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Isolation of a Gene Encoding a Novel Spectinomycin Phosphotransferase from *Legionella pneumophila*

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A gene capable of conferring spectinomycin resistance was isolated from *Legionella pneumophila*, the agent of Legionnaires' disease. The gene (*aph*) encoded a 36-kDa protein which has similarity to aminoglycoside phosphotransferases. Biochemical analysis confirmed that *aph* encodes a phosphotransferase which modifies spectinomycin but not hygromycin, kanamycin, or streptomycin. The strain that was the source of *aph* demonstrated resistance to spectinomycin, and Southern hybridizations determined that *aph* also exists in other legionellae.

Legionella pneumophila is the agent of Legionnaires' disease, a potentially fatal form of pneumonia for immunocompromised individuals (30). This gram-negative bacterium survives in water systems as a parasite of protozoans (9). Infection results via the inhalation of contaminated aerosols, and disease occurs after the bacteria parasitize alveolar macrophages (5, 14, 30). Fortunately, legionellae, unlike other aquatic microbes such as pseudomonads, have not shown significant degrees of innate antibiotic resistance (3, 8). Mutants resistant to erythromycin, rifampin, and ciprofloxacin have been isolated in the lab but not from clinical or environmental sources (3, 7, 20). Thus, erythromycin and the fluoroquinolones continue to be effective for the treatment of legionellosis (8). In the course of an unrelated study which involved a selection for spectinomy-

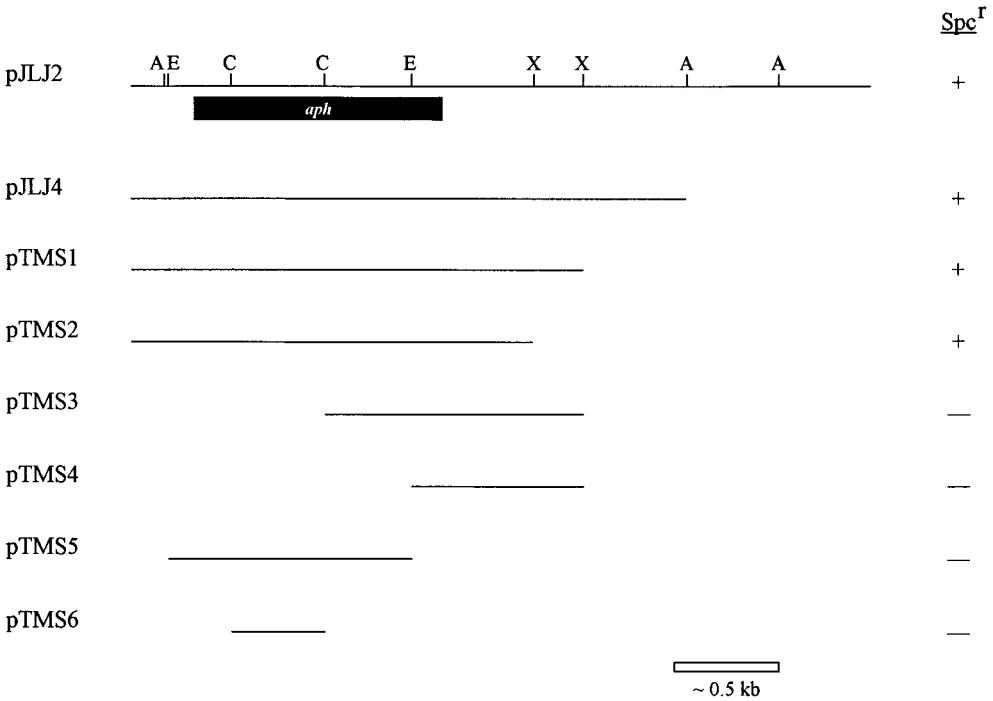

FIG. 1. Restriction map and spectinomycin resistance phenotypes encoded by pJLJ2 and its subclones. For pJLJ2 and pJLJ4, *L. pneumophila* DNA was cloned into pBR322. For pTMS1 to pTMS4, the DNA was cloned into pUC19. For pTMS5 and pTMS6, *Legionella* sequences were cloned into pBluescript. Plus signs indicate that *E. coli* transformants carrying this plasmid were resistant to spectinomycin (Spc). Minus signs indicate that the clones were unable to grow on media containing spectinomycin. The approximate location of the *L. pneumophila* spectinomycin-modifying gene (*aph*) is indicated. Recognition sites for restriction enzymes *Ava*I (A), *Cla*I (C), *Eco*RV (E), and *Xba*I (X) are indicated. The locations of the 5′ and 3′ ends of the pJLJ2 insert as well as of the 5′ ends of the pJLJ4, pTMS1, and pTMS2 inserts resulted from the ligation of a *Legionella Sau*3AI fragment into the *Bam*HI site of pBR322. Note, also, that the 5′ end of the pTMS5 insert was an *Eco*RV, not an *Ava*I, site.

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cin resistance, we uncovered a *L. pneumophila* gene that promotes the phosphorylation of spectinomycin, an aminocylcitol closely related to the aminoglycosides.

The identification of the spectinomycin-modifying enzyme was the fortuitous outcome of a failed attempt at identifying a *mip* regulator within our genomic library of *L. pneumophila* serogroup 1 strain 130b (24). A pBR322-based library had been introduced into *Escherichia coli* HB101 containing a chloramphenicol-resistant vector, and ampicillin-, chloramphenicol-, and spectinomycin-resistant clones were selected. All triply-resistant clones harbored the same spectinomycin-resistant plasmid, but to our knowledge, there had been no previous indication of spectinomycin resistance in *L. pneumophila*. Thus, we performed three additional experiments. First, we directly screened the library for a locus that conferred spectinomycin resistance upon HB101. DNA preparations from seven spectinomycin-resistant clones contained a plasmid (pJLJ2) that was identical to the one previously obtained (see Fig. 1). Next, to confirm that the insert DNA contained within pJLJ2 was of *Legionella* origin, a portion of it was used as a probe in a Southern hybridization with whole-cell DNA from strain 130b. As expected, the probe hybridized with *Legionella* DNA (data not shown). Finally, to ascertain whether spectinomycin resistance was manifest in *L. pneumophila*, strain 130b was plated onto buffered charcoal-yeast extract agar containing various amounts of the antibiotic (6). Although *L. pneumophila* was incapable of forming colonies on media containing 200 μg of spectinomycin per ml, it readily grew on buffered charcoal-yeast extract agar containing either 25 or 100 μg of spectinomycin per ml. Indeed, the number of CFU formed on these plates was identical to that obtained on medium without drug. These data indicated that strain 130b contains a gene that is capable of inactivating spectinomycin.

To gain insight into the mechanisms of *Legionella* spectinomycin resistance, we determined the sequence of the cloned locus. To that end, a partial restriction map of pJLJ2 was derived, and subclones were made (Fig. 1). Based upon the phenotypes of the subclones, the putative spectinomycin resistance gene was localized to a 1.8-kb *Sau*3AI-*Xba*I fragment (Fig. 1). Figure 2 presents the sequences for the open reading frame (ORF) associated with spectinomycin resistance as well as its flanking regions. The ORF began at an ATG codon and ended 993 bp downstream at a TAA codon. This ORF would encode a protein of 331 amino acids and with a size of 36.4 kDa. The ORF was neither preceded by an obvious ribosome binding site and a −10 or −35 promoter region nor followed by an apparent transcription termination signal, suggesting that it is part of an operon. GenBank searches indicated that the predicted protein has similarity to bacterial aminoglycoside phosphotransferases (the APH enzymes) as well as to several eukaryotic kinases. It did not, however, bear any homology with previously characterized spectinomycin-modifying enzymes. From two perspectives, this information represented a potentially unique finding. First, none of the 21, previously identified APHs act on spectinomycin, and second, spectinomycin resistance genes, present in *Staphylococcus aureus* and members of the family *Enterobacteriaceae*, inactivate the drug via adenylylation, not phosphorylation (13, 21, 26). BLASTP results from a GenBank search specified three regions of similarity between the *Legionella* protein and the phosphorylating enzymes (Fig. 2 and 3). The first of these areas spanned residues 198 to 223 and displayed homology with four APHs whose targets include hygromycin, kanamycin, neomycin, paromomycin, and ribostamycin (Fig. 3A). The average amino acid identity and similarity between the *Legionella* protein and the APHs were 34 and 56%, respectively. This region of homology

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EcoRV    .         .         .         .         .         .
gatatcgaaatgctttagacaacgctcggaatgatacgaaagaatcttcggcagcggttg     60

aagaaggggtaggaaaagataattcgccttcttcttttcaaactaataaatagtatgatt    120

atactaatttccaaacaataattgacttttATGCTAAAACAACCAATTCAAGCTCAACAA    180
                              M  L  K  Q  P  I  Q  A  Q  Q      10

CTTATCGAACTTTGAAAGTGCATTATGGAATTGATATTCATACAGCACAATTCATCCAG    240
 L  I  E  L  L  K  V  H  Y  G  I  D  I  H  T  A  Q  F  I  Q     30

GGTGGTGCTGATACGAATGCATTTGCATATCAAGCAGATTCAGAATCCAAGTCTTATTTC    300
 G  G  A  D  T  N  A  F  A  Y  Q  A  D  S  E  S  K  S  Y  F     50
                                       ClaI
ATAAAGCTAAAATACGGCTATCATGATGAAATTAATTTATCGATAATCCGTCTTTTACAT    360
 I  K  L  K  Y  G  Y  H  D  E  I  N  L  S  I  I  R  L  L  H     70

GATTCTGGAATAAAAGAAATTATTTTTCCTATCCATACACTTGAAGCAAAATTATTCCAG    420
 D  S  G  I  K  E  I  I  F  P  I  H  T  L  E  A  K  L  F  Q     90

CAACTAAAGCATTTTAAAATAATTGCGTATCCATTTATTCATGCGCCCAATGGTTTCACC    480
 Q  L  K  H  F  K  I  I  A  Y  P  F  I  H  A  P  N  G  F  T    110

CAAAATTTAACAGGAAAACAGTGGAAACAGCTTGGAAAAGTATTAAGACAAATTCATGAA    540
 Q  N  L  T  G  K  Q  W  K  Q  L  G  K  V  L  R  Q  I  H  E    130

ACATCAGTTCCCATCTCGATTCAACAACAATTAAGAAAAGAAATATACTCCCCTAAATGG    600
 T  S  V  P  I  S  I  Q  Q  Q  L  R  K  E  I  Y  S  P  K  W    150

CGTGAAATAGTCAGATCCTTTTATAATCAAATTGAATTTGATAATTCAGATGATAAGCTC    660
 R  E  I  V  R  S  F  Y  N  Q  I  E  F  D  N  S  D  D  K  L    170
                                       ClaI
ACGGCTGCCTTTAAATCTTTTTTTAACCAAAATAGTGCTGCAATTCATCGATTAGTTGAT    720
 T  A  A  F  K  S  F  F  N  Q  N  S  A  A  I  H  R  L  V  D    190

ACTTCAGAAAAACTATCTAAAAAAATTCAACCTGATTTAGATAAATACGTACTATGTCAT    780
 T  S  E  K  L  S  K  K  I  Q  P  D  L  D  K  Y  V  L  C  H    210

TCTGATATACATGCGGGCAATGTGTTAGTCGGTAATGAAGAGTCGATTTACATTATTGAT    840
 S  D  I  H  A  G  N  V  L  V  G  N  E  E  S  I  Y  I  I  D    230

TGGGATGAGCCTATGTTAGCTCCAAAAGAACGTGATTTGATGTTCATAGGTGGTGGCGTT    900
 W  D  E  P  M  L  A  P  K  E  R  D  L  M  F  I  G  G  G  V    250

GGTAATGTATGGAATAAACCCCATGAAATCCAATATTTTTATGAAGGTTATGGTGAAATA    960
 G  N  V  W  N  K  P  H  E  I  Q  Y  F  Y  E  G  Y  G  E  I    270
                                                 EcoRV
AATGTCGATAAAACAATTTTGTCTTATTACAGGCATGAACGAATTGTCGAAGATATCGCA   1020
 N  V  D  K  T  I  L  S  Y  Y  R  H  E  R  I  V  E  D  I  A    290

GTATACGGGCAAGACTTGCTTTCACGTAATCAAAACAATCAGTCCAGACTTGAAAGTTTT   1080
 V  Y  G  Q  D  L  L  S  R  N  Q  N  N  Q  S  R  L  E  S  F    310

AAATATTTTAAAGAAATGTTTGATCCAAACAACGTTGTTGAAATAGCTTTTGCTACAGAG   1140
 K  Y  F  K  E  M  F  D  P  N  N  V  V  E  I  A  F  A  T  E    330

CAGTAAgctatgaccactgaacccttaccaatggggctacgaataccttacaattgtaaa   1200
 Q  -                                                           331

ccctcaaacaccaaatttgaggcaacctaatcaatggttgtataatatcaccattg       1256
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FIG. 2. Nucleotide and predicted amino acid sequences of a *L. pneumophila* gene associated with spectinomycin resistance. Cloned *L. pneumophila* DNA was sequenced from double-stranded plasmids by the dideoxy chain termination method by using ^{35}S-dATP and Sequenase version 2.0 (Amersham Life Science, Arlington Heights, Ill.) (2). Both strands of DNA were sequenced and then analyzed with PCGene (Intelligenetics, Mountain View, Calif.). Nucleotide sequences comprising the coding region of the gene are presented in uppercase letters, and the flanking bases are in lowercase characters. Key restriction enzyme recognition sites are boldface type. The lines above the sequences denote the three regions of homology between the *Legionella* protein and previously described antibiotic phosphotransferases (Fig. 3).

contains a site (motif 1) that bears a consensus sequence, V--HGD----N, which is believed to play a functional role in the APHs (19, 26, 28). Indeed, replacement of the invariant histidine abolishes enzyme activity (4, 19, 26). The *Legionella* protein contained the sequence V--HSD----N as its residues 207 through 217 (Fig. 3A). The second region of the cloned protein that bore a resemblance to APHs stretched from residues 224 to 246 (Fig. 3B). It showed 25% identity and 45% similarity to a segment of a hygromycin phosphotransferase and 50% identity and 60% similarity to a region of a modifier of streptomycin. In the previously defined APHs, this region contains a string of residues (motif 2) that has been implicated as a nucleotide binding site (4, 19, 26). The *Legionella* protein exhibited a rather modest conservation of the motif 2 consensus sequence (Fig. 3B). The final region of homology between the

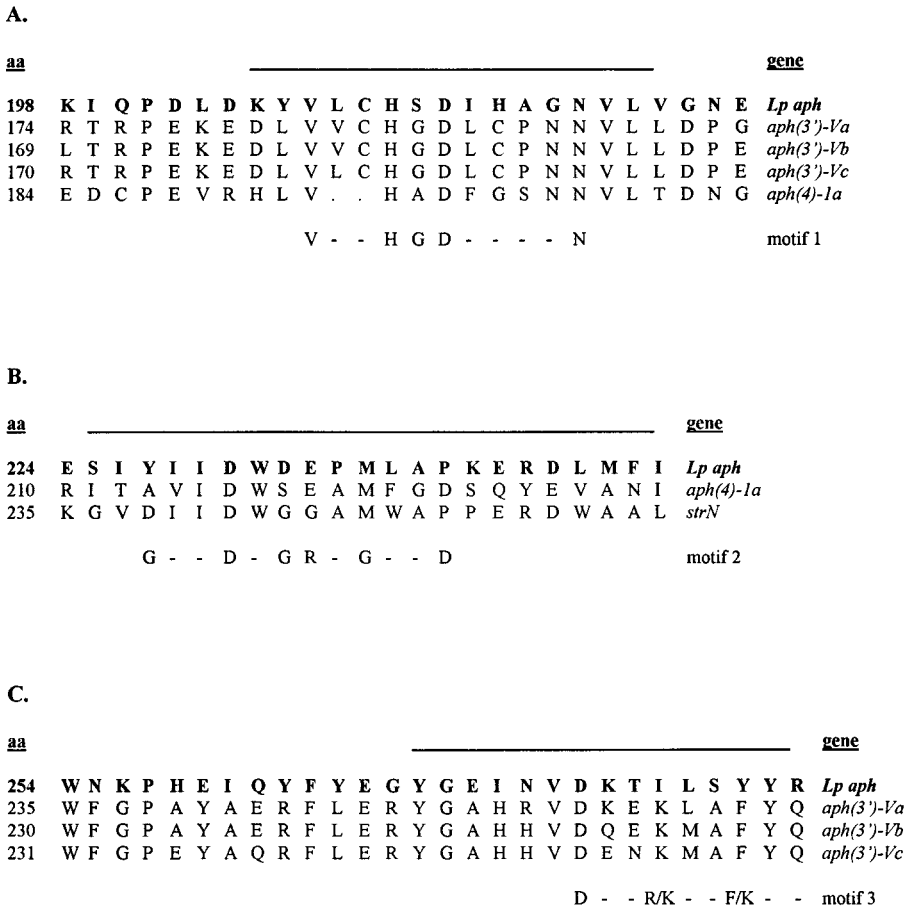

FIG. 3. Three regions of similarity between the cloned *L. pneumophila* (*Lp*) protein and APH enzymes. The first region contains motif 1 (A), the second contains motif 2 (B), and the third contains motif 3 (C). The left column indicates the amino acid (aa) residue numbers at which the homologies begin. The *Legionella* protein sequences appear, in boldface, on the top line; below them are the amino acids of the other APHs. The corresponding gene designations appear in the right column. The alignment of the APH sequences is as previously reported (26). The lines above the sequences denote three of the eight segments that had been deemed conserved among the 21 known APHs. The consensus residues for motifs 1 through 3 appear below the protein sequences. Note that motif 3 extends beyond the third region of homology. *aph(3′)-Va* is derived from *Streptomyces fradiae* (27), *aph(3′)-Vb* is derived from *S. ribosidificus* (15), *aph(3′)-Vc* is derived from *Micromonospora chalcea* (25), *aph(4)-Ia* is derived from *E. coli* (16), and *strN* is derived from *S. griseus* (23).

Legionella protein and APHs spanned residues 254 to 281 and averaged 35% identity and 56% similarity (Fig. 3C). However, the *Legionella* protein did not contain an internal site (motif 3) that is hypothesized to facilitate ATP hydrolysis (4, 19, 26). These data, particularly the motif 1 homology, suggested that the *L. pneumophila* gene encodes a phosphotransferase.

To determine whether *L. pneumophila* indeed possesses a novel APH, we assayed the ability of the cloned gene to mediate the phosphorylation of spectinomycin. More specifically, osmotic shockates of HB101(pTMS2) (Fig. 1) were tested for their capacity to transfer ^{32}P from labeled ATP to the antibiotic (12). As a control, we confirmed that extracts from HB101 containing *aph(3′)-Ia* of Tn*903* mediate the phosphorylation of kanamycin but not spectinomycin (22). In four trials (the results of three are shown in Table 1), the HB101(pTMS2) extract promoted significant incorporation of label into spectinomycin ($P < 0.001$, Student's t test). Importantly, the phosphorylation reactions required both the antibiotic substrate and the cloned gene, since no ^{32}P incorporation was observed with extract alone or with extracts from a vector control (Table 1). The phosphorylating activity was abolished by boiling or by proteinase K treatment. These data confirm that the *Legionella* gene encodes a phosphotransferase. We have designated it *aph*, for aminoglycoside/aminocyclitol phosphotransferase. The identification of *L. pneumophila aph* represents the first demonstration of a gene encoding a spectinomycin phosphotransferase. Although we do not know how the *L. pneumophila* APH phosphorylates spectinomycin, it did have the greatest homology with the APH(3′) enzymes (Fig. 3). To ascertain whether

TABLE 1. Phosphorylation of antibiotics by HB101(pTMS2) extracts

Trial[b]	Phosphate incorporation (cpm)[a] with antibiotic substrate				
	None	Spectinomycin	Streptomycin	Kanamycin	Hygromycin
I	7.2 ± 0.8	635.5 ± 29.9	25.9 ± 16.5	21.1 ± 3.8	16.1 ± 0.5
II	21.9 ± 0.6	580.1 ± 23.2	19.4 ± 3.7	32.6 ± 4.1	20.3 ± 1.7
III	ND[c]	12,606 ± 480[d]	ND	89.9 ± 5.5	ND

[a] Osmotic shockates were incubated with 200 μM antibiotic for 20 min at 37°C. All samples were tested in triplicate; results are means ± standard deviations.
[b] Trials I and II were done on different days but utilized the same cell extract. Trial III employed a second, more concentrated osmotic shockate.
[c] ND, not determined.
[d] Extracts from control HB101(pUC19) cells were negative for phosphotransferase activity, exhibiting an incorporation of 75.3 ± 2.2 cpm.

aph facilitates the phosphorylation of previously described targets for APHs, we repeated the assay with hygromycin, kanamycin, and streptomycin as substrates (Table 1). The *Legionella* APH did not modify any of the aminoglycosides, suggesting that it is specific for spectinomycin. In support of these data, HB101 and C600 clones containing *aph* were sensitive to hygromycin, kanamycin, and streptomycin. Limited substrate specificity is not uncommon among APHs; e.g., *aph(6)-I* targets only streptomycin (26). It has been suggested that differences in substrate specificity are due to variations in motifs 2 and 3 (17). Ultimately, the *Legionella* APH may not prove to be the only spectinomycin phosphotransferase; a recent GenBank submission, obtained after the completion of our work, presents an unpublished sequence for a putative spectinomycin phosphotransferase in *Streptomyces flavopersicus* (1). One final note about the predicted amino acid sequence of *aph* involves the presence of 10 TTA triplets. In *Streptomyces*, the availability of tRNAs for generally rare codons seems to play a role in resistance expression (18).

We strongly suspect that *aph* is the basis of spectinomycin resistance in strain 130b. To determine if *aph* exists in other *L. pneumophila* strains, Southern hybridizations were done with digoxigenin-labeled pTMS6 (Fig. 1) and DNAs from 10 other clinical isolates. The *aph*-specific probe hybridized under high-stringency conditions (i.e., 10% base pair mismatch allowed) with samples from serogroup 2 (SG2) strain ATCC 33154, SG3 strain ATCC 33155, SG4 strain ATCC 33156, SG7 strain ATCC 33823, SG8 strain ATCC 35096, and SG14 strain 1169-MN-H, as well as strains of SG9 to SG12 from our collection (data not shown) (6). In each case, the *Eco*RI hybridization pattern was unique, suggesting restriction site polymorphism in or around *aph*. This study represents the first genetic analysis of *Legionella* antibiotic resistance. Previous analyses have been biochemical characterizations of β-lactamases produced by *L. pneumophila* and *Legionella gormanii* (10, 11, 29). It is logical that *Legionella* organisms should possess mechanisms for inactivating antibiotics. Given their aquatic and terrestrial niches, they are likely exposed to producers of antibiotics, such as *Streptomyces* spp. which synthesize spectinomycin (23, 27). Fortunately, the innate resistance profiles of *L. pneumophila* strains have not hampered disease management. Nevertheless, the identification of any antibiotic resistance raises two questions, i.e., what the source of the resistance gene was and whether that source provide resistance to clinically relevant antimicrobials. For several reasons, we suspect that *aph* either originated within the legionellae or was obtained from another organism in the distant past. First, *aph* had a GC content and a codon usage that was typical of a *Legionella* gene. Second, the gene was in *L. pneumophila* strains that were isolated at different times and from different geographical locations. Third, the *aph* gene was present on differently sized restriction fragments; i.e., a conserved *aph* fragment would suggest linkage with a discrete, mobile genetic element.

Nucleotide sequence accession number. The *L. pneumophila* *aph* sequence has been deposited in the GenBank database at the National Center for Biotechnology Information under accession no. U94857.

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