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Mutations in APH(3')-IIIa Increase Activity Against Amikacin

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Mutations in APH(3')-IIIa Increase Activity Against Amikacin

By

Joseph Kramer
B.S.E., Tulane University, 2008

Advisor: Ichiro Matsumura, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science
in Graduate Division of Biological and Biomedical Science
Genetics and Molecular Biology
2013

Abstract

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By Joseph Kramer

Aminoglycoside modifying enzymes have evolved the ability to catalyze reactions with a wide array of substrates. This evolution has not been documented in clinical settings. Here we have simulated conditions to determine whether the aminoglycoside phosphotransferase APH(3')-IIIa is capable of evolving increased enzymatic activity using the aminoglycoside amikacin as a substrate. APH(3')-IIIa was subjected to four rounds of mutation: two rounds of error-prone PCR and two rounds of staggered extension process. After four rounds of selection, we observed a 60-fold decrease in susceptibility to amikacin. Purified mutant APH(3')-IIIa protein had an increased catalytic efficiency in reaction with both amikacin and kanamycin, its native substrate. *E. coli* expressing the mutant APH(3')-IIIa also had a decreased ability to form colonies on non-selective plates. The catalytic function of the mutant had no impact on its colony forming ability, but expression of the mutant protein did have an effect on the colony forming ability. The results suggest a possible mechanism through which resistant alleles of APH(3')-IIIa fail to spread in clinical *E. coli* isolates even under constant exposure to high levels of aminoglycoside antibiotics.

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INTRODUCTION

Antibiotic discovery and advancement over the last 85 years has drastically improved the ability for people to fight off infections. However, the wide-spread use of antibiotics has promoted resistance in bacteria, for example, by evolving enzymes that can chemically modify the antibiotics and render them nonfunctional. Aminoglycoside modifying enzymes are a class of such enzymes, including aminoglycoside acetyltransferases (AAC), aminoglycoside nucleotyltransferases (ANT), and aminoglycoside phosphotransferases (APH). Each of these enzymes catalyzes the same reaction with a distinct set of substrates (22).

Mutations in aminoglycoside modifying enzymes are not commonly observed. Sequence similarity among the same enzymes from different bacteria suggests that these enzymes are capable of evolving (22). However, in clinical isolates of the same bacterial species, no mutants of these enzymes have been identified (24). This is unusual because enzymes involved in antibiotic resistance are well known for their malleability, as demonstrated in numerous experiments of directed evolution. Also being frequently found are mutations that can improve the secondary activities of antibiotic resistance enzymes, and thus can broaden their specificities. This class of mutations often come with no negative effects to the primary activity (2). In particular, aminoglycoside modifying enzymes have expanded their range of substrates with increased resistance in a laboratory setting (24). Therefore, the lack of mutants from clinical environments begs an explanation.

Aminoglycoside phosphotransferase (3') type IIIa (APH(3')-IIIa) is a member of the APH family, which has enzymes demonstrating 11 known resistance mechanisms

(22). APH(3')-IIIa functions by adding a phosphate group from ATP to the 3' hydroxyl group of a number of aminoglycoside antibiotics. APH(3')-IIIa has been found in clinical samples tested for aminoglycoside resistance (11, 20). The primary substrates of APH(3')-IIIa include kanamycin, neomycin B, paromycin, and lividomycin (27). It has, however, only a weak ability to phosphorylate amikacin which is probably the reason that aminoglycoside phosphorylation by APH(3')-IIIa is not known to be involved in amikacin resistance in clinical settings (5, 8, 26). Like the high malleability of other antibiotic resistance enzymes, APH(3')-IIIa might have the potential to evolve increased secondary activity to phosphorylate amikacin (10). In my thesis research, APH(3')-IIIa was first mutated to reach a higher level of amikacin resistance, then followed by characterization of these novel mutants. In the end, the limitations of the directed evolution of APH(3')-IIIa are examined to provide some insights into why this enzyme has not been evolved for amikacin resistance in clinical settings.

MATERIALS AND METHODS

Mutagenesis

(i) Error-Prone PCR

The APH(3')-IIIa gene was cloned from the pQBAV3c vector (4). Primers 1 and 2 were used to perform a mutagenic PCR (Table 1). The final 50 μL PCR was performed including 1.25 μL mutagenic buffer (8 mM dTTP, 8 mM dCTP, 4.8 mM $MgCl_2$, 0.5 mM $MnCl_2$). The product was purified and digested with EcoRI and SalI. The product of the correct size was purified from the excised gel band, and ligated into the pQBAV3c vector, which was digested at its EcoRI and SalI restriction sites.

(ii) Staggered Extension Process (StEP)

Staggered extension process was used to combine mutations obtained from random mutagenesis (28). Primers 1 and 2 were used to perform a PCR on the random mutagenesis products. A short annealing/extension time was used to prevent full replication of the APH(3')-IIIa gene (only 15 seconds at 60°C, compared to the standard 30 seconds at 60°C and 2 minutes at 72°C for the random mutagenesis) with a higher number of cycles (80 cycles vs 35 cycles). The product was purified and digested at the EcoRI and SalI restriction sites. The product was purified after digestion, and ligated into the EcoRI and SalI-cut pQBAV3c vector.

(iii) Site-Directed Mutagenesis

Primers 3 and 4 were used in combination with primers 1 and 2 in multiple PCRs to introduce the D190A mutation into APH(3')-IIIa. The first PCR used primers 1 and 4 and the second PCR used primers 2 and 3 with APH(3')-IIIa in pQBAV3c as the template. These products were both purified, and then combined in a PCR that used

primers 1 and 2. The product of this final PCR was digested at the EcoRI and SalI restriction sites. The product was purified after digestion, and ligated into the EcoRI and SalI-cut pQBAV3c vector.

(iv) Cloning into pET28

The APH(3')-IIIa gene was cloned into a pET28 vector that was modified with an ampicillin resistance gene . Primers 5 and 6 were used to perform a PCR on the APH(3')-IIIa gene in pQBAV3c. The product was purified and digested at the NdeI and HindIII restriction sites. The product was purified after digestion, and ligated into the modified pET28 vector, which was digested at its NdeI and HindIII restriction sites. The result of this ligation placed the APH(3')-IIIa gene in a configuration that would place a His-tag on the N-terminus of the protein.

Selection for decreased amikacin susceptibility

Four rounds of mutagenesis of APH(3')-IIIa were carried out with error-prone PCR (rounds 1 and 3) and StEP (rounds 2 and 4). *E. coli* InvαF' containing the modified pQBAV3c plasmid with APH(3')-IIIa were tested after each round of mutagenesis to select for mutants with a decreased susceptibility for amikacin (Sigma #a1774). After the first round of mutageneis, cells were plated (at approximately 1000 CFU/plate) on LB with an initial concentration of amikacin at 18 μg/mL. Colonies that formed were then grown to saturation. The cultures were diluted 1:100,000, and 50 μL of the diluted broth was plated on LB plates containing 22, 26, 30, 35, 40, 45, 50 μg/mL amikacin. A silimar procedure was repeated after the second round of mutagenesis, only with different concentrations of amikacin in the plates (initial concentration of amikacin 80 μg/mL; dilutions plated on amikacin concentrations of 160, 170, 180, 200 μg/mL). After the third

round, cells were plated (at approximately 1000 CFU/plate) on LB with 220 μg/mL amikacin. Colonies that formed were then grown to saturation, and 50 μL of the diluted broth (1:50,000) was plated on LB plates containing 250, 280, 310, 340, 370, 400, 425, 450, 500, 550, 600, 650, 700, 750, 800, 825, 850, 875. 900, 950, 1000, 1050, 1100, 1150, 1200, 2000 μg/mL amikacin. Finally, after a fourth round of mutagensis, cells were plated (at approximately 1000 CFU/plate) on LB with 1200 μg/mL amikacin. The reported susceptibility of each mutant is the concentration at which it was unable to form any colonies.

Purification of APH(3')-IIIa enzyme and mutants

E. coli BL21(DE3) containing the modified pET28 plasmid with APH(3')-IIIa were grown up overnight in a 5 mL culture of LB broth (EMD #1.10285.5007) with ampicillin (Sigma #a0166). The starter culture was then added to a 500 mL culture of LB broth with ampicillin , grown for 4.5 hours, inoculated with 500 μL of 1 M IPTG (GoldBio #I2481C50), and grown overnight at 37 °C.

The preparation and purification of APH(3')-IIIa was performed at 4 °C. The 400 mL of the fully grown cultures were spun down at 4000 rpm for 10 minutes. The cells were resuspended in 30 mL of 50 mM Tris pH 7.5 and sonicated on ice for 5 minutes (5 cycles of 30 seconds on/ off sonication at 6 watts) (Misonix Sonicator 3000). The cell lysate was spun down at 16,000 rpm for 40 minutes, and the supernatant was saved.

A His-trap column connected to an AKTA purifier (GE Healthcare) was equilibrated with buffer B (50 mM Tris pH 7.5, 400 mM imidazole) before the supernatant was run through the column. The column was washed with buffer A (50 mM Tris pH 7.5) before applying a stepped gradient of buffer B to elute off the APH(3')-IIIa

enzyme. Fractions containing the enzyme were dialyzed (Spectra #132720) in 50 mM Tris pH 7.5 (Sigma #t4661) and run on a polyacrylamide gel to verify purity.

Enzyme Kinetics

A coupled assay with pyruvate kinase/lactate dehydrogenase was used to measure the phosphorylation of aminoglycosides (6, 15). The assay was performed in a buffer with concentrations of 50 mM Tris pH 7.6, 40 mM KCL (Sigma #p9541), 10 mM $MgCl_2$ (Sigma #m0250), 0.25 mg/mL NADH (Sigma #n4505), 2.5 mM PEP (Sigma #p7252), and 1 mM ATP (Sigma #a26209). The buffer (975 µL) was mixed with 5 µL of pyruvate kinase/lactate dehydrogenase (Sigma #p0294) and 10 µL of the appropriate concentration of aminoglycoside, then incubated at 37 °C for 15 minutes. The reaction was initiated by the addition of 10 µL of a stock solution of APH(3')-IIIa (1µM). The oxidation of NADH was measured in a quartz cuvette (Fisher #14-385-914A) by the continuous monitoring of absorbance at 340 nm using a spectrophotometer (Shimadzu UV-1601) for a period of 5 minutes. The rate of oxidation of NADH was used to determine the initial velocity of the phosphorylation of APH(3')-IIIa. The result were obtained using Kaleidagraph v3.5 (Synergy Software), fitting the data to the equations:

$$v = V_{max}[S]/(K_m + [S])$$

$$v = V_{max}[S]/(K_m + [S] + [S]^2/K_i)$$

Fitness Testing

(i) pqBAV3c

E. coli InvαF' containing the pQBAV3c plasmid with APH(3')-IIIa was grown overnight to saturation in 2 mL LB broth with 34 µg/mL chloramphenicol. A 100 µL sample of each culture was mixed with 900 µL of LB broth in a cuvette, and the optical

density of each culture was measured at 600 nm. An aliquot of each culture was combined with LB broth to create a 1 mL sample that had an optical density at 600 nm of 1.0. The 1 mL sample was then serially diluted twice, each time with 10 µL of the sample being added to 990 µL of LB broth. A 50 µL volume of the final serial dilution was plated onto LB plates containing 34 µg/mL chloramphenicol. Plates were grown at 37 °C overnight, and visibly formed colonies were counted.

(ii) pET28 in BL21(DE3)

E. coli BL21(DE3) containing the modified pET28 plasmid with APH(3')-IIIa was grown overnight to saturation in 2 mL LB broth with 100 µg/mL ampicillin. A 100 µL sample of each culture was mixed with 900 µL of LB broth in a cuvette, and the absorbance of each culture was measured using to determine the optical density at 600 nm. An aliquot of each culture was combined with LB broth to create a 1 mL sample that had an optical density at 600 nm of 0.5. The 1 mL sample was then diluted with 10 µL of the sample being added to 990 µL of LB broth. A 50 µL volume of the final serial dilution was plated onto LB plates containing 100 µg/mL ampicillin. Plates were grown at 37 °C overnight, and visibly formed colonies were counted.

(iii) pET28 in TG1

E. coli TG1 containing the modified pET28 plasmid with APH(3')-IIIa was grown overnight to saturation in 2 mL LB broth with 100 µg/mL ampicillin. A 100 µL sample of each culture was mixed with 900 µL of LB broth in a cuvette, and the absorbance of each culture was measured using to determine the optical density at 600 nm. An aliquot of each culture was combined with LB broth to create a 1 mL sample that had an optical density at 600 nm of 0.5. The 1 mL sample was then serially diluted twice,

with 10 μL of the sample being added to 990 μL of LB broth, and then 100 μL of the dilution being added to 900 μL of LB broth. A 50 μL volume of the final serial dilution was plated onto LB plates containing 100 μg/mL ampicillin. Plates were grown at 37 °C overnight, and visibly formed colonies were counted.

RESULTS

Selection of *E. coli* InvαF' with Increased Resistance to Amikacin

The APH(3')-IIIa gene was mutated in order to study the increase in its ability to confer resistance to amikacin. Four rounds of mutagenesis were performed on APH(3')-IIIa to create a version of the enzyme that confers resistance to high levels of amikacin. All mutants were sequenced (Table 2). Mutants are referenced as APH(# round of mutagenesis).(# mutant number). In the first round, random mutagenesis was performed using error-prone PCR and mutants were selected for their ability to form colonies on LB plates containing 18 μg/mL amikacin. Preliminary testing had shown the inability of *E. coli* InvαF' containing the pQBAV3c plasmid with APH(3')-IIIa to grow on this high of a concentration (data not shown). Random mutagenesis led to the selection of 43 separate mutants from the approximately 10000 plated. The sequencing revealed that of the 43 mutants, most contained just 1 or 2 nucleotide mutations in the open reading frame (ORF) (27/43 mutants). These mutants were able to grow on concentrations that were double the lethal level for wildtype *E. coli* expressing APH(3')-IIIa. These results are consistent with previous studies showing that single amino acid changes are capable of significant changes in secondary enzyme activity (25).

The pQBAV3c plasmids containing the separate 43 mutants were pooled, along with the plasmid containing the wildtype APH(3')-IIIa, and used as templates for staggered extension precess (StEP) mutagenesis. The goal of StEP mutagenesis is to combine mutations from the error-prone PCR mutants, in a process that mimics recombination (1). Mutants from the StEP mutagenesis were selected for their ability to form colonies on LB plates containing 80 μg/mL amikacin. This resistance level resulted

in the selection of 7 mutants from the approximately 10000 colonies screened. This mutagenesis was intended only to combine mutations previously selected after error-prone PCR. However, two new mutations (I40T and D193N), arose and were seen in multiple mutants. The S194R and K255R mutations were also seen in multiple mutants. Recurring mutations suggest an importance of these residues in increasing the ability of APH(3')-IIIa to confer resistance to amikacin. Surprisingly, most of the selected mutants contained only a single residue mutation (4/7 mutants). Some of these missense mutations were seen in the previous round. It is possible that the increase in amikacin resistance of these mutants in this round was due to mutations outside of the ORF (as with APH2.4 and APH2.5 compared to APH1.18) or the loss of secondary mutations (as with APH2.3 compared to APH1.27 or APH1.33).

Another round of error-prone PCR was performed on the 7 selected mutants from the StEP mutagenesis that grew on 80 μg/mL amikacin. Mutants were now selected for their ability to form colonies on LB plates containing 220 μg/mL amikacin, a resistance level that resulted in the selection of 4 mutants from the approximately 10000 colonies screened. All four mutants selected contained the S194R and three of the four mutants (APH3.1, APH3.3, APH3.4) also had a mutation in the 40th residue, although there were two different mutations at this position - I40V and I40T. These mutations, which were prevalent in round 2, remained after round 3. Additionally, the D193N mutation, contained in two mutants from round two, was lost. Due to the close proximity of the D193N and S194R, it is likely that these mutations were affecting the enzyme in the same manner, but the S194R allowed for the ability to confer higher levels of amikacin resistance, and was able to be selected in later rounds.

Another round of StEP was performed with the 4 mutants from round 3 and wildtype APH(3')-IIIa as template. The resulting mutant, APH4.1, had the ability to form colonies on LB plates containing 1200 μg/mL amikacin, higher than any previous mutants, and well over an order of magnitude higher than the wildtype. APH4.1 had the three mutations that appeared, in earlier rounds, to be important for APH(3')-IIIa to confer increased resistance: I40T, S194R, and K255R.The role of these mutations can be estimated by determining their location within the protein (Figure 1). None of these mutations were in residues known to directly interact with aminoglycoside and none were in the aminoglycoside binding loop of the protein (7). Based on their peripheral protein locations, the I40T and K255R probably contribute to protein stability. The S194R is close to the aminoglycoside binding site of APH(3')-IIIa. This mutation may have an effect on the ability of a substrate to bind within the protein.

Biochemical Charaterization of APH(3')-IIIa and the APH4.1 mutant

Kinetic parameters were determined for both APH(3')-IIIa and APH4.1 using a coupled pyruvate kinase/lactate dehydrogenase assay (Table 3, Figure 2). The K_m and k_{cat} of wildtype APH(3')-IIIa were similar to previous characterizations, indicating the His-tag did not have an impact on ezymatic activity (16). The parameter measurements of APH4.1 indicate a significant evolution in substrate specificity and molecular interaction. APH4.1 had both a higher k_{cat} and lower K_m when catalyzing the phosphorylation of amikacin, when compared to the wildtype APH(3')-IIIa. This led to a k_{cat}/K_m value that was very close to its catalytic efficiency with kanamycin. Similar k_{cat}/K_m for both aminoglycoside in APH4.1 contrasts with the wildtype APH(3')-IIIa, which had a catalytic efficiency that was an order of magnitude higher with kanamycin, when

compared to amikacin. APH4.1 also has an extreme change in its interaction with kanamycin, when compared to the wildtype. The sharp peak at the left of Michaelis-Menten plot indicates substrate inhibition at a very low *K*i value. The phenotypic cost and benefits of this substrate inhibition is unknown. Similar enzyme inhibition is seen in laboratory settings, and often ignored (18). The overall effects on the enzyme-substrate interaction did not greatly change the k_{cat} /K_m of the phosphotransferase with kanamycin, but did change the k_{cat} /K_m with amikacin. This is notable, since k_{cat}/K_m (catalytic efficiency) is the single kinetic parameter shown to have a significant relationship to the antibiotic susceptibility for APH(3')-IIIa (16). This may help explain why the inhibition did not alter the conferred resistance phenotype toward kanamycin in APH4.1.

Role of biochemical activity of APH4.1 on colony forming ability

The plating of *E. coli* InvαF' containing either the APH(3')-IIIa or APH4.1 gene on the pQBAV3c plasmid revealed significant differences in the cells to form colonies (Figure 3). When plated under non-selective conditions for increased resistance, the *E. coli* cells expressing the wild type APH(3')-IIIa formed colonies much more readily than the cells with APH4.1 form of the gene. Initial speculation was that the altered enzymatic activity of APH4.1 was the cause of the decreased ability of cells expressing the protein to formed colonies. The promiscuous activity acquired by APH4.1 from the evolution may have unintended biochemical interactions in the cell. To test this hypothesis, a single point mutation was made in the 190th residue of APH(3')-IIIa and APH4.1 changing the aspartic acid into alanine. This well characterized mutation has no significant effect on the structure or stability of APH(3')-IIIa, but is shown to eliminate any observable activity for the phosphorylation of kanamycin (9). The D190A mutation

in both APH(3')-IIIa and APH4.1 resulted in the inability of these proteins to grown in kanamycin, and the inability of APH4.1 to grow in amikacin (data not shown).

If the enzymatic activity of APH4.1 was inhibiting colony forming ability, the D190A mutation should reverse this inhibition. The addition of this mutation, however, resulted in no change in decreased colony forming ability. The D190A mutation was not completely harmless; the addition of this mutation to APH(3')-IIIa resulted in a significant decrease in the ability of cells expressing the enzymes to form colonies. These results can exclude the evolved promiscuous activity of APH4.1 as the cause of a decrease in colony forming ability. However, the significant decrease in colony forming ability of APH(3')-IIIa with the D190A mutation suggests that it is not inconsequential. This mutation has the ability to impact the colony forming ability of cells of cell expressing APH(3')-IIIa.

Colony forming ability of APH(3')-IIIa mutations

The difference in the ability to form colonies between the wildtype and APH4.1 version of APH(3')-IIIa was further investigated using intermediate mutants that were created during the error-prone PCR and StEP mutagenesis. The intermediate mutants that shared a residue mutation with APH4.1 were grown, diluted to equal concentrations, and plated under non-selective conditions for increased amikacin resistance. The results show that the mutants of APH(3')-IIIa, after round 2, all have a significantly decreased ability to form colonies (Figure 4). Some variation in the level of colony forming ability between the round 2 and the round 3 mutants existed, but there is still an obvious inverse correlation between the susceptability to amikacin and colony forming ability. Curiously, some of the intermediate mutants had a more severe loss of colony form ability (APH2.3,

APH3.1, APH3.4) compared to wildtype APH(3')-IIIa than APH4.1. Whether this is due to deleterious mutations in these intermediates that are not present in APH4.1, beneficial mutations in APH4.1 that are not present in the intermediates, or another cause is unknown.

Role of plasmid stability on colony forming ability

The loss of the pQBAV3c plasmid in *E. coli* InvαF' could explain the differences seen in colony forming ability between APH(3')-IIIa and its mutants. This type of loss could be due to mutations in APH4.1 causing instability in the pQBAV3c plasmid. If this were the case, a disparity in colony forming ability would be revealed by growing *E. coli* InvαF' containing pQBAV3c on plates both with and without selection for plasmid retention. However, this experiment revealed that there was little to no plasmid loss in cells expressing either wildtype APH(3')-IIIa or APH4.1 (Table 4). Both sets of cells had greater than 90% plasmid retention. It also appears that a difference in plasmid stability is not the cause of decreased colony forming ability in APH(3')-IIIa or APH4.1 with the D190A mutation, since neither of these had a significant difference in plasmid retention compared to the version of the gene without the D190A mutation.

Role of protein expression level on colony forming ability

An explanation for the disparity in colony forming ability of cells expressing APH(3')-IIIa and its mutants is differences in protein expression level. Protein expression has been shown to have a fitness cost to the growth of *E coli* (23). The constitutive promoter for APH(3')-IIIA in the pQBAV3c plasmid was part of the fragment of DNA affected during the mutagenesis process. It is possible that mutations in the promoter could lead to an over-expression of APH(3')-IIIa. In order to minimize differences in

expression, the APH(3')-IIIa and APH4.1 were cloned into a pET28 plasmid, which contains a T7 promoter under a LacI repressor. Expression of both these genes, under identical promoters, was observed in *E. coli* BL21(DE3) Gold. The result of controlling expression level was actually an increase in the difference of colony forming ability between cells expressing APH(3')-IIIa and cells expressing APH4.1 (Figure 5). The difference in the colony forming ability between APH(3')-IIIa and APH4.1 went from an approximately 2-fold difference observed in InvαF' to an approximately 10-fold difference observed in BL21(DE3) Gold. The increased difference in colony forming ability compared to APH(3')-IIIa was also seen in APH4.1 with the D190A mutation. APH(3')-IIIa with the D190A mutation retained its approximate 10-fold difference in colony forming ability; in the pQBAV3c plasmid, it already had the same promoter as the wildtype APH(3')-IIIa gene.

Role of transcription of RNA on colony forming ability

Elimination of transcription allows for the characterization of transcription and translation in the ability of cells to form colonies. The pET28 constructs containing APH(3')-IIIa and APH4.1 were transformed into TG1 cells. These cells do not contain the T7 RNA polymerase required to transcribe the phosphotransferase protein in the pET28 plasmid. Elimination of transcription did decrease the difference in colony forming ability between APH(3')-IIIa and APH4.1, as compared to the difference between these plasmids in BL21(DE3) cells (Figure 6). However, this still did not completely eliminate the difference. TG1 cells with the pET28 plasmid containing APH(3')-IIIa formed a significantly higher number of colonies compared to TG1 cells with the pET28 plasmid containing APH4.1; the difference between APH(3')-IIIa and

APH4.1 was approximately 2-fold, compared to the approximately 10-fold difference in BL21(DE3) cells. This result indicates that there is some factor in colony forming ability that is unrelated to the transcription and translation of APH(3')-IIIa and APH4.1, but somehow affected by the differences in their genetic sequence.

DISCUSSION

The use of antibiotics for a successful treatment is dependent on the antibiotics to be lethal to the infectious microbe, while at a level that remains non-toxic to the patient being treated. Antibiotics may no longer be a feasible option if the accumulation of mutations raise the minimal susceptible concentration of antibiotics to such high levels as to be harmful to humans. Aminoglycoside modifying enzymes are already able to confer high levels of resistance to a wide range of antibiotics (22). Fortunately, these enzymes are effective to only a limited range of specific substrates, thus allowing a selection of antibiotics for treating individual infections with little or no resistance. However, these experiments have demonstrated that the range of the enzyme's substrate specificity can be broadened. In this study, single and double mutations in APH(3')-IIIa greatly increased the minimal concentration, up to ~30 μg/mL, of amikacin to which *E. coli* were susceptible. It is worth noting that 35 μg/mL of amikacin is the highest serum level recommended in humans (12). With more (3-6) mutation, APH(3')-IIIa was able to confer 60-fold higher level of amikacin resistance than the wildtype. This increased resistance to amikacin, a secondary substrate of APH(3')-IIIa, did not come with a noticeable decrease in resistance to its primary substrate, kanamycin. The results in this study are consistent with previous similar experiments of directed evolution, in that increased promiscuity does not necessarily accompany impaired primary activity (2).

The specific results of these mutations are not trivial. It has been demonstrated that mutations found through *in vitro* evolution can be predictors of antibiotic resistance that are observed in a clinics (17). Should APH(3')-IIIa mutations be found to confer resistance to amikacin in clinics, it is likely that they would be among those observed in

this study. In this study APH(3')-IIIa mutations conferring increased resistance to amikacin also reduced the ability of *E. coli* to form colonies under non-selective conditions. This observation per se, however, does not mean that these mutations cannot or will not arise in a clinical setting. Mutations that confer antibiotic resistance often result in a fitness decrease (3, 19). They are able to be maintained in clinical populations, because they do provide a growth advantage under selective conditions, and are accompanied by compensatory mutations that select against the loss of this new advantage (13, 14, 21). In the case of amikacin, a mutated APH(3')-IIIa is not the only aminoglycoside modifying enzymes that can confer resistance. AAC(6')-1ad, ANT(4')-II, and APH(3')-VI have all been observed to provide amikacin resistance in the clinic, therefore all three forms of aminoglycoside modification – acetylation, adenylylation, and phosphorylation – can be used for amikacin resistance (5, 8, 26). It is possible that due to negative consequences to colony forming ability of mutating APH(3')-III, these modes of resistance become much more prevalent and outcompete APH(3')-III in a clinical setting.

The impaired colony forming ability by mutated APH(3')-IIIa might be partially explained by the expression of the mutated protein, since the effect was increased in cells that transcribe and translate the mutant proteins. But the effect was not entirely accounted for by protein expression, since colony formation was still different between *E. coli* TG1 cells hosting APH(3')-IIIa and APH4.1. The promiscuous activity of APH4.1, originally thought to be the most likely cause of negative externalities to the cell, appeared to have little impact. Elimination of functional activity with the D190A mutation resulted in no restoration of colony forming ability.

This data suggests that APH(3')-IIIa may have an intolerance to mutations that negatively affects the ability of cells to form colonies. Further elucidation of this effect may offer insights into the evolutionary potential of aminoglycoside modifying enzymes.

FIGURES

Primer	Name	Sequence
1	pQBAV3_MCS_-48_ecori_F	gcaaacaaaccaccgctggtagcg
2	pQBAV3_MCS_+278_psti_R	gccgtaatatccagctgaacggtctggttatag
3	aph_D190A_for	cttgtcttttcccacggcgccctgggagac
4	aph_D190A_rev	gtctcccagggcgccgtgggaaaagacaag
5	aph_pet28_F_ndeI_nhis	ggggtatctttaaatactgtagaaaagaggaaggaaataacatatggct
6	aph_pet28_R_hindIII_nhis	gtctgcagcggccgctactaaagcttttaaaacaattcatc

TABLE 1: Primers used for experimentation

Round of Mutagenesis	Mutant	Missense Mutations	Silent Mutations	Amikacin susceptibility Level (μg/mL)
1	1	H78Y, **V96A**		30
1	2	N38D		26
1	3	I254M	**R211**	26
1	4	**Q236R**		30
1	5	**E9K**, **K12R**, L136I		30
1	6	**K12E**	D261	35
1	7	D193N		35
1	8	**V96I**, K248E		30
1	9	F79Y, **Q236R**	**D167**	30
1	10			30
1	11	M1V	G69	26
1	12	I258T		30
1	13	Y102C	E235	30
1	14	D167G		30
1	15	E16G, **K255R**		26
1	16	K21E, V198A	L244	26
1	17			26
1	18	**S194R**		35
1	19	L136I	E80	26
1	20	T177A, D231G, K248E	**S27**, L259	26
1	21	**I40T**	**L10**	26
1	22		**L41**	30
1	23	E15K	L140	26
1	24	**E160D**		30
1	25		E234	26
1	26	E103G	L175, V185	35
1	27	**I112V**, V198M		45
1	28			30
1	29	E181G	K21	30
1	30	**E9G**, K179R, K255R	P28	30
1	31		**E9**, D153	30
1	32	D104V		26
1	33	**I112V**, E161G	**E234**	26
1	34	H78T, A152V	**E24**, **L41**	50
1	35	N38S	T55, **D144**	26
1	36	G36R, **E234G**, **K255E**	D190	26
1	37		**R211**	26
1	38		D94	30
1	39			30
1	40		D137	26
1	41	A2T	K43	26
1	42		K253, E262	26

1	43			30
2	1	**I40T**, **K255R**		160
2	2	**I40T**, **S194R**, **K255R**	**L10**, **V73**	200
2	3	**I112V**		160
2	4	**S194R**		160
2	5	**S194R**		160
2	6	**D193N**		170
2	7	**D193N**	**E24**	160
3	1	K3R, I6M, **I40T**, **D144G**, **E160G**, **K176R**, **S194R**, **I196F**, **K255R**	**E9**, **L10**, **S27**, **V73**, V96	280
3	2	**I112V**, **S194R**		700
3	3	**E24V**, **I40V**, **S194R**	E68, L140	1200
3	4	K11N, **I40T**, **R120K** , **C156R**, **S194R**, **K255R**	**L10**, **V73**	500
4	1	**E24V**, **I40T**, **R120K**, **C156R**, **K176R**, **S194R**, **I196F**, Y219H, **K255R**	**V73**, V76	-

TABLE 2: Amikacin Susceptibility of APH(3')-IIIa mutants. APH(3')-IIIa underwent mutagenesis and was selected for the ability to grow on increased levels of amikacin. Mutants were sequenced, and their susceptibility to amikacin was determined. Bold mutations were residues where a mutation was observed in multiple mutant variants.

	APH(3')-IIIa				APH4.1			
Substrate [a]	k_{cat} (s^{-1})	K_m (μM)	K_i (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	K_i (μM)	k_{cat}/K_m (M^{-1} s^{-1})
KAN	2.05±0.1	11.1±2.9	n.d. [b]	$1.9x10^5$	141±12 2	331±23 3	0.71±0.4	$4.3x10^5$
AMK	2.23±0.3	362±101	n.d. [b]	$6.1x10^3$	6.97±0.7	39.4±8.6	2069±466	$1.8x10^5$

TABLE 3: Kinetic Parameters of APH(3')-IIIa and APH4.1. APH(3')-IIIa and APH4.1 were purified and their kinetic paramenter with kanamycin and amikacin were determined using a pyruvate kinase/lactate dehydrogenase coupled assay.

[a] KAN = kanamycin; AMK = amikacin

[b] n.d. = not detected

	No Plasmid	APH(3')-IIIa	APH(3')-IIIa (D190A)	APH4.1	APH4.1 (D190A)
CFU/OD/mL (LB only)	1.41±0.19 x10^8	1.82±0.31 x10^8	2.93±1.82 x10^7	9.71±2.41 x10^7	1.09±0.33 x10^8
CFU/OD/mL (Chloramphenicol)	0	1.75±0.24 x10^8	1.65±1.14 x10^7	8.93±2.53 x10^7	1.04±0.56 x10^8
Plasmid Retention	-	97±9.0%	69±38%	92±8.4%	95±32%

TABLE 4: Plasmid stability of APH(3')-IIIa and APH4.1. Equal amounts of *E. coli* InvαF' with the pQBAV3c containing APH(3')-IIIa and APH4.1 were plated on LB plates with and without chloramphenicol. This was done for cells with versions of APH(3')-IIIa and APH4.1 containg a D190A mutation. No group showed significant changes in plasmid retention compared to cells with the APH(3')-IIIa plasmid.

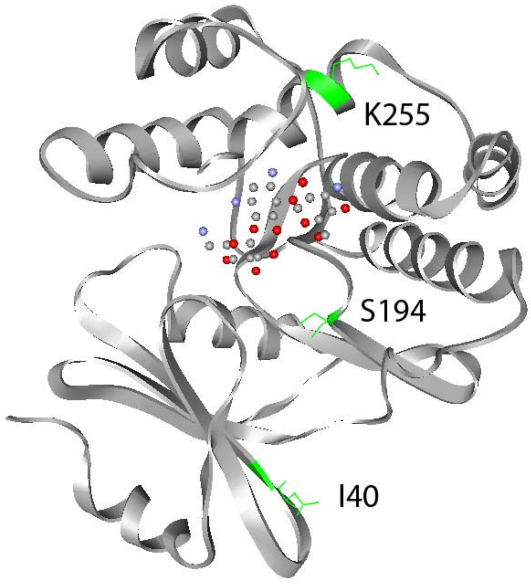

FIGURE 1: 3-D model representation of mutation location in APH(3')-IIIa. This model shows the location of the three residues that were mutated in APH4.1 (colored green). These three residues (I40, S194, K255) were found to be changed in multiple mutants during the four rounds of mutagenesis.

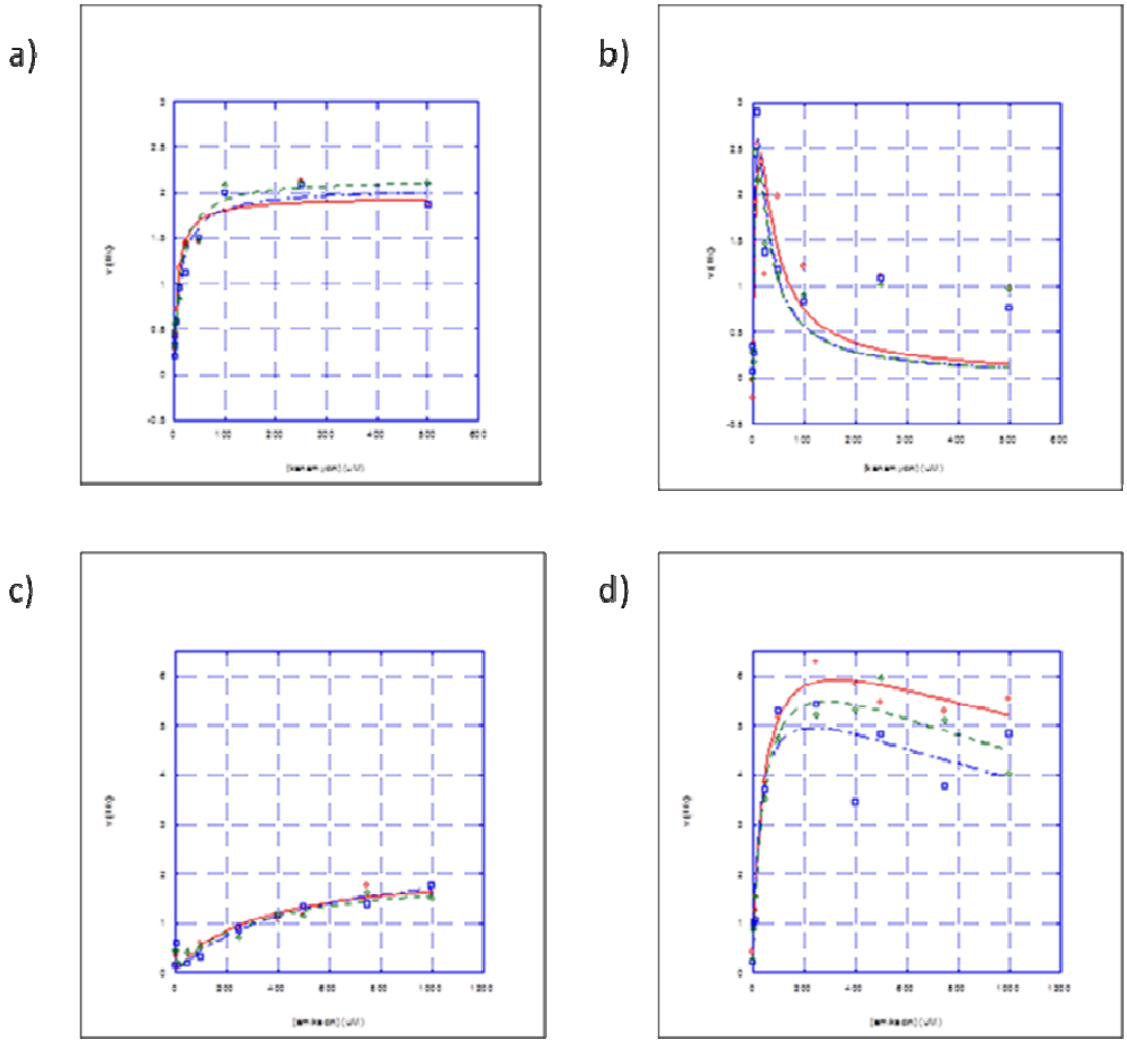

FIGURE 2: Michaelis-Menton plot of APH(3')-IIIa and APH4.1. The plots show the kinetic data for a) APH(3')-IIIa with kanamycin substrate b) APH4.1 with kanamycin substrate c) APH(3')-IIIa with amikacin substrate d) APH4.1 with amikacin substrate.

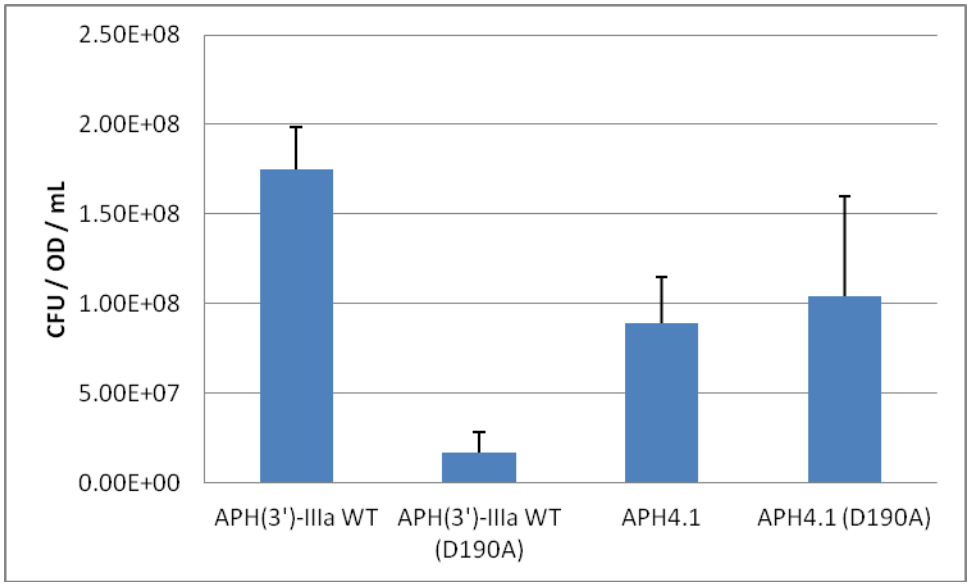

FIGURE 3: Growth of *E. coli* InvαF' containing the pQBAV3c plasmid expressing APH(3')-IIIa and APH4.1. Equal amounts of cells with a plasmid containing APH(3')-IIIa and APH4.1 were plated on LB plates with chloramphenicol. This was done for cells with versions of APH(3')-IIIa and APH4.1 containg a D190A mutation. The cells with the APH(3')-IIIa plasmid were able to form colonies at a significantly higher rate than cells with the APH4.1 plasmid. Introduction of the D190A mutation into APH4.1 did not significantly change its rate of colony formation.

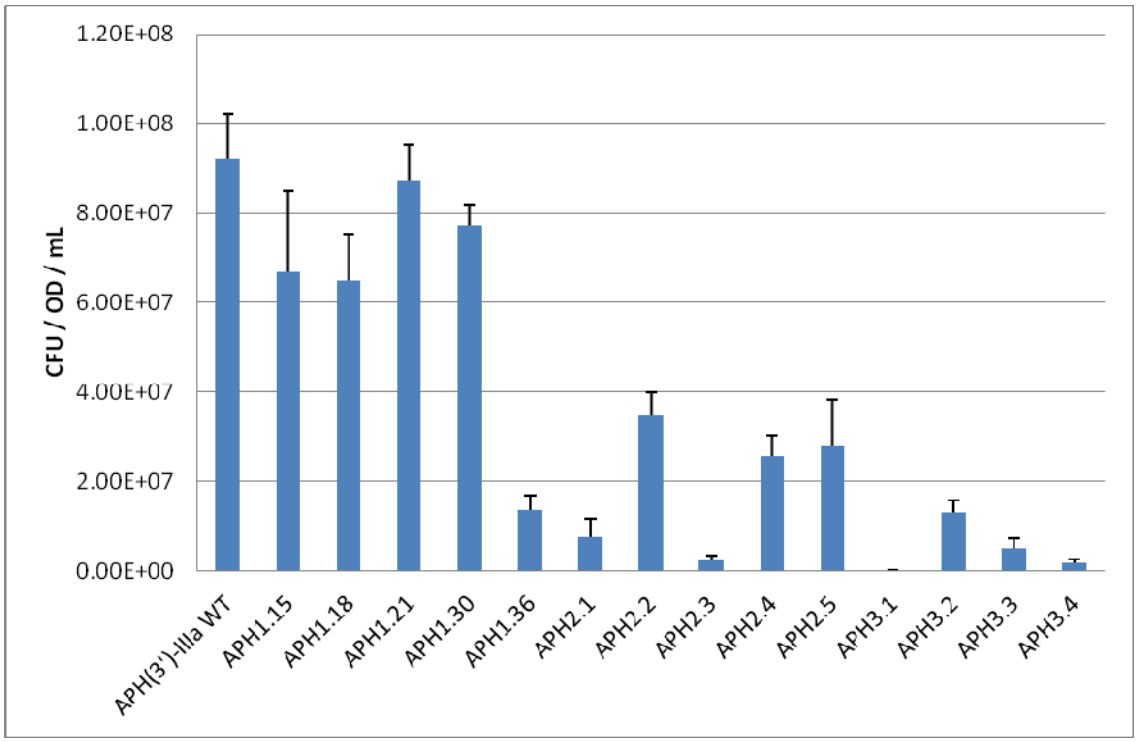

FIGURE 4: Growth of *E. coli* InvαF' containing the pQBAV3c plasmid with APH(3')-IIIa and its mutants. Equal amounts of cells with a plasmid containing APH(3')-IIIa and several APH mutants were plated on LB plates with chloramphenicol. The cells with the plasmid containing APH1.36, APH2.1, APH2.2, APH2.3, APH2.4, APH2.5, APH3.1, APH3.2, APH3.3, and APH3.4 all had a significantly decreased ability to form colonies compared to APH(3')-IIIa.

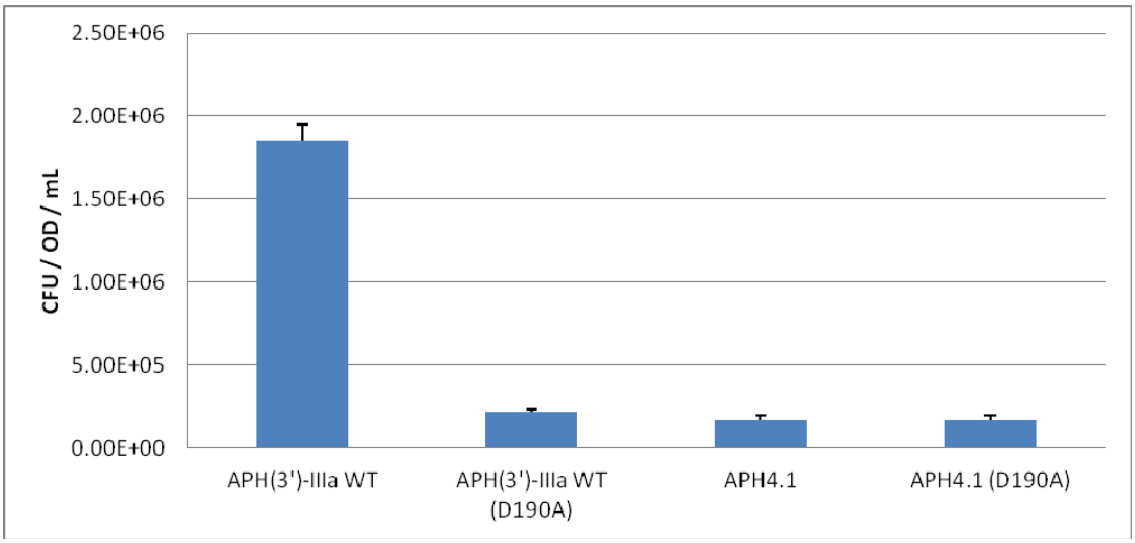

FIGURE 5: Growth of *E. coli* BL21(DE3) containing the modified pET28 plasmid with APH(3')-IIIa and its mutants. Equal amounts of cells with a plasmid containing APH(3')-IIIa and APH4.1 were plated on LB plates with ampicillin. This was done for cells with versions of APH(3')-IIIa and APH4.1 containg a D190A mutation. The cells with the APH(3')-IIIa plasmid were able to form colonies at a significantly higher rate than cells with the APH4.1 plasmid. Relative to the colony forming ability of APH(3')-IIIa, APH4.1 had a decreased rate of forming colonies in BL21(DE3) compared to InvαF' (Figure 3). Introduction of the D190A mutation into APH4.1 did not significantly change its rate of colony formation.

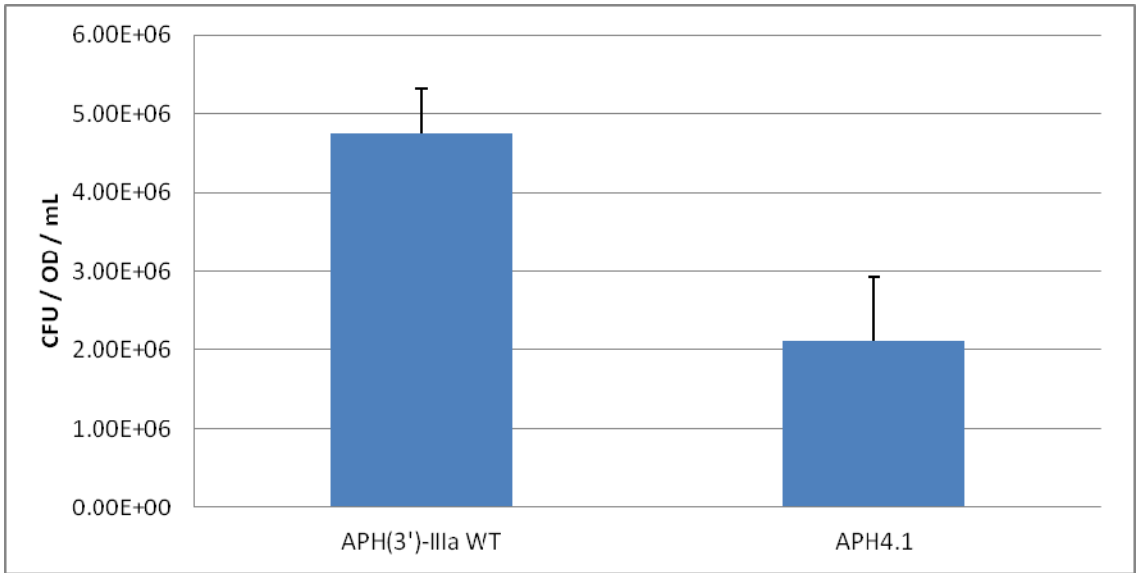

FIGURE 6: Growth of *E. coli* TG1 containing the modified pET28 plasmid with APH(3')-IIIa and its mutants. Equal amounts of cells with a plasmid containing APH(3')-IIIa and APH4.1 were plated on LB plates with ampicillin. The cells with the APH(3')-IIIa plasmid were able to form colonies at a significantly higher rate than cells with the APH4.1 plasmid. Relative to the colony forming ability of APH(3')-IIIa, APH4.1 had an increased rate of forming colonies in TG1 compared to BL21(DE3) (Figure 5).

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