Hydrolysis of ATP by Aminoglycoside 3’-Phosphotransferases

AN UNEXPECTED COST TO BACTERIA FOR HARBORING AN ANTIBiotic RESISTANCE ENZyME

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Choonkeun Kim1, Joo Young Cha1, Honggao Yan2, Sergei B. Vakulenko1, and Shahriar Mobashery3‡1
From the 1Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556 and the 3Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824

Aminoglycoside 3’-phosphotransferases (APH(3’)-s) are common bacterial resistance enzymes to aminoglycoside antibiotics. These enzymes transfer the γ-phosphoryl group of ATP to the 3’-hydroxyl of the antibiotics, whereby the biological activity of the drugs is lost. Pre-steady-state and steady-state kinetics with two of these enzymes from Gram-negative bacteria, APH(3’)-Ia and APH(3’)-IIa, were performed. It is demonstrated that these enzymes in both ternary and binary complexes facilitate an ATP hydrolysis activity (ATPase), which is competitive with the transfer of phosphate to the antibiotics. Because these enzymes are expressed constitutively in resistant bacteria, the turnover of ATP is continuous during the lifetime of the organism both in the absence and the presence of aminoglycosides. Concentrations of the enzyme in vivo were determined, and it was estimated that in a single generation of bacterial growth there exists the potential that this activity would consume as much as severalfold of the total existing ATP. Studies with bacteria harboring the aph(3’)-Ia gene revealed that bacteria are able to absorb the cost of this ATP turnover, as ATP is recycled. However, the cost burden of this adventitious activity manifests a selection pressure against maintenance of the plasmids that harbor the enzyme. It would appear that these resistance enzymes place an additional price burden of this ATPase activity. The pre-steady-state kinetics for transfer of the γ-phosphoryl group of ATP to the aminoglycoside antibiotics, we observed that APH(3’)-s from Gram-negative bacteria also catalyze hydrolysis of ATP (12, 13). At the time we deemed this reaction as insignificant, believing the transfer of the γ-phosphoryl group of ATP to the aminoglycosides as considerably more favorable. That these enzymes were capable of ATP hydrolysis was not a surprising finding, as the enzyme binds MgATP for the purpose of facilitating γ-phosphoryl transfer as its normal function. This phosphoryl group is merely being transferred to a water molecule in the ATPase activity. The pre-steady-state kinetics for transfer of the γ-phosphoryl group of ATP to kanamycin, neamine, and water, investigated in this report, proves surprising. We disclose that the cost of this ATPase activity is seemingly significant, but at first glance at the phenotypic level, the organisms appear to absorb this cost. However, in the absence of selection, bacteria harboring APH(3’)-s experience the loss of the plasmid that encodes for the enzyme. It would appear that these resistance enzymes place an additional burden on the bacterial host above and beyond the standard replicational burden. This goes counter to the aforementioned concept on stable retention of the resistance determinants in the long run, at least for the case of APH(3’)-s (8–11).

EXPERIMENTAL PROCEDURES

Plasmid Construction—To construct plasmid pUC-APH, the aph(3’)-Ia gene was excised from plasmid pUC4K (Amersham Biosciences) with BamHI endonuclease and inserted into the BamHI site of plasmid pUC19. Then, the blaTEM gene encoding TEM-1 β-lactamase was removed from the construct by digestion with restriction endonucleases AatII and DraI and religation. Plasmid pUC-TEM was constructed by substituting the aph(3’)-Ia gene in the plasmid pUC-APH with the blaTEM gene. This was performed by PCR-amplification of the pUC-APLah plasmid with primers, PUC-S (5’-AACACCCCTTGATATACGGCG-3’) and PUC-E (5’-CTGGCAGATCATTTTACGCTGACTTGACCCGGA-3’; BglII site underlined) that would amplify the entire plasmid except for the aph(3’)-Ia gene. The blaTEM gene was subsequently amplified from the plasmid pUC19 with two primers TEM-5 (5’-ATGAGATTAAACATGTCGG-3’) and TEM-E (5’-AAACTAGATCTGACGATTTAC-
CAATGCTTAA-3' (BglII site underlined). PCR products were digested with BglII, gel-purified, and ligated to produce plasmid pUC-TEM. Plasmids pACYC-APH and pACYC-TEM were prepared in a similar way. The plasmid pACYC-APH containing only the aph(3')-Ia gene was constructed by removing the blaTEM-1 gene with endonucleases AatII and DraI from the commercial plasmid pACYC177 harboring both the aph(3')-Ia and blaTEM-1 genes (New England Biolabs, Beverly, MA). The plasmid pACYC-TEM was completed by the ligation of two PCR-generated fragments. The first fragment was produced by PCR-amplification of the plasmid pACYC-APH with the following oligonucleotide primers, PACYC-5' (5'-AACACCCCGGTATAAGGGGAGGTCGGAATCG-3') and PACYC-E (5'-AACACCCCGGTATAAGGGGAGGTCGGAATCG-3'); BglII site underlined). The second fragment was the same PCR-amplified blaTEM-1 gene that was used to construct the plasmid pUC-TEM (see above). The sequences of all constructs were confirmed by DNA sequencing. In all constructs both the blaTEM-1, and the aph(3')-Ia genes were controlled by the same aph(3')-Ia gene promoter.

Plasmid pACYCD216W, which harbors the gene for the D216W mutant variant of aph(3')-Ia, was generated by site-directed mutagenesis. The plasmid pUC-APH was amplified by Pfu Turbo DNA polymerase and two mutagenic primers, D216WD (5'-GGAATATAGGGTTGATTTGGGGACGATCGGAATCG-3'; mutation site underlined) and D216WR (5'-GGAATATAGGGTTGATTTGGGGACGATCGGAATCG-3'; mutation site underlined). The PCR products were cleaned with the gel extraction kit (Qiagen) and treated with restriction enzyme DpnI to remove methylated template plasmids. The amplified plasmids were then transformed into Escherichia coli JM83. The nucleotide sequence was verified for the entire gene. The mutated plasmid, pUC-mAPH, was digested with BamHI and HindIII. The fragment containing the mutation site was ligated into the corresponding site of pACYC177, and the product of ligation (pACYCD216W) was used to transform E. coli K-12.

**Determination of the Concentration of APH(3')-Ia in Vivo**—Using the following parameters: cell length (L), 2 μm; cell diameter (D), 0.8 μm; outer membrane thickness (O), ~10 nm; inner membrane thickness (I), 8 nm; periplasm thickness (P), 10 nm; nuclear volume (N), 0.16 × 10^{-15} L, we first calculated that the cytoplasmic volume (C) for a single E. coli cell at 0.67 × 10^{-15} L. Calculations were made according to Equation 1 (15, 16),

\[
C = \pi [L - 2(O + I + P)][(D - 2(O + I + P))/2] - N \tag{Eq. 1}
\]

Subsequently, we determined the in vivo concentration of APH(3')-Ia produced from the pACYC-APH and pUC-APH plasmids in E. coli K-12 cells using SDS-PAGE and ImageQuant 5.2 software from Amersham Biosciences. E. coli K-12 strain carrying no plasmids was used as a negative control. Strains were grown until the absorbance of 0.2. One milliliter of each strain was centrifuged at 10,000 × g for 30 s. The pellets were resuspended in 1 ml of washing buffer (50 mM HEPES, pH 7.5 and 1 mM EDTA) and 150 μl of each was used for protein separation on a 12% SDS-PAGE; several concentrations of purified APH(3')-Ia were used as standards (0.6, 1.2, 3.1, 6.2, and 12.5 μg/lane). Proteins were stained with Coomassie Blue and scanned, and the amount of the APH(3')-Ia was quantified using the ImageQuant 5.2 program. The quantity of APH(3')-Ia from E. coli K-12 strains producing APH(3')-Ia was calculated by subtracting the band corresponding to the small amount of protein of E. coli K-12 strain not harboring any plasmid from that of corresponding location in the gel for APH(3')-Ia from the same organism harboring plasmid pACYC-APH or pUC-APH. Simultaneously, several dilutions of cultures were made, and cells were plated onto LB (Luria-Bertani) agar plates to count numbers of cells in 150 μl of each culture. Concentration of APH(3')-Ia in vivo was evaluated by dividing the amount of APH(3')-Ia in 150 μl of cell culture by both the number of cells in 150 μl and by the cytoplasmic volume of a single E. coli cell. The absorbance of the cultures was measured with a Cary 50 spectrophotometer from Varian Inc. (Palo Alto, CA) at 600 nm (A600 = 1; 1 × 10^9 cells/ml) (17, 18). Doubling times during log phase were determined by Equations 2 and 3.

\[
\log N_2 = \log N_1 + n \log 2 \tag{Eq. 2}
\]

\[
g = (t_2 - t_1)/n \tag{Eq. 3}
\]

where N₂ is the number of bacteria at time t₂, N₁ is the number of bacteria at time t₁, n is the number of doublings, and g is the doubling time.

**Pairwise Competition Experiments**—Growth cultures of each strain (E. coli K-12, E. coli JM83, or E. coli DH10B) harboring either the aph(3')-Ia or the blaTEM-1 genes with the high or low copy plasmids were diluted and mixed to 1:1 ratio to ~10^9 cells/ml. Cells were grown with shaking (180 rpm) for 24 h in either LB broth or glucose minimal medium without antibiotics. When E. coli JM83 and E. coli DH10B were grown in the glucose minimal medium, the medium was supplemented with proline or leucine, respectively. The number of cells carrying either the aph(3')-Ia or the blaTEM-1 genes was counted on LB agar plate containing kanamycin or ampicillin, respectively.

**Bacterial Growth Curves**—Overnight cultures of (30 μl) of E. coli strains (E. coli K-12, E. coli JM83, and E. coli DH10B) harboring the aph(3')-Ia or the blaTEM-1 gene were inoculated separately into 3 ml of LB broth containing kanamycin or ampicillin, respectively. Cells were grown for 2 h at 37 °C and then diluted to reach an A600 of 0.1. Cell cultures were further diluted 1000-fold and were grown with shaking (180 rpm) at 37 °C in the glucose minimal medium without antibiotics. The medium was supplemented with proline and leucine for E. coli JM83 and E. coli DH10B, respectively. The absorbance of the cultures was measured at 600 nm at various time intervals.

**Plasmid Loss Analysis**—Overnight cultures of E. coli K-12 strains harboring the aph(3')-Ia or the blaTEM-1 genes on the low or high copy plasmids were diluted 1000-fold and grown in LB medium in the absence of antibiotics with agitation (180 rpm) at 37 °C. Every 12 h, the cultures were diluted 100-fold in fresh LB broth. Every 5 days cultures were diluted 10^5-10^7-fold and plated on LB agar plates without antibi-

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3 redpoll.pharmacy.ualberta.ca/CCDB.

4 www2.uta.edu/vanwaasbergen2/micro/growth.html.
RESULTS AND DISCUSSION

The work commenced with in vitro investigations with purified APH(3′)-Ia and APH(3′)-IIa, both of which have been purified to homogeneity in our laboratory (20). Quench-flow experiments for the evaluation of microscopic rate constants were carried out with [γ-32P]ATP, as described earlier (20). The results of a representative pre-steady-state experiment for the two activities of APH(3′)-Ia, phosphoryl transfer to kanamycin A and to water, are given in Fig. 2. The reactions consisted of two phases, a burst phase and a steady-state phase in both cases as shown in Fig. 2.

The microscopic kinetic values for the type Ia APH(3′) [APH(3′)-Ia] are given in Table 1. Those for the type IIa enzyme [APH(3′)-IIa] are given in Table 2. The values compare the phosphate transfer reactions in the ternary (APH(3′)-Iakanamycin A·ATP and APH(3′)-IIakanamycin A·ATP) and the binary (APH(3′)-IaATP and APH(3′)-IIaATP) complexes. The ATPase activity is seen whether the aminoglycoside is bound to the active site or not. The transfer of the γ-phosphoryl group (k3) to a water molecule by APH(3′)-Ia was 7- and 19-fold slower than that to kanamycin A and neamine, respectively. The data for APH(3′)-IIa are quite similar to those of APH(3′)-Ia. Invariably the slow event appears to be the dissociation of one of the products from the enzymes, probably governed by a conformational change in the

![Pre-steady-state kinetics of APH(3′)-Ia catalyzed reaction.](image)

**TABLE 1**

Pre-steady-state kinetic parameters of APH(3′)-Ia

| Phosphate acceptor | k₃ | k₋₃ | k₄ | k₋₄ |
|--------------------|----|-----|----|-----|
| Kanamycin          | 28.4 ± 3.6 | 33.9 ± 1.1 | 40.0 ± 0.3 | 1.7 ± 0.1 |
| Neamine            | 77.9 ± 3.1 | 62.4 ± 4.4 | 11.4 ± 0.8 | 5.9 ± 0.1 |
| H₂O (+ kanamycin)  | 4.0 ± 0.1 | 20.4 ± 3.2 | 0.38 ± 0.04 | 0.06 ± 0.01 |
| H₂O (+ neamine)    | 6.4 ± 0.8 | 24.4 ± 4.6 | 0.32 ± 0.06 | 0.07 ± 0.01 |
| H₂O without aminoglycoside | 4.1 ± 0.5 | 64.0 ± 7.0 | 0.35 ± 0.05 | 0.02 ± 0.003 |

**TABLE 2**

Pre-steady-state kinetic parameters of APH(3′)-IIa

| Phosphate acceptor | k₃ | k₋₃ | k₄ | k₋₄ |
|--------------------|----|-----|----|-----|
| Kanamycin          | 41.4 ± 1.6 | 30.7 ± 1.4 | 7.8 ± 0.2 | 4.0 ± 0.1 |
| Neamine            | 73.2 ± 4.0 | 62.6 ± 1.7 | 24.4 ± 2.3 | 11.1 ± 0.7 |
| H₂O (+ kanamycin)  | 9.4 ± 0.9 | 20.5 ± 2.4 | 0.24 ± 0.02 | 0.08 ± 0.01 |
| H₂O (+ neamine)    | 8.1 ± 0.9 | 28.7 ± 3.8 | 0.33 ± 0.05 | 0.07 ± 0.02 |
| H₂O without aminoglycoside | 10.4 ± 1.5 | 71.6 ± 8.6 | 0.37 ± 0.06 | 0.05 ± 0.01 |

apers. Then, one hundred colonies from each culture were transferred onto LB agar plates containing kanamycin or ampicillin, and the newly formed colonies that retained plasmids were counted.

Another plasmid loss test with E. coli K-12 harboring either plasmid pACYC177 or pACYCD216W was performed as described above. Cultures were plated on LB agar plates not containing antibiotics every 4 days, and the colonies harboring plasmids were selected with ampicillin for both pACYC177 and pACYCD216W. All the experiments for plasmid-loss were performed three times.
structure of the enzyme, and reflected in the slower $k_4$, compared with $k_5$ values.

Because APH(3')s are expressed in bacteria constitutively, and ATP is ubiquitous, hydrolysis of ATP by these enzymes takes place continuously whether bacteria are exposed to aminoglycoside antibiotics or not. As such, harboring the genes for APH(3')s should exact a price on bacteria, a factor that we set out to evaluate.

We have used both high (pUC19, >200 copies per cell) and low copy (pACYC177, 10–12 copies per cell) plasmids (21). The plasmids were elaborated to possess the aph(3')-Ia gene (pUC-APH and pACYC-APH) or the blaTEM-1 gene (for the TEM-1 β-lactamase; a resistance enzyme for β-lactam antibiotics; pUC-TEM and pACYC-TEM) as a control, each under the aph(3')-Ia promoter sequence. The two genes are essentially the same size (816 bp and 792 bp for the aph(3')-Ia and blaTEM-1 genes, respectively). The four constructed plasmids were introduced by transformation into the antibiotic susceptible strain E. coli K-12 and into its two laboratory derivatives, E. coli JM83 and E. coli DH10B. The presence of various chromosomal mutations in the two laboratory strains results in their lower growth rates (E. coli DH10B has the lowest rate, as discussed later) in comparison to the parental E. coli K-12 strain. One might expect that the extra burden imposed by production of resistance enzymes (and the attendant ATPase reaction of APH(3')s) would produce a more profound effect on strains that are already attenuated in their growth rates. The low and high copy plasmids were expected to produce concentrations of APH(3')-Ia within the range for expression of the enzyme by clinical strains in vivo (22). We determined the in vivo concentrations of APH(3')-Ia by a slight modification of a known methodology (23). Bacteria not harboring the plasmid and purified APH(3')-Ia were used as a negative control and as an external standard, respectively (Fig. 3). Concentrations of APH(3')-Ia in vivo were evaluated at 400 ± 20 μM and 1800 ± 50 μM for the E. coli K-12 strains harboring the low and the high copy plasmids, respectively. These concentrations are not atypical for an important enzyme in bacteria (24, 25).

The doubling times (e.g. generation times) for E. coli K-12 harboring low and high copy plasmids were 29 and 35 min, respectively. Doubling times were the same whether the plasmids contained the aph(3')-Ia or the blaTEM-1 genes, hence no measurable effect on growth was attributable to the ATPase activity of APH(3')-Ia. The amounts of ATP hydrolyzed by APH(3')-Ia in the absence of an aminoglycoside in E. coli harboring pACYC-APH and pUC-APH during a single doubling time were evaluated to be 1.8 ± 0.5 mM and 201 ± 30 mM, respectively (from concentration of APH(3')-Ia in vivo × slowest rate constant ($k_{-4}$) × doubling time). The effect on the ATPase activity is 3-fold or larger in the presence of aminoglycosides (Tables 1 and 2). Like for many other critical metabolites, concentrations of ATP in bacteria have been evaluated at high values of 3–5 mM (26–30). These determinations indicate that the mere adventitious hydrolysis of ATP by the constitutively expressed APH(3')-Ia has the potential of consuming as much as several-fold of the existing pool of ATP during a single bacterial doubling time.

### TABLE 3

| E. coli strains | Ratio of strains harboring the aph(3')-Ia gene to those with the blaTEM-1 gene |
|----------------|--------------------------------------------------------------------------------|
|                | 0 h  | 24 h (LB) | 24 h (minimal medium) |
| K-12 (high copy plasmid) | 1.2 ± 0.1 | 1.2 ± 0.1 | 1.2 ± 0.1 |
| K-12 (low copy plasmid) | 1.1 ± 0.1 | 1.1 ± 0.1 | 1.0 ± 0.1 |
| JM83 (high copy plasmid) | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.4 ± 0.1 |
| JM83 (low copy plasmid) | 1.0 ± 0.1 | 0.9 ± 0.1 | 1.0 ± 0.1 |
| DH10B (high copy plasmid) | 0.9 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.1 |
| DH10B (low copy plasmid) | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.0 ± 0.1 |

**FIGURE 3. Determination of the in vivo concentrations of APH(3')-Ia in E. coli K-12.**

C, E. coli K-12 strain without any plasmid (negative control); D, E. coli K-12 strain harboring the low copy plasmid pACYC-APH; 2, E. coli K-12 strain carrying the high copy plasmid pUC-APH; M, the purified APH(3')-Ia → as external standards (0.6, 1.2, 3.1, 6.2, and 12.5 μg). The arrow indicates APH(3')-Ia protein.

**FIGURE 4. Growth curves of E. coli strains harboring the aph(3')-Ia or the blaTEM-1 gene in the glucose minimal medium not containing antibiotics.**

A, E. coli K-12 strains harboring plasmid pACYC-APH ( ), pACYC-TEM ( ), pUC-APH ( ), and pUC-TEM ( ); B, E. coli JM83 strains carrying plasmid pACYC-APH ( ), pACYC-TEM ( ), pUC-APH ( ), and pUC-TEM ( ); C, E. coli DH10B strains containing plasmid pACYC-APH ( ), pACYC-TEM ( ), pUC-APH ( ), and pUC-TEM ( ).
ATPase Activity of APH(3’)-Ia

Clearly, bacteria absorb much of this cost in the loss of ATP, largely because of the ability of ATP to be regenerated by the metabolic process. However, we assumed that the cost could not be inconsequential.

The effect of the ATP hydrolase activity of APH(3’)-Ia on the bacterial growth was determined with all strains harboring low (pACYC-APH or pACYC-TEM) and high copy plasmids (pUC-APH or pUC-TEM) in the glucose minimal medium not containing an aminoglycoside (Fig. 4). Surprisingly, we could not see any significant differences in growth between the strains harboring the aph(3’)-Ia and blaTEM-1 genes, the latter of which serving as a control. The naïve strain harboring the high copy plasmids, regardless of which resistance gene, grew slower than those harboring the low copy plasmids. This would be expected, since replication of the extra genetic materials and synthesis of larger amounts of the resistance enzyme are called for in the case of the high copy plasmid.

Subsequently, the potential cost of the ATPase activity of APH(3’)-Ia was measured by pairwise competition experiments. Mixtures (1:1) of strains carrying aph(3’)-Ia gene and blaTEM-1 gene were incubated for 24 h in both LB broth and the glucose minimal medium, followed by selective plating on kanamycin- and ampicillin-containing agar for counting of the cells. There was no significant difference (Table 3).

We wondered whether in light of the ATPase activity there might be a selection pressure for the loss of the plasmid that harbors the aph(3’)-Ia gene. An analysis for the loss of plasmid (14, 19) was performed. Each strain harboring the aph(3’)-Ia or blaTEM-1 genes was grown in LB medium in the absence of antibiotics with continuous subculture. The dilutions of cultures were spread on LB agar plates not containing antibiotics every 5 days. Then, one hundred colonies of each strain were transferred to LB agar plates containing kanamycin and ampicillin, respectively.

We could not estimate the effect of ATPase activity of APH(3’)-Ia on the plasmid maintenance with E. coli K-12 strains harboring pUC-APH and pUC-TEM, because both strains completely lost their plasmids within 100 generations of growth, indicative of the high cost of high copy plasmid maintenance. Significantly, E. coli K-12 strain harboring the low copy pACYC-APH lost its plasmid more rapidly than that containing pACYC-TEM. The former lost ~50% of the plasmids in 1500 generations in contrast to 10% loss from the latter strain (Table 4). It is important to restate that plasmids and promoters are identical in both cases and the only difference is the nature of the resistance genes, which are of the same size.

As yet another control, we attempted the same experiments with a mutant variant of APH(3’)-Ia. We chose to introduce a tryptophan within the active site (D216W) of the enzyme near the ATP-binding site, based on the x-ray structure. At saturating concentration of ATP, the activity of the enzyme was reduced to 30% of the wild type. The plasmid pACYC216W was expelled from the host more slowly (47% loss in 1500 generations) than the wild-type plasmid pACYC177 (71% loss in as many generations), as shown in Table 5. The bacterium lost pACYC177 more rapidly than pACYC-APH because the former is larger (Fig. 5).

Attenuation of the ATPase activity of the D216W mutant enzyme resulted in reduction of the rate of the loss of the plasmid. The difference in 1500 generations is 4.8-fold (ratio of 48%/10%, Table 4) preference for the loss of the plasmid when the wild-type APH(3’)-Ia was being compared with the TEM-1 β-lactamase (a protein of the same size without the ATPase activity as a control) versus 1.5-fold (ratio of 71%/47%, Table 5) preference for the loss between the wild-type APH(3’)-Ia and the D216W mutant enzyme. Whereas the results from enzymologic determination of activity in vitro do not necessarily have to translate exactly to effects in experiments with whole bacteria, our findings here do. An attenuation of enzymologic ATPase activity for the D216W mutant...
APH(3’)-Ia to 30% parallels closely a proportional effect (from 4.8-fold to 1.5-fold) on the propensity of the corresponding plasmids that were lost from the bacteria that harbor them.

Our kinetics of hydrolysis of ATP by the constitutively expressed APH(3’)-Ia suggests a consequence to the bacteria that harbor this resistance enzyme. However, measurements of the rate of growth of bacteria expressing these proteins did not reveal any difference, indicative of the fact that bacteria tolerate this metabolic demand in the short run. However, there would appear to be a selective pressure for the loss of the plasmid that bears the aph(3’)-Ia gene over a number of generations in the absence of an aminoglycoside, a selection pressure that is 5-fold greater than what is measured for the organism that harbors the gene for the β-lactamase in the absence of a β-lactam antibiotic. As phosphorylation is a common mechanism for antibiotic resistance (1–4), there might be generality of our observations to other resistant bacteria dependent on plasmid-borne, ATP-consuming resistance enzymes.

The larger implication is that, in the absence of selection, bacteria harboring an enzyme that catalyzes the consumption of key metabolites (ATP in our case) could experience the loss of the plasmid that encodes for the given enzyme. As a final note we would like to acknowledge that our studies do not rule out the possibility that the fitness effect could also have to do with the longevity of the RNA or slightly altered rates of replication of the pACYC-APH plasmid. If one of these were the case, the bacteria have learned to cope with this significantly wasteful and adventitious reaction very well. Our observations suggest that periodic removal of aminoglycosides from hospital formularies may mitigate the dissemination of these resistance enzymes among pathogens. Such policies on rotation of antibiotics in hospital formularies are in place in a few countries presently and perhaps should be more widely practiced.

REFERENCES

1. Wright, G. D., Berghuis, A. M., and Mobashery, S. (1998) Adv. Exp. Med. Biol. 456, 27–69
2. Wright, G. D. (1999) Curr. Opin. Microbiol. 2, 499–503
3. Vakulenko, S. B., and Mobashery S. (2003) Clin. Microbiol. Rev. 16, 430–450
4. Magnet, S., and Blanchard, J. S. (2005) Chem. Rev. 105, 477–497
5. Kim, C., and Mobashery, S. (2005) Bioorg. Chem. 33, 149–158
6. Gillespie, S. H., and McHugh, T. D. (1997) Trends Microbiol. 5, 337–339
7. Lenski, R. E. (1997) Antibiotic Resistance: Origins, Evolution, Selection, and Spread, pp. 131–151, Wiley, Chichester, NY
8. Schrag, S., Perrot, V., and Levin B. R. (1997) Proc. R. Soc. London 264, 1287–1291
9. Salzler, A. A., and Amabili-Cuevas, C. F. (1997) Antimicrob. Agents Chemother. 41, 2321–2325
10. Andersson, D. I., and Levin, B. R. (1999) Curr. Opin. Microbiol. 2, 489–493
11. Andersson, D. I. (2003) Curr. Opin. Microbiol. 6, 452–456
12. Siregar, J. J., Lerner, S. A., and Mobashery, S. (1994) Antimicrob. Agents Chemother. 38, 641–647
13. Siregar, J. J., Miroshnikov, K., and Mobashery, S. (1995) Biochemistry 34, 12681–12688
14. Smith, M. A., and Bidochka, M. J. (1998) Can. J. Microbiol. 44, 351–355
15. Goodsell, D. S. (1991) Trends Biochem. Sci. 16, 203–206
16. Nanninga, N. (1985) Molecular Cytology of Escherichia coli, pp. 161–197, Academic Press, London, UK
17. Guihard, G., Bénédicti, H., Besnard, M., and Letellier, L. (1993) J. Biol. Chem. 268, 17775–17780
18. Bagel, S., Hüllen, V., Weidemann, B., and Heisig, P. (1999) Antimicrob. Agents Chemother. 43, 868–875
19. Becker, E. C., and Meyer, R. J. (1997) J. Bacteriol. 179, 5947–5950
20. Kim, C., Haddad, J., Vakulenko, S. B., Meroueh, S. O., Wu, Y., Yan, H., and Mobashery, S. (2004) Biochemistry 43, 2373–2383
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 1.3–1.6, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Reisbig, M. D., Hossain, A., and Hanson, N. D. (2003) J. Antimicrob. Chemother. 51, 1141–1151
23. Golerni, D., Maveyraud, L., Vakulenko, S., Samama, J.-P., and Mobashery, S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14280–14285
24. Louie, T. M., Xie, X. S., and Xun, L. (2003) Biochemistry 42, 7509–7517
25. Seyer, K., Lessard, M., Piette, G., Lacroix, M., and Saucier, L. (2003) Appl. Environ. Microbiol. 69, 3231–3237
26. Diego-Gonzalez, F., and Russell, J. B. (1997) FEMS Microbiol. Lett. 151, 71–76
27. Goss, S. R., Spice, A. B., and Nichols, W. W. (1988) FEMS Lett. 228, 245–248
28. Lasko, D. R., and Wang, D. I. C. (1996) Biotechnol. Bioeng. 52, 364–372
29. Rohwer, J. M., Jensen, P. R., Shinohara, Y., Postma, P. W., and Westerhoff, H. V. (1999) Eur. J. Biochem. 235, 225–230
30. Rozkov, A., and Elford, S. -O. (1999) Biotechnol. Bioeng. 62, 730–738