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Cloning and Sequencing of the *rph* Gene Encoding RNase PH from *Legionella pneumophila*

Se Jin Kim, Jong-Seok Lim, Nicholas P. Cianciotto[1], and Yong-Kyung Choe*

Korea Research Institute of Bioscience and Biotechnology, Taejon 305-600, Korea;
[1]Department of Microbiology-Immunology, Northwestern University, Chicago, IL 60611, USA

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***Legionella pneumophila*, the cause of Legionnaires' disease, is able to survive intracellularly in eukaryotic cells such as monocytes, macrophages, and protozoan organisms. During protein biosynthesis, the *rph* gene encodes ribonuclease (RNase) PH which functions as a phosphorolytic nuclease that removes nucleotides following the CCA terminus of tRNA and as a nucleotidyltransferase which adds nucleotides to the ends of RNA molecules by using nucleoside diphosphates as substrates. In this study, the *rph* gene was screened in pUC19 library employing a DNA probe which was constructed from PCR based on a consensus pattern of multiple alignment of RNase PH. The encoded protein consists of 235 amino acid residues with a calculated molecular weight of 26,112 Daltons. The RNase PH signature domains are completely conserved.**

Key words: Exo-ribonuclease, *Legionella pneumophila*, RNase PH, *rph* gene

Legionella pneumophila is a ubiquitous Gram-negative bacterium that is widely distributed in natural and man-made fresh-water habitats (11, 27). *L. pneumophila*, a facultative intracellular pathogen, can cause severe pneumonia in humans called Legionnaires' disease and the less severe Pontiac fever (21). In the environment, *L. pneumophila* can infect and replicate intracellularly in protozoan organisms such as *Acanthamoeba castellanii* (14), *Hartmannella vermiformis* (10), *Naegleria fowleri* (24), and *Tetrahymena pyriformis* (9). *L. pneumophila* is capable of infecting and replicating within several different mammalian cell types and cell lines at varying efficiencies. However, mice are highly resistant to infection from *L. pneumophila*, and most mouse peritoneal macrophages are nonpermissive to growth of this pathogen, including BDF1, DBA/2, C3H/HeN, C57BL/6, and BALB/c (29). Legionella infection occurs after inhalation of aerosolized bacteria. The bacteria enter the human lungs, where they are capable of invading and proliferating in alveolar macrophages and blood monocytes. The ability of *L. pneumophila* to cause disease is dependent on its ability to survive and replicate within a specialized membrane-bound phagosome in human macrophages and epithelial cells (1, 8). Following formation of this replicative phagosome, bacterial replication is initiated, which eventually leads to killing of the host cell and release of intracellular bacteria. Alteration of bacterial gene expression during the intracellular infection may play a role in the formation of the replicative phagosome and adaptation of the bacteria to the new intracellular niche.

tRNA and its derivatives, aminoacyl-tRNA and peptidyl-tRNA, play a central role in the process of protein biosynthesis (13). RNA molecules are generally synthesized as long precursors that consequently undergo a series of processing reactions to remove extra residues and thus generate mature functional forms (5). In *Escherichia coli*, where our knowledge of the process is most advanced, tRNA maturation consists of removal of precursor-specific residues from both 5' and 3' termini of the RNA chain (6). Although the single-step reaction for 5' processing by RNase P has been well established (25), limited information suggests that 3' processing may be a multistep reaction. Six exo-ribonucleases are known which are able to remove nucleotides from the 3' end of tRNA precursors *in vitro* (4). These are polynucleotide phosphorylase (PNPase), RNase II, RNase D, RNase BN, RNase T, and RNase PH. Two of them, RNase T and RNase PH, are the major enzymes responsible for final 3' trimming of most tRNAs (19, 20), whereas RNase II and PNPase constitute the major mRNA degradation activities (7). The smallest active form of RNase PH is a $\alpha 2$ (16). RNase PH is both a phosphorolytic nuclease that removes nucleotides following the CCA terminus of

* To whom correspondence should be addressed.
(Tel) 82-42-860-4186; (Fax) 82-42-860-4593
(E-mail) ykccoruk@mail.kribb.re.kr

tRNA and a nucleotidyltransferase which adds nucleotides to the ends of RNA molecules by using nucleoside diphosphates as substrates (15, 17). Cells lacking different exo-ribonucleases display varying degrees of deficiency in both cell growth and tRNA processing. In the extreme case, cells lacking RNase II, D, BN, T, and PH are inviable (16), whereas double mutants of RNase PH and PNPase are cold sensitive and defective in ribosome synthesis (30).

We have been interested in the smallest active form of RNase PH, because *L. pneumophila* is one of the common etiologic agents of pneumonia with mortality rates of up to 50% (22). Despite its increasing medical significance, insufficient genetic and molecular biological information have been accumulated on *L. pneumophila*. RNase PH, related to mature tRNA metabolism and ribosome synthesis, could be used as a molecular target to develop novel agents that can specifically control the growth of pathogenic cells. In order to clone the gene encoding RNase PH, degenerate PCR primers for the RNase PH were designed and synthesized from the conserved regions identified from the multiple sequence alignment of RNase PH from bacteria. We report identification of the *rph* analog from *L. pneumophila* and show the similarity between its deduced amino acid sequence and those of several organisms.

Materials and Methods

Bacterial strains, chemicals, and culture conditions

Legionella pneumophila 130b strain was grown on buffered charcoal-yeast extract (BCYE) agar plate or in buffered yeast extract (BYE) broth. *Escherichia coli* DH5α was used for transformation and propagation of recombinant plasmids derived from the vector pUC19. *E. coli* was cultivated in Luria-Bertani (LB) medium. Antibiotic used for selection of marker in *E. coli* was ampicillin (50 μg/ml). Restriction enzymes and T4 DNA ligase were purchased from Promega LKB, Boehringer Mannheim GmbH (Germany), and New England Biolabs (USA). Taq polymerase was purchased from Promega LKB, and radiochemicals were supplied by Amersham (UK).

DNA manipulation

L. pneumophila chromosomal DNA was isolated by the standard method (26). Plasmid DNA isolation was performed by using a Qiagen (Chatsworth, CA, USA) plasmid kit according to the manufacturer's recommandations. DNA fragments were isolated from the agarose gel with the GENECLEAN kit (Bio 101; La Jolla, CA, USA) as described by the manufacturer and ligated into pUC19. *E. coli* cells were transformed by the $CaCl_2$ method.

Construction of an *L. pneumophila* library

A genomic library of *L. pneumophila* was constructed as described previously (18) with a slight modification. High molecular mass *L. pneumophila* 130b DNA (50 μg) was partially digested with *Sau*3A1 to generate fragments predominantly 1-3 kb in length. The resulting genomic DNA fragments and molecular mass standards (1 kb ladder) were separated by electrophoresis through a 1% (w/v) agarose gel. Following electrophoresis, the appropriate portion of the gel containing 1-3 kb length was excised with a clean razor, and purification was performed with a GENECLEAN kit as above. Vector DNA (pUC19) was prepared as described above. Following digestion with *Bam*HI, linearized pUC19 was dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim). Phosphatase was removed from the reaction mixture by heating at 70°C in the presence of 0.01 M EDTA for 15 min, followed by successive extraction with TE-saturated phenol and then chloroform-isoamyl alcohol (24:1, v/v). Dephosphorylated pUC19 and partially digested chromosomal DNA were mixed at a molar ratio of 1:2 (vector : insert) with a Rapid DNA ligation kit (Boehringer Mannheim), and incubated for 5 min at room temperature. Portions of the ligation mixture were used to transform $CaCl_2$-treated *E. coli* DH5α. Transformants were selected on LB agar plates containing ampicillin.

Cloning and amplification of the *L. pneumophila rph* gene

The degenerate PCR primers for the *L. pneumophila* RNase PH gene were designed and synthesized from the conserved regions identified from the multiple sequence alignments of various bacterial RNase PH nucleotide and the protein sequences. Primer sequences were as follows; forward primers 5'-GGYTG-GDTNACNGCNGARTA-3' and reverse primers 5'-GTNCCNGGRTGNGCYTG-3' (where D=G+A+T, N=A+G+C+T, R=A+G, Y=C+T). PCR was conducted using the isolated genomic DNA as a template with the degenerate primers and cloned into TA cloning vector, pCR2.1 (Original TA Cloning Kit, Invitrogen). The probe was labeled with [α-^{32}P]ATP, and colony hybridization was performed with the pooled colonies of pUC19 library. The sequencing of the positive clones and PCR products were carried out as described by the manufacturer with the T7 polymerase sequencing kit from Amersham Life Science (USA).

Computer analysis

For the PCR primers construction, the homology of the multiple sequence alignment of various RNase PH

genes with the nucleotide and amino acid sequences was performed by Clustal W and Multiple alignment of Antheprot (Ver 4.0) of a personal computer. The sequence alignment and ORF searching of the PCR products and positive clones were analyzed by BLASTP and ORF search of the National Center for Biotechnology Information protein databases.

Results and Discussion

Construction of PCR fragment with genomic DNA of *L. pneumophila*

To isolate the part of gene encoding RNase PH from *L. pneumophila*, the degenerate primers were synthesized from the multiple alignment of the known RNase PH nucleotide and protein sequences. A pair of the primers generated the specific PCR product of about 210 bp (data not shown). The PCR products were cloned into TA cloning vector, pCR2.1. The cloned gene fragments were sequenced, and data base searches revealed that the encoded polypeptide sequence is very similar to RNase PH sequence from other organisms. To obtain the complete RNase PH gene from *L. pneumophila* DNA, colony hybridization was carried out with radioisotope-labeled PCR fragments on the 1-3 kb *Sau*3A1 fragments of the pUC19 library. Plasmid DNA with the strongest signal for positive clones were purified as described above, and PCR and sequencing were performed. Two kinds of

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   1 GATCTTCAACATATGCCTTTAATGACCTGCCTTCCCCAGCTCTGACTTCACACAATTTAT   60
  61 CGATACTTTTCTGAAATAACTCCAAAGCAATTTGAGACAATTCCTCCATATCCAGTAGAT  120
 121 TAGCCTCCACAACGCCTGGCCATGAAAGCACTTGACTGACAGTCATATCATTAGCCAGAT  180
 181 GATGAGAAGCAGATAATTTACTGCTTAAATCAAGTAATGCATTAATCGTACCTATATTTA  240
 241 TAAGCATAGACCGATTATCACAACTTGTATCCTTGTACTTTAGATGGCACTCCAGTTTAC  300
 301 CCCGATGTATCTTATCCCGAAGCAAAGTTCTTAATTCTGCTTCAAGAAATCGAAAGGACT  360
 361 CCGGCAAACGAAAAGAGACATCGAGATAGCGATGGTTAACAGACCTGATTTCCCAACAAA  420
 421 AATCACCTACATCAAACTGCTTTTGTATTCGAGAAAAAGCCGTCATACTATGAATCATAA  480
 481 AGAATACCTACTTTTTAACCTTGCGCGAATATACCTGAAATTAAAAGAATTGCACAGGGA  540
 541 TTGCTCATCAATTAATTGCAACAAACTTCTATATCAGGTCCTGAAAGTCAGGCCTTAAAC  600
 601 CAAACTTGAAACTTAAAACAAACTGTTTCTAACGTAAAGAATGGAAACAAATCGAATGGT  660
 661 TTGATTAAAAATATTTTTCAGCAAAAATAGAGAGGAACGTACAAATAGCGTATGTATTTA  720
 721 GGGTGCAACTCAAGTATTAAAGAGTTATACTTGGGCATTTTACAGAGGAGTATCTCTAAT  780
   1                                                           M    1
 781 GCGTCCCAGTAATCGTGAACATGATCAACTTCGTCCCGTTACAATAACACGTAATTTTAC  840
   2   R  P  S  N  R  E  H  D  Q  L  R  P  V  T  I  T  R  N  F  T   21
 841 CAATTATGCCGAAGGCTCTGTATTAGTCGAATTTGGTCAGACAAAAGTGATTTGTAACGC  900
  22   N  Y  A  E  G  S  V  L  V  E  F  G  Q  T  K  V  I  C  N  A   41
 901 ATCAATCGTCGAAGGAGTTCCCCGGTTTTTAAAAGGTAAAAACCAAGGGTGGATAACTGC  960
  42   S  I  V  E  G  V  P  R  F  L  K  G  K  N  Q  G  W  I  T  A   61
 961 TGAATATGGCATGCTCCCACGTGCAACTCATAGCCGTACCGAAAGGGAAGCCAGTAAAGG 1020
  62   E  Y  G  M  L  P  R  A  T  H  S  R  T  E  R  E  A  S  K  G   81
1021 TAAGCAAGGTGGCAGAACATTGGAAATTCAACGTCTCATAGGGCGCTCATTGAGAGCATG 1080
  82   K  Q  G  G  R  T  L  E  I  Q  R  L  I  G  R  S  L  R  A  C  101
1081 TATCGATTTAAAGGTTTTGGGCGAAAATACTATTACTCTTGATTGTGATGTTATTCAAGC 1140
 102   I  D  L  K  V  L  G  E  N  T  I  T  L  D  C  D  V  I  Q  A  121
1141 GGATGGAGGAACCAGAACAGCAGCTATAACTGGCAGTTGTGTGGCTATGCGAGATGCGAT 1200
 122   D  G  G  T  R  T  A  A  I  T  G  S  C  V  A  M  R  D  A  I  141
1201 TCACTGGATGGTTCAACGTGAAAAAATAAAGAAGATGCCTGCTTTTAATTATGTAGCTGC 1260
 142   H  W  M  V  Q  R  E  K  I  K  K  M  P  A  F  N  Y  V  A  A  161
1261 TGTATCTGTAGGTATTTACAGGGGTCAACCCGTGCTGGATTTGGATTATGCAGAAGATGT 1320
 162   V  S  V  G  I  Y  R  G  Q  P  V  L  D  L  D  Y  A  E  D  V  181
1321 TCTTGCTGAAACAGATATGAATGTGGTTATGAATGAACAAGGACATTTTATTGAAGTTCA 1380
 182   L  A  E  T  D  M  N  V  V  M  N  E  Q  G  H  F  I  E  V  Q  201
1381 AGGCACCGCTGAAGATAACTCCTTTAACCGGGAACAACTGAACAGCATGTTGTCATTGGC 1440
 202   G  T  A  E  D  N  S  F  N  R  E  Q  L  N  S  M  L  S  L  A  221
1441 TGAAATTGGTATTCCTCAATTGATTGAAATCCAAAAAAATGCCTGAGAACAATAACCCGG 1500
 222   E  I  G  I  P  Q  L  I  E  I  Q  K  N  A  *                 235
1501 CATAAACAGTCGCCCGCTATAGAAATTAATACTTCAAACCTGAACAAAAGATC        1553
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Fig. 1. Nucleotides sequence of *L. pneumophila rph* gene, flanking regions, and translated amino acid sequence of RNase PH. The deduced amino acid sequence is shown below the nucleotide sequence in single letter code. An asterisk indicates the stop codon, and the putative ribosome binding sites (Shine-Dalgarno sequence) is underlined. *Sau*3A1 restriction sites are represented by dot dash.

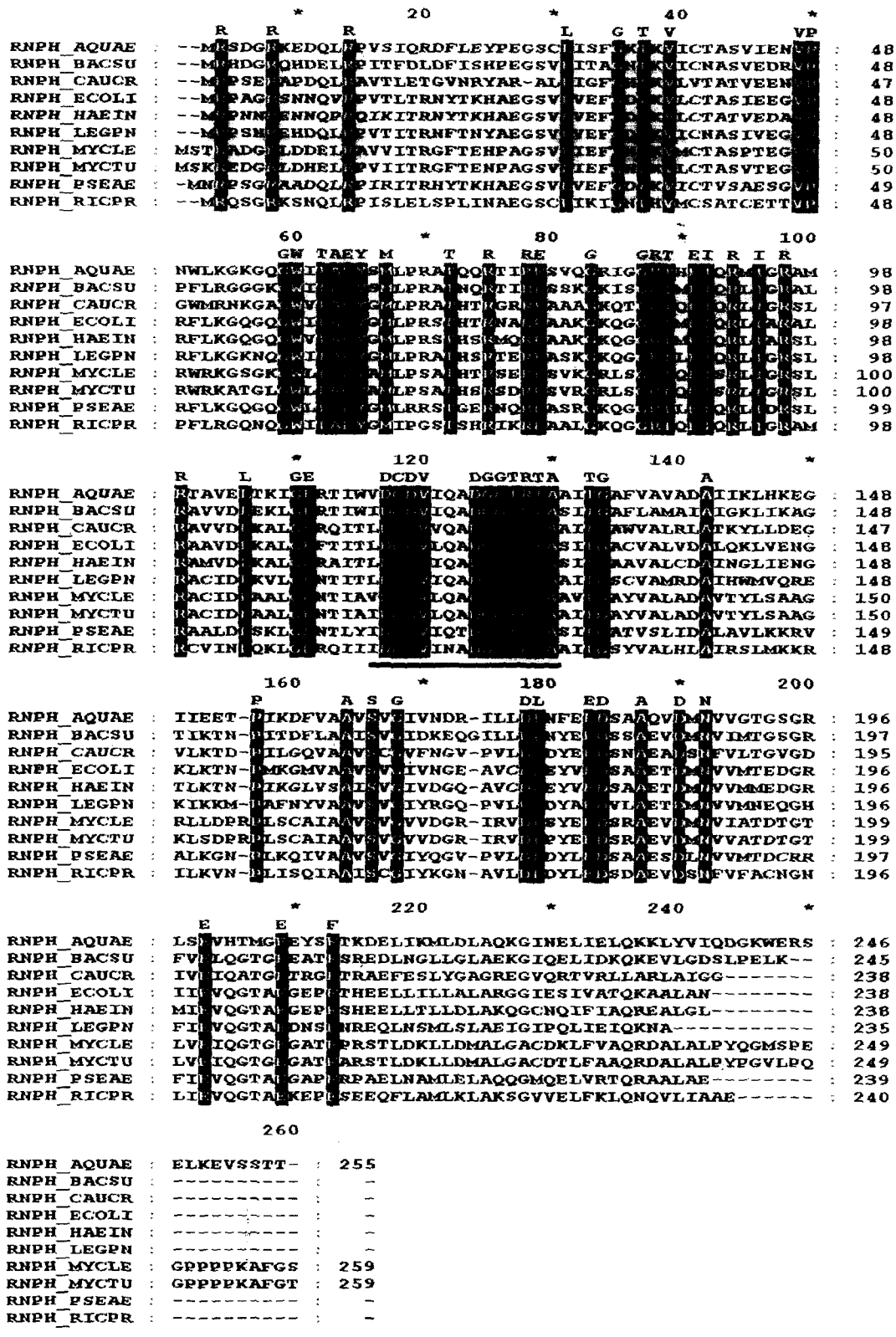

Fig. 2. Alignment of RNase PH from *Aquifex aeolicus* (AQUAE), *Bacillus subtilis* (BACSU), *Caulobacter crescentus* (CAUCR), *Escherichia coli* (ECOLI), *Haemophilus influenzae* Rd (HAEIN), *Legionella pneumophila* 130b (LEGPN), *Mycobacterium leprae* (MYCLE), *Mycobacterium tuberculosis* (MYCTU), *Pseudomonas aeruginosa* (PSEAE), and *Rickettsia prowazekii* (RICPR). Conserved RNase PH signature sequence is presented by underline. Black box and capital letters indicate conserved sequence.

*Sau*3A1 genomic DNA fragments from the pUC19 library were selected. The sequences of these fragments were assembled to generate a complete gene structure (Fig. 1).

Primary structure and characteristics of *rph* gene of *L. pneumophila*

The positive clones of pUC19 library were determined with the nucleotide sequence of the 1,553 bp. The nucleotide sequence (GenBank accession number, AF120720) and its deduced polypeptide sequence are shown in Fig. 1. The *rph* gene was preceded by a Shine-Dalgarno sequences (AGGAG) located 13 bp upstream from the ATG start codon and terminated by a TGA stop codon within the entire 708 bp in length. The *L. pneumophila* RNase PH deduced from the nucleotide sequence consists of 235 amino acid residues with a calculated molecular weight of 26,112 Daltons. Based upon its deduced amino acid sequence, RNase PH is an acidic protein with a pI=6.13. Whereas acidic amino acid residues are distributed throughout the protein, there is a particularly high positive charge density in the N-terminal region. According to previous report (3), RNase PH requires Mg^{2+}, with maximal activity in the range 3-10 mM. Its optimum pH is about 8, although activity is relatively constant between pH 6.8 and pH 9.5. RNase PH is dramatically stimulated by P_i or arsenate with optimum activity at about 10 mM for each (3). Analysis of the deduced amino acid sequence of RNase PH also indicates that it is a relatively hydrophilic protein. Secondary structure analysis of the RNase PH sequences by the method of Garnier *et al.* (12) suggested that the protein is relatively rich in helix-coil-helix (70%). Interestingly, the C-terminal of the protein is almost entirely α-helix. Alignment of *L. pneumophila* RNase PH with all sequences available in GenBank indicated that the entire sequence is highly conserved (Fig. 2). Especially, the N-terminal segment around the RNase PH signature sequence is highly conserved among all RNase PHs (Fig. 2).

Phylogenetic relationship and homology matrix score with the RNase PH sequences

The phylogenic location of the *L. pneumophila* RNase PH was determined by sequence comparison using an unweighted Clustal W method (Fig. 3). The resulting phylogenetic tree of these proteins could be divided into the two groups. One group was consisted of *L. pneumophila*, *E. coli*, *Haemophilus influenzae* Rd, *Pseudomonas aeruginosa*, *Rickettsia prowazekii*, and *Caulobacter crescentus*. The other group contained *Bacillus subtillis*, *Aquifex aeolicus*, *Mycobacterium tuberculosis*, and *Mycobacterium leprae*. Statistically significant matches to several members of the RNase PH proteins in the various organisms were found. Among the most homologous were the RNase PHs encoded by *E. coli* (79% similarity and 66% identity), *H. influenzae* Rd (78% and 63%), *P. aeruginosa* (76% and 63%), *B. subtilis* (76% and 59%), *M. leprae* (72% and 57%), *A. aeolicus* (72% and 56%), *R. prowazekii* (71% and 53%), *M. tuberculosis* (70% and 56%), and *C. crescentus* (67% and 53%). Alignment of the amino acid sequences of the *E. coli* and *B. subtilis* open reading frames reveals 132 identical amino acids of 238 positions (56% identity) (2). Mian has shown that RNase PH sequences comprise a complete domain which is also present in PNPase from bacteria (23). Since both RNase PH and PNPase are 3'-5' phosphorolytic nucleases, the presence of a common domain suggests that they have a similar structure and/or a mechanism of action. PNPase has been shown to be involved in 3' adenylation-mediated degradation of mRNA (28), whereas RNase PH can catalyse the major tRNA maturation, and these RNase are needed for ribosome biogenesis (30). RNase PH and PNPase have similar catalytic properties to process tRNA precursors.

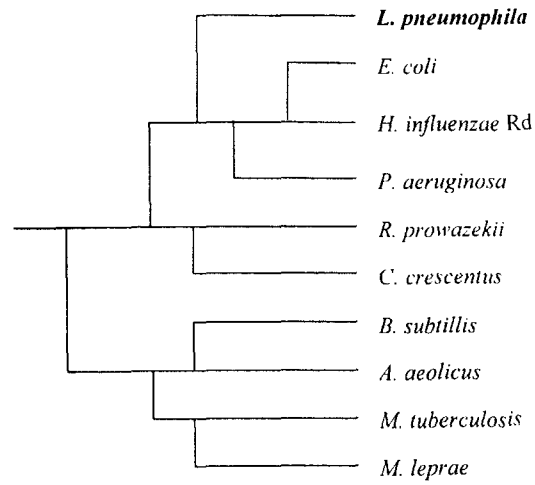

Fig. 3. Phylogenetic relationship of *L. pneumophila* RNase PH. The phylogenetic tree consisting of 10 RNase PH sequences was made by Clustal W method in Antheprot (version 4.0).

This study is the first to report the nucleotide sequence of *rph* encoding RNase PH (tRNA nucleotidyltransferase) from *Legionella pneumophila* 130b. Our data suggest the conservation of RNase PH signature sequence as revealed by the multiple sequence alignment of RNase PH including *E. coli* and other organisms. Thus, we propose that RNase PH relatedness to the mature tRNA formation and ribosome synthesis could be used as a molecular target for the growth control of pathogenic bacteria.

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