted the examination of the 16S rRNA gene sequences from multiple isolates. It seemed plausible that piscirickettsiosis might be a disease with an extended evolutionary history resulting in phylogenetic diversity between isolates from varied geographic origins and hosts. The information reported here shows relatively little nucleotide sequence variation between strains LF-89 and EM-90, but does provide clear evidence of differences between the isolates.

The use of ITS sequence divergence between isolates or strains belonging to the same species can help to clarify relationships within a species because ITS regions are intrinsically more variable than rRNA

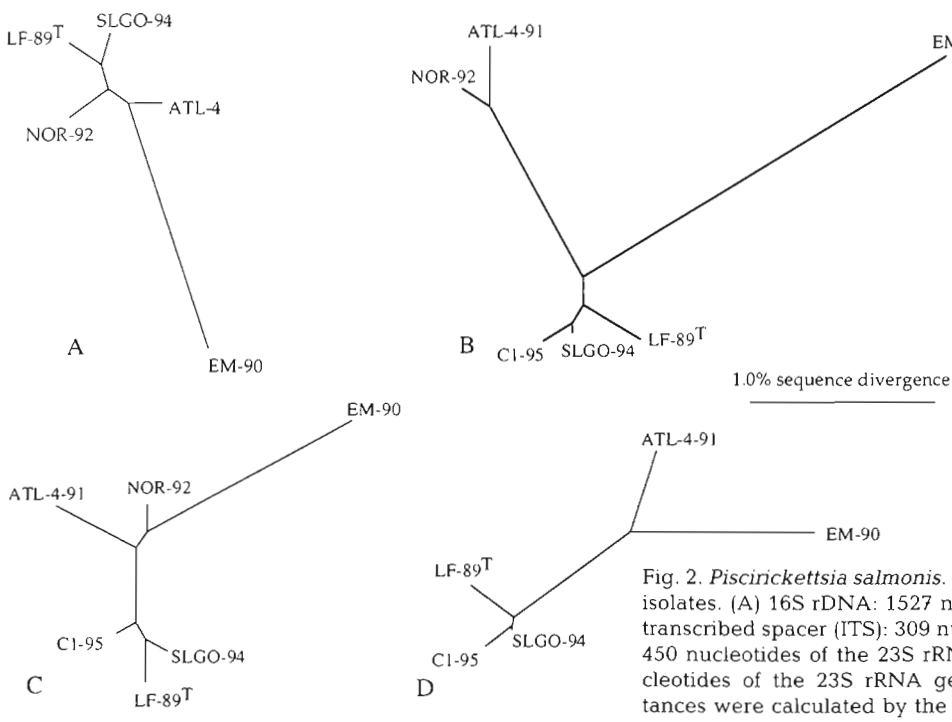

Fig. 2. *Piscirickettsia salmonis*. Phylogenetic relationships among isolates. (A) 16S rDNA: 1527 nucleotides compared. (B) internal transcribed spacer (ITS): 309 nucleotides compared. (C) The first 450 nucleotides of the 23S rRNA gene compared. (D) 1902 nucleotides of the 23S rRNA gene compared. Evolutionary distances were calculated by the method of Jukes & Cantor (1969) and trees inferred by the distance method of De Soete (1983)

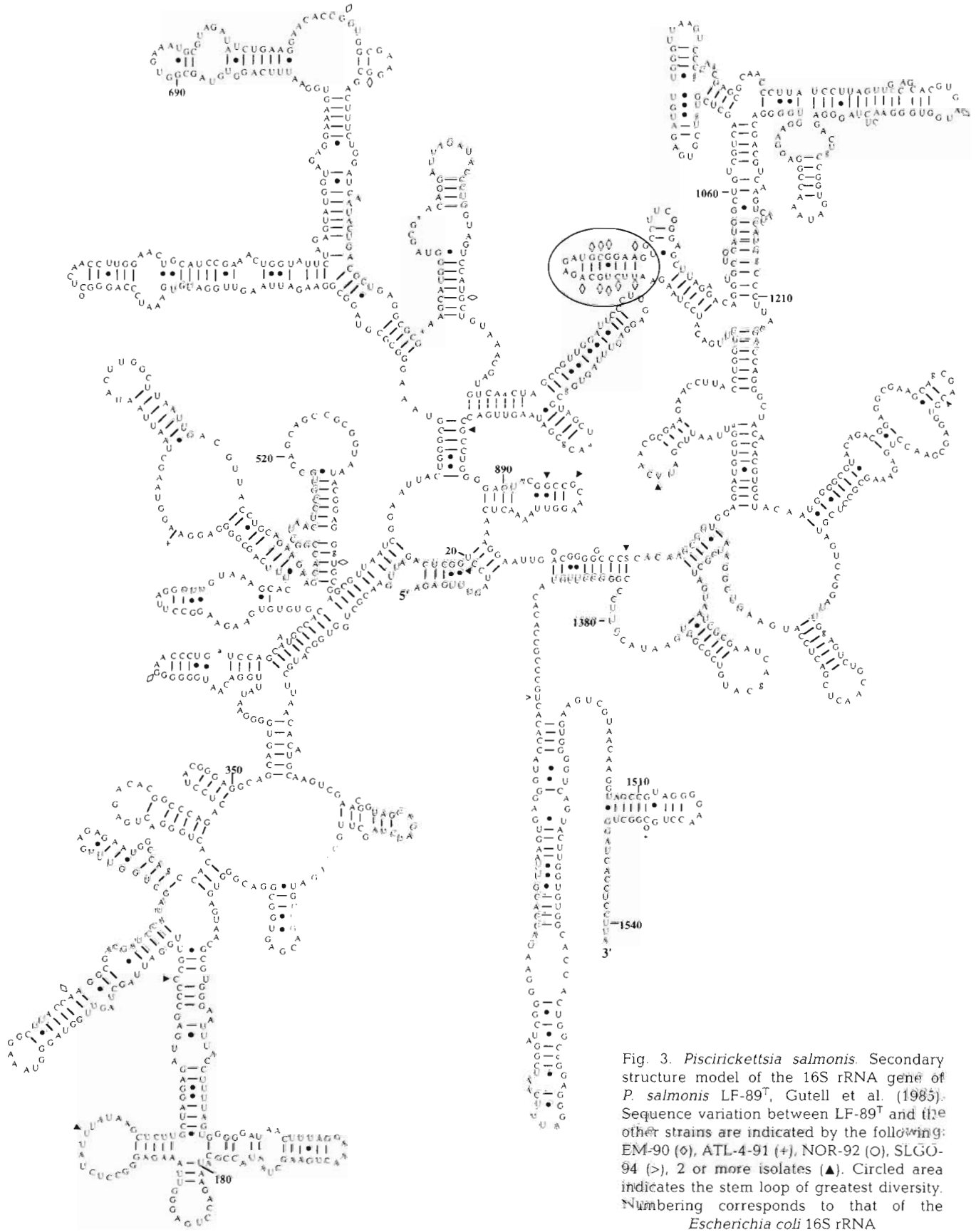

Fig. 3. *Piscirickettsia salmonis*. Secondary structure model of the 16S rRNA gene of *P. salmonis* LF-89^T, Gutell et al. (1985). Sequence variation between LF-89^T and the other strains are indicated by the following: EM-90 (◇), ATL-4-91 (+), NOR-92 (○), SLGO-94 (>), 2 or more isolates (▲). Circled area indicates the stem loop of greatest diversity. Numbering corresponds to that of the *Escherichia coli* 16S rRNA

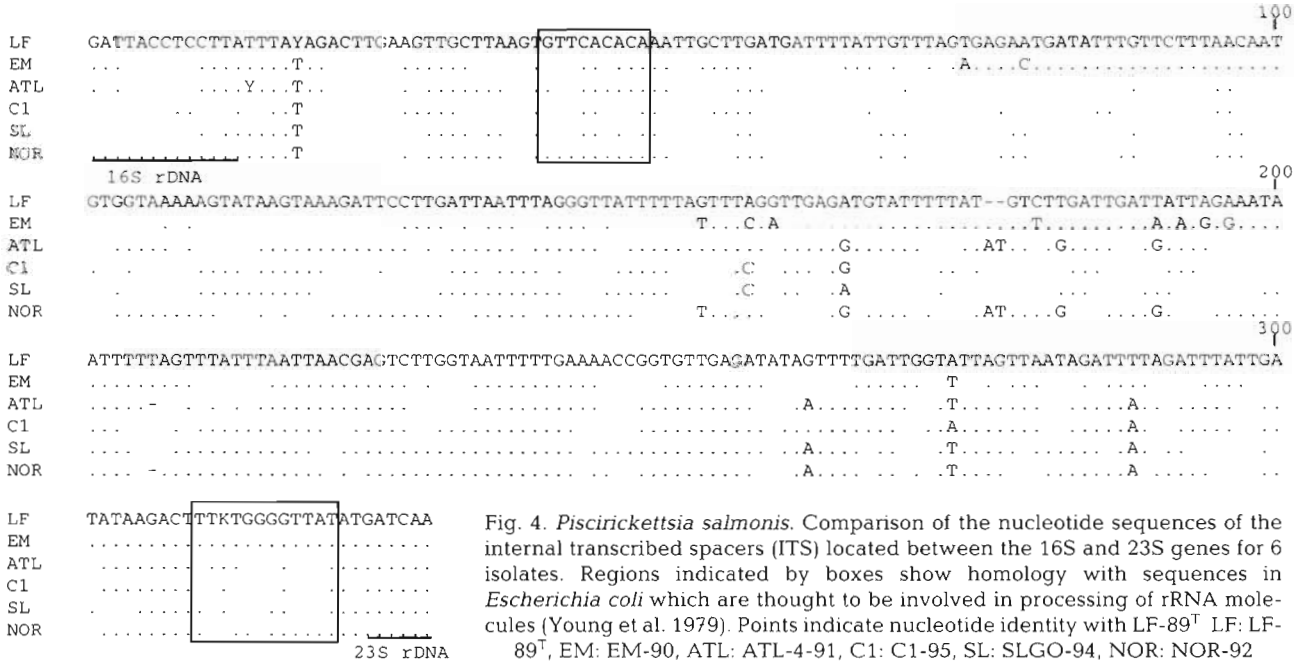

Fig. 4. *Piscirickettsia salmonis*. Comparison of the nucleotide sequences of the internal transcribed spacers (ITS) located between the 16S and 23S genes for 6 isolates. Regions indicated by boxes show homology with sequences in *Escherichia coli* which are thought to be involved in processing of rRNA molecules (Young et al. 1979). Points indicate nucleotide identity with LF-89^T LF: LF-89^T, EM: EM-90, ATL: ATL-4-91, C1: C1-95, SL: SLGO-94, NOR: NOR-92

Table 3. Sequence similarities of the internal transcribed spacer (ITS; 309 nucleotide positions were used for the comparison after elimination of ambigous nucleotides), the first 457 nucleotides of the 5' end of the 23S rDNA (450) and 1902 nucleotides of the 23S rDNA (1902) sequence between 6 isolates of *Piscirickettsia salmonis*

Isolate	% similarity to (ITS/450/1902)				
	LF-89	EM-90	ATL-4-91	C1-95	SLGO-94
LF-89^T					
EM-90	96.5/97.6/97.9				
ATL-4-91	97.8/98.7/98.5	95.2/98.5/98.5			
C1-95	99.4/99.3/99.5	96.5/97.8/97.9	97.8/98.9/98.5		
SLGO-94	99.1/99.6/99.6	96.9/97.6/98.1	98.1/98.7/98.7	99.7/99.8/99.8	
NOR-92	97.8/99.1/NA	95.9/98.5/NA	99.4/98.7/NA	97.8/99.3/NA	98.1/99.1/NA

genes. However, there are limitations to the applications of ITS regions for strain differentiation. The use of ITS sequencing for the phylogenetic analysis of species that have more than 1 rRNA operon can be complicated because of sequence variation between the operons (Frothingham & Wilson 1993). However, ITS regions are well suited for comparison of slow-growing organisms that often have 1 copy of the rRNA operon (Frothingham & Wilson 1993). Only 1 ITS sequence was obtained for each of the *Piscirickettsia salmonis* isolates, suggesting the presence of a single rRNA operon.

Processing of rRNA into the ribosomal subunits involves cleavage with RNase III (Maidak et al. 1994). In *Escherichia coli* 1 position at which this cleavage occurs is downstream of the sequence GCUCACACA, 33 nucleotides from the 3' end of the 16S rRNA gene (Woese 1987). In *Piscirickettsia salmonis* this sequence is present with only 1 base change (GUUCACACA) and is located 1 position farther from the 16S rRNA gene than in *E. coli*. This sequence is also found in *Aeromonas hydrophila* (East & Collins 1993) and *Plesiomonas shigelloides* (East et al. 1992), which have RNase III cleavage sites identical to the sequence found in *P. salmonis*.

According to Leblond-Bourget et al. (1996), as an approximation, most species groups of bacteria exhibit 16S-23S sequence divergence of <13%. The maximum divergence in this study was 4.8% (Table 3). In previous research with obligate intracellular parasites differences in 16S rDNA similarity ranging from 99.9 to 84% have been used as criteria for assigning strains of novel species (Anderson et al. 1991). Wen et al. (1995b) suggested that 2 strains of *Ehrlichia risticii* may represent new species on the basis of distinct antigenic profiles and similarity values of 99.2 to 99.3% (Wen et al. 1995a). However, Fox et al. (1992) states that while 16S rDNA data is a powerful tool for determining what spe-

cies a strain belongs to, it may not by itself be enough to define a species. According to Stackebrandt & Goebel (1994) a polyphasic approach is needed to separate a bacterial species from its phylogenetic neighbors and DNA reassociation is the superior method when separating bacteria at the species level. The strength of 16S rRNA sequence analysis is to determine if the organisms are related enough (similarities >97%) that DNA reassociation studies need to be performed.

The diversity within the 16S, ITS and 23S rDNAs reported here is not sufficient to split the genus *Piscirickettsia* into several species, although additional research on the biology and biochemistry of the bacterium might eventually support such a taxonomic change. Systematic studies often link DNA reassociation, virulence, phenotypic, biochemical and antigenic characteristics with 16S rDNA data as criteria in establishing a novel species; information on the genus *Piscirickettsia* is still emerging.

The sequence differences between EM-90 and the other 4 *Piscirickettsia salmonis* sequences are of practical value. These data allowed the development of isolate-specific PCR assays that were used to differentiate EM-90 from LF-89, ATL-4-91, NOR-92 and SLGO-94 (Mauel et al. 1996). It was also found that *EcoR*I and *Pst*I restriction sites located in the variable stem-loop region permitted the use of restriction fragment length polymorphism to differentiate EM-90 from the other strains (Mauel et al. 1996).

In conclusion, comparative 16S, ITS, and 23S rDNA sequence analyses of *Piscirickettsia salmonis* indicate that all isolates form a monophyletic group within the gamma Proteobacteria, and that isolate EM-90 has diverged genetically from the other *P. salmonis* isolates. While variation between isolates from the different geographic regions was observed it is based on single isolates from 2 of the regions (Norway and British Columbia, Canada). Sequences from additional isolates should be determined and compared before phylogenetic conclusions can be reached concerning regional variation within *P. salmonis*.

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