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CLONING AND EXPRESSION OF STREPTOMYCIN INACTIVATING ENZYMES APH(6)-Ia AND APH(6)-Id

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Abstract- Discovered in the 1940s by Selman Waksman, the aminoglycoside antibiotic streptomycin is clinically important in the treatment of tuberculosis worldwide. However, strains of *Mycobacterium tuberculosis* and other pathogenic bacteria have become resistant to streptomycin. One mechanism by which this can occur is through the action of phosphotransferases that attach a phosphate group to position 6 of the streptidine ring of streptomycin, thereby inactivating it. Two such phosphotransferases are APH(6)-Ia from producer strain *Streptomyces griseus*, and APH(6)-Id found in animal, plant and human pathogenic isolates. Here, we report the subcloning and expression in *Escherichia coli* of soluble recombinant APH(6)-Ia and –Id enzymes. Sequencing of *aph(6)-Ia* revealed a one-nucleotide disagreement with the published sequence, such that the amino acid at position 262 is an alanine instead of a serine. The sequence of *aph(6)-Id* is identical to that of the gene found in transposon *Tn5393* of plant pathogen *Erwinia amylovora*. The successful expression of soluble forms of these enzymes now paves the way for experiments to study their structure and function by using site-directed mutagenesis.

Keywords: streptomycin, aminoglycoside phosphotransferase, expression

INTRODUCTION

Since its discovery in the 1940s, the aminoglycoside antibiotic streptomycin has been clinically useful in the treatment of a variety of bacterial infections, most notably those due to *Mycobacterium tuberculosis*, one of the three major causes of death--along with malaria and HIV/AIDS--in developing countries. Aminoglycoside antibiotics, which include kanamycin, neomycin, gentamycin, paromomycin and others in addition to streptomycin, each contain a six-membered aminocyclitol ring along with two or three carbohydrate moieties that are typically aminosugars. Their mechanism of action involves binding to the A site of the bacterial 30S ribosomal subunit (7); this inhibits translational processivity and causes miscoding during protein synthesis.

Over the years, many pathogenic bacteria, including *Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, have developed resistance to aminoglycosides. Although resistance in some cases is caused by mutations in genes for ribosomal proteins, in many clinical cases resistance is caused by enzymatic modification (phosphorylation, adenylation or acetylation), hence inactivation, of the antibiotic. The genes encoding these enzymes (24) are typically acquired through plasmid- or transposon-mediated gene transfer. The origin of the resistance genes found in clinical isolates is not always clear, but in some cases can be traced back to soil-dwelling antibiotic-producing bacteria that harbor the genes (1, 4). Because of the possible environmental origin of resistance, it is of interest to understand the function of resistance gene products that occur in natural as well as in clinical settings. The long-range goal of this work is to elucidate the structure and function of a pair of streptomycin phosphotransferases, one of which is found in the natural environment (APH(6)-Ia) and the other of

Abbreviations: **kDa**, kilodaltons; **IPTG**, isopropylthiogalactoside

which has been isolated from the clinical setting (APH(6)-Id).

Aminoglycoside phosphotransferases are classified and named according to the regiospecificity of the phosphate transfer, i.e. the position on the aminoglycoside of the particular hydroxyl group that is phosphorylated. Currently, there are seven classes: APH(3'), APH(2''), APH(3''), APH(7), APH(6), APH(4) and APH(9) (16, 25). The APH(6) phosphotransferase class comprises four members; these catalyze the transfer of the γ-phosphate group of ATP to the hydroxyl at position 6 of the streptidine ring of either streptomycin (APH(6)-Ia, -Ic, and –Id) or hydroxystreptomycin (APH(6)-Ib) (16). Fig. 1 displays the APH(6)-catalyzed reaction.

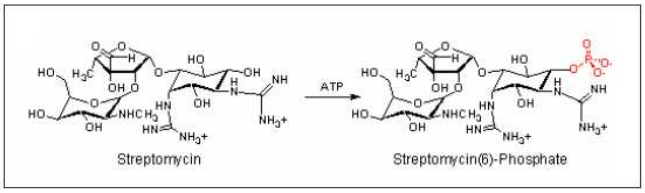

Figure 1. The Streptomycin 6-phosphotransferase-catalyzed reaction. Modified from ref. 25.

The gene for APH(6)-Ia is found within the streptomycin biosynthetic gene cluster of the soil bacterium *Streptomyces griseus* (18, 14). The entire gene cluster, including *aph(6)-Ia*, has been cloned and sequenced (5). Previously, restriction fragments derived from this cluster and containing *aph(6)-Ia* (which is also known as *aphD* or *strA*) were cloned into pBR322-based vectors; these plasmids, when transformed into *E. coli* cells, conferred resistance to streptomycin and directed expression of a protein of a molecular weight (34.5 kDa) consistent with APH(6)-Ia (11). The native APH(6)-Ia enzyme has been purified from *S. griseus* and characterized (19).

The gene for APH(6)-Id, also known also as *strB*, typically is linked with *strA* (note that this *strA* gene is different from *aph(6)-Ia*; it encodes a APH(3")-Ib phosphotransferase) as a *strA-strB* pair which, in turn, has been found on mobile DNA elements in a number of plant, animal and human pathogenic bacterial isolates (21, 17, 20, 8). Chiou and Jones (3) demonstrated that *aph(6)-Id* (*strB*) encodes a 28 kDa enzyme that catalyzes the production of streptomycin-6-phosphate from streptomycin. Interestingly, the *strB* (*aph(6)-Id*) gene is only capable of conferring high-level resistance to streptomycin when it is co-expressed with its partner, *strA* (*aph(3'')-Ib*). Amino acid sequence alignment indicates that there is 47% similarity between the APH(6)-Id and APH(6)-Ia proteins (16).

This paper presents the subcloning of the *aph(6)-Ia* and *aph(6)-Id* genes, and their expression as His-tagged fusion proteins in *Escherichia coli*. The work reported here represents the first step in the process of investigating the structure and function of the two enzymes.

MATERIALS AND METHODS

PCR-Amplification and Subcloning of Genes

aph(6)-Ia. The *Streptomyces griseus aph(6)-Ia* gene was PCR-amplified from a plasmid (pJDM40) contained within a *Streptomyces lividans* culture generously provided by Professor Wolfgang Piepersberg of Bergische Universitaet Wüppertal, Germany. Plasmid pJDM40 contains the entire streptomycin biosynthetic operon, including the gene *aph(6)-Ia* (also called *strA* or *aphD*). The pJDM40-bearing *S. lividans* cells were grown in TGY (0.8% Bacto-tryptone, 0.1% glucose and 0.4% Bacto-yeast) broth containing thiostreptone (25 μg/ml) for selection, and the pJDM40 plasmid was isolated from the culture using a Wizard Plus SV Minipreps kit (Promega). Purified plasmid DNA then was used as a template in a PCR amplification that employed native *Pfu* DNA polymerase. Forward and reverse primers containing Nde I and Xho I restriction sites, respectively, were designed to allow cloning into a pET expression vector (Novagen). Their sequences were:
5'—GTAAGGAAATTTCATATGAGTTCGTCGGACC—3' (forward) and
5'—GCTTCAGTGCTTCTCGAGTTCAGGGCTTCG—3' (reverse; restriction sites underlined). The following cycling parameters were used: 1 min. at 95°C; followed by 30 cycles of (95°C for 45 sec., 60°C for 60 sec., and 72°C for 3 min); followed by 10 min. at 72°C and indefinite hold at 4°C. The 0.9 kilobase-pair PCR product was purified from a low-melting agarose gel using a PCR Preps kit (Promega), and then ligated into pET-15b, which allows for isopropylthiogalactoside (IPTG)-induced expression of an N-terminal His-tagged fusion protein that can be subsequently purified by nickel affinity chromatography. Ligation mixtures containing recombinant *aph(6)-Ia*/pET-15b plasmids were transformed into NovaBlue (Novagen) cells and plated onto LB plates containing ampicillin (100 □g/ml). Plasmid DNA was isolated from transformants, and was checked by restriction digestion to see if *aph(6)-Ia* was present. Plasmid DNA from a single positive transformant was used as a template for nucleotide sequencing through the entire coding region of the gene, and was transformed into Rosetta (DE3) pLysS *E. coli* cells for expression. In a separate set of experiments, the *aph(6)-Ia* gene was PCR-amplified directly from *S. griseus* genomic DNA isolated from a culture obtained from ATCC (#10137, derived from Waksman's original streptomycin-producing strain 3463). The cycling conditions were the same as for the pJDM40 template mentioned above, except that dimethylsulfoxide (DMSO) at 5% was added to the PCR to help insure the removal of secondary structure in the template (15). The

product from this second PCR amplification was gel purified and analyzed directly by DNA sequencing.

aph(6)-Id. A plasmid (pSM1) containing the *strA-strB* gene pair from the plant pathogen *Pseudomonas syringae* was kindly provided by Dr. George Sundin of Oklahoma State University, U.S.A. The *aph(6)-Id* (*strB*) gene was PCR-amplified from pSM1 using native *Pfu* DNA polymerase and cycling parameters similar to those used for *aph(6)-Ia*, except that the annealing temperature was 52°C instead of 60°C. Forward and reverse PCR primers contained Nde I and Bam HI restriction sites, respectively, for subcloning into pET-15b, which was performed essentially as described above. The sequences of the primers were: 5'—CTTGGGGCATATGTTCATGCCGCCTG—3' (forward) and 5'—GTCGCTTGGATCCTAGTATGACGTCTG—3' (reverse; restriction sites underlined). The entire coding region of the resulting *aph(6)-Id*/pET-15 plasmid was sequenced to verify that no mutations were introduced at the PCR amplification step. The recombinant plasmid was transformed into Rosetta (DE3) pLysS cells for expression.

Expression

Both APH(6)-Ia and APH(6)-Id proteins were expressed similarly. Rosetta (DE3) pLysS cells bearing either recombinant *aph(6)-Ia*/pET-15b or *aph(6)-Id*/pET-15b plasmid were streaked from a -80°C frozen glycerol stock onto LB plates containing ampicillin (amp; 100 μg/ml) and chloramphenicol (cam; 34 μg/ml). A single colony was picked and used to grow an overnight culture. The following day, an aliquot of this culture was diluted 1:500 into fresh LB/amp/cam media and grown at 37°C to a cell density of OD_{600} = 0.6, at which point the culture flask was moved to a 25°C incubator and isopropylthiogalactoside (IPTG) was added to a concentration of 1 mM to induce expression of the recombinant protein. After 3-4 hours of induction, cells were pelleted and washed with 20 mM Tris, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell pellets were frozen at -80°C overnight, thawed—at which point they lysed due to the presence of plasmid-derived lysozyme—and then resuspended in 1x His-Bind Buffer (Novagen) containing 2.5 U/100ul of Benzonase™ nuclease (Novagen), which was added to degrade DNA and thereby reduce viscosity. The cell suspension was incubated at room temperature for 15 min followed by gentle vortexing. The lysate was centrifuged, and divided into supernatant (soluble) and pellet (insoluble) fractions. The soluble and insoluble fractions were analyzed by SDS-PAGE and by Western blotting using a His-tag specific antibody. Protein concentrations were determined using the Bio-Rad protein reagent.

Western Blotting

Proteins on SDS-PAGE gels were transferred to nitrocellulose membranes. The membranes were blotted first with a penta-His-tag monoclonal primary antibody (Novagen), and then with a goat anti-mouse IgG secondary antibody linked to horseradish peroxidase (HRP) that was part of a His-Tag HRP Lumiblot kit (Novagen). Detection was by chemiluminescent exposure of Kodak Bio-Max XAR film. After processing, the image on the film was captured using an Alpha Imager 2200 system (Alpha Innotech).

RESULTS AND DISCUSSION

DNA Sequencing

aph(6)-Ia. The nucleotide sequence of the *aph(6)-Ia* gene (also called *strA* or *aphD*) that was PCR-amplified from plasmid pJDM40 and cloned into pET-15b agrees with the sequence of the gene originally submitted to Genbank (Accession # Y00459; ref. 6) except at a single nucleotide. At nucleotide position 784, there is a clear "G" instead of a "T" in each of three sequencing reactions using different primers. To confirm that this difference was not simply due to an error introduced in the initial PCR, the *aph(6)-Ia* gene was PCR-amplified directly from *S. griseus* genomic DNA. This time, the organic reagent DMSO (5%) was added to insure disruption of possible secondary structure in the template (15). The resulting sequence of this PCR product also had a "G" at position 784. Adding further evidence to the conclusion that the "G" we obtained was not a PCR-generated error is that fact that a BLAST search using the *aph(6)-Ia* gene as a query found nine different partial sequences that all had a "G" instead of a "T" at position 784. One possible explanation for the discrepancy between our sequence and the original sequence is that an error was made in the original Genbank submission. Another possibility is that the strain we obtained and the one whose sequence was originally reported (Accession # Y00459) are from two closely-related, yet nonidentical, strains of *S. griseus*. This nucleotide difference translates into a replacement of serine-262 with an alanine. (See the sequence alignment in Fig. 2, in which Ala-262 is indicated with an asterisk.) It is not known if this amino acid difference (alanine versus serine at position 262) would affect the function of the enzyme; this question can be addressed in the future using site-directed mutagenesis. The nucleotide sequence of *aph(6)-Ia* has been deposited in Genbank (Accession # AY971801).

aph(6)-Id. The sequence of the *aph(6)-Id* gene (also called *strB*) that was sequenced and subcloned here (originally from *Pseudomonas syringae)* matches that of *strB* from the transposon *Tn5393* present in *Erwinia amylovora* (Accession # M96392; ref. 3), lending support for the two *aph(6)-Id* genes having a common origin in gene transfer events involving this DNA mobile element. The nucleotide sequence of *P. syringae aph(6)-Id* reported here has been submitted to Genbank (Accession # AY997127).

Expression

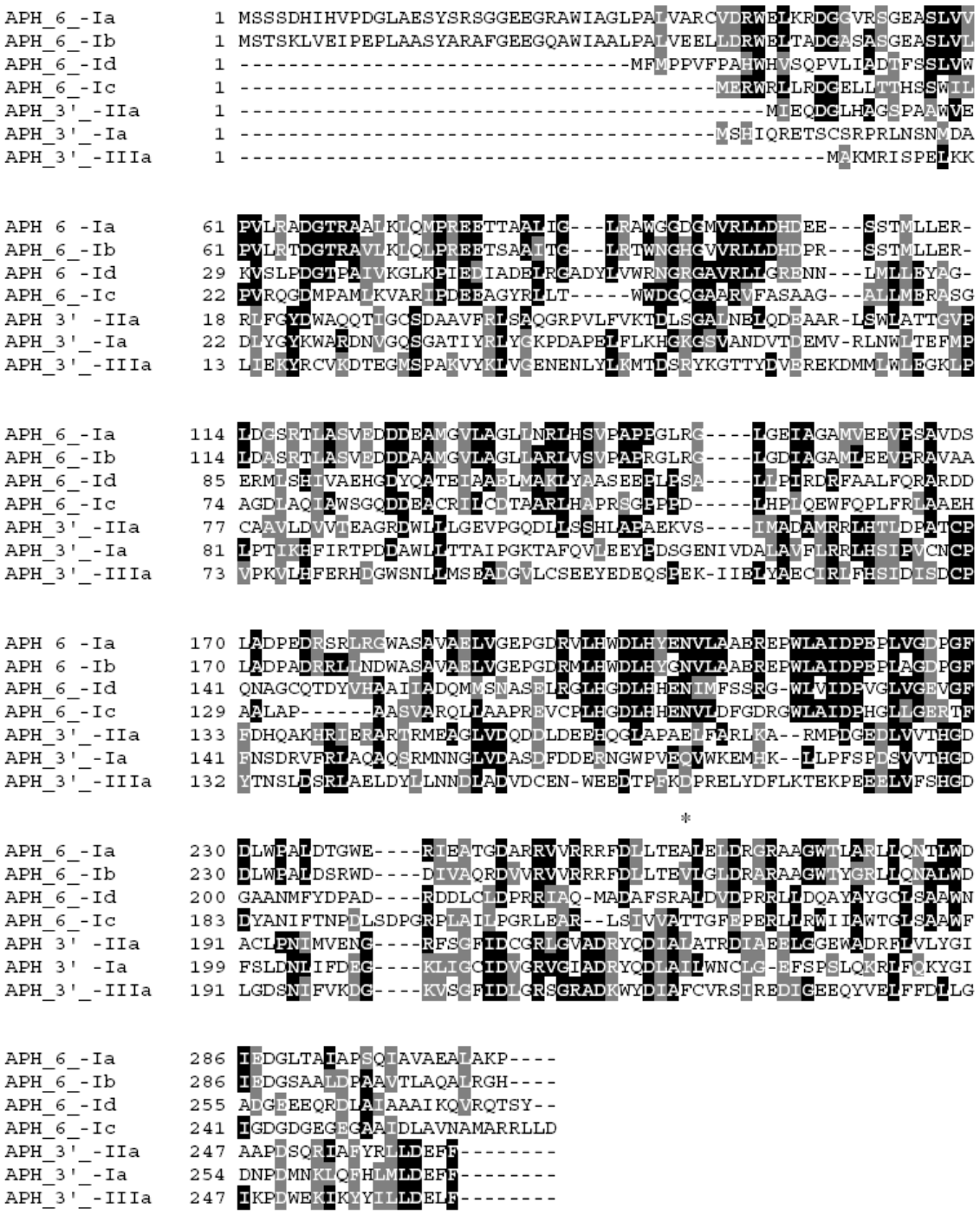

Figure 2. Multiple Sequence Alignment among APH(6) and APH(3') enzymes using ClustalW. Conserved amino acid residues are shaded gray; identical residues are shaded black. The position of Ala-262 in APH(6)-Ia is indicated with an asterisk. Among the enzymes whose sequences are displayed, APH(3')-IIa and APH(3')-IIIa have been studied in the most depth in terms of their kinetic (12), structural (9, 13), and structure-function (22, 2, 23, 10) characteristics.

Upon induction of expression and cell lysis, the majority of the expressed recombinant APH(6)-Ia and APH(6)-Id proteins were found in inclusion bodies in insoluble fractions (not shown). Nevertheless, for each, some soluble recombinant protein was produced. Figure 3 presents Western blotting analysis, using the anti-His-tag antibody, of expressed soluble recombinant N-terminal His-tag fusions of APH(6)-Ia and APH(6)-Id. Based on its amino acid sequence and the size of its His tag, a molecular weight of 34 kDa is predicted for 6xHis-APH(6)-Ia. Fig. 3 shows that a protein band of approximately this size was present upon induction of expression (lane 4). The 34 kDa band was absent from Rosetta (DE3) pLysS cells lacking the expression vector and absent from cells containing the vector but not induced with IPTG (lanes 2 and 3). These results indicate specific expression of a His-tagged protein consistent with APH(6)-Ia. Additional faint bands are also seen in lane 4; these likely represents minor proteolytic digestion products derived from the 34 kDa protein.

Fig. 3 shows that APH(6)-Id was also expressed in soluble form, albeit less well than APH(6)-Ia. A band for a His-tag-containing protein approximately 30 kDa in size is seen in the induced cells (lane 6); this agrees well with the size of 32 kDa predicted for His-tagged APH(6)-Id based on its amino acid sequence. The ~30 kDa band is absent in the uninduced cells (lane 5), indicating specific expression of a His-tagged protein consistent with APH(6)-Id. The lower yield of soluble APH(6)-Id compared to the –Ia enzyme, combined with the observation that the total yields of recombinant protein (soluble plus insoluble; not shown) were similar, indicates that the former is less soluble than the latter under the expression conditions used.

Conclusion

In this paper, we have reported the cloning and expression of two related enzymes, APH(6)-Ia and APH(6)-Id, that phosphorylate, and thereby inactivate, the antibiotic streptomycin. Sequencing of *aph(6)-Ia* reveals that its nucleotide sequence differs from a previously published sequence at a single nucleotide (G versus T at position 784); this means that the amino acid residue at position 262 in the primary structure is an alanine rather than a serine as previously indicated (6). Sequencing of *aph(6)-Id*, which was obtained originally from a streptomycin-resistant strain of plant pathogen *P. syringae*, gives an exact match to that of *aph(6)-Id* (*strB*) found within transposon *Tn5393* from a similarly streptomycin-resistant strain of *E. amylovora*, suggesting an origin in a horizontal gene transfer event.

The successful expression of soluble forms of APH(6)-Ia and APH(6)-Id reported here is an essential prerequisite for the purification, characterization, and analysis of the structure and function of these two enzymes, one from the streptomycin-producer strain *S. griseus* and the other found in clinical and other bacterial isolates. The eventual hope is that an understanding of the active sites of APH(6)-Ia and –Id, combined with what is known about the structure and function of similar aminoglycoside phosphotransferases (2, 9, 10, 12, 13, 22, 23), will help to elucidate the active site of aminoglycoside phosphotransferases in general.

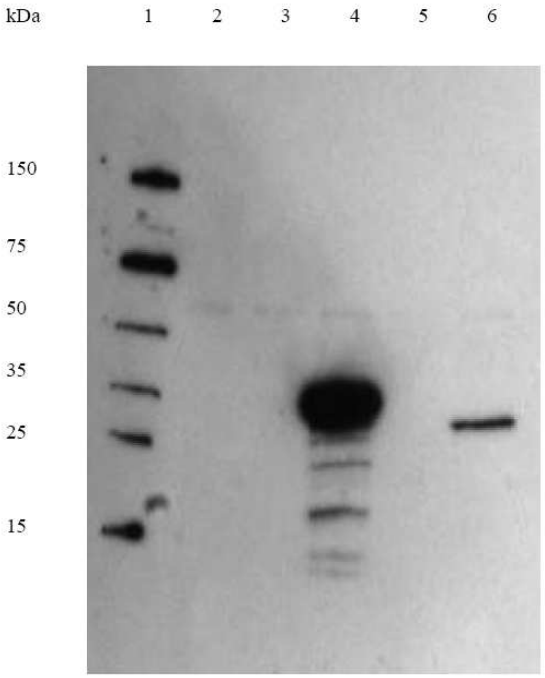

Figure 3. Western blotting analysis of the expression of soluble recombinant APH(6)-Ia and APH(6)-Id. Expression conditions are described in the Materials and Methods section. An anti-polyHis-tag antibody was used. *Lane* 1, molecular weight markers (sizes in kDa indicated on the left). Crude extracts from: *Lane 2*, mock-induced Rosetta (DE3) pLysS cells lacking expression vector; *Lanes 3 and 4*, cells containing the *aph(6)-Ia* expression vector that were either not induced (*lane 3*) or induced (*lane 4*) with 1 mM IPTG; and *Lanes 5 and 6*, cells containing the *aph(6)-Id* expression vector that were either not induced (*lane 5*) or induced (*lane 6*). Ten micrograms of total protein were loaded in each lane.

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