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Decreased Susceptibility to 4′-Deoxy-6′-*N*-Methylamikacin (BB-K311) Conferred by a Mutant Plasmid in *Escherichia coli*

MICHAEL H. PERLIN[1] AND STEPHEN A. LERNER[1,2]*

Departments of Medicine[1] and Microbiology,[2] University of Chicago, Chicago, Illinois 60637

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Escherichia coli MP6 contains a plasmid that encodes aminoglycoside 3′-phosphotransferase II, which phosphorylates kanamycin and confers high-level kanamycin resistance. Amikacin is a minor substrate of this enzyme, but MP6 is susceptible to amikacin. Strain MP10 has a spontaneous mutation in the plasmid of MP6 that increases the aminoglycoside 3′-phosphotransferase II activity not only against kanamycin but also against amikacin. This mutation is also responsible for the appearance of resistance to amikacin in MP10. Resistance to 4′-deoxy-6′-*N*-methylamikacin (BB-K311) by enzymatic modification has not been reported previously. As with amikacin, MP6 was susceptible to BB-K311 and its aminoglycoside 3′-phosphotransferase II did not phosphorylate this amikacin derivative appreciably. We found that the plasmid-borne mutation in MP10, however, localized by being cloned with a 3.7-megadalton *Hin*dIII fragment containing the aminoglycoside 3′-phosphotransferase II gene, resulted in increased phosphorylation of BB-K311 and resistance to it. Thus, the mutation distinguishing MP6 and MP10 has increased the activity of an existing aminoglycoside-modifying enzyme and produced new bacterial resistance to two previously minor substrates of the enzyme.

The substitution of hydroxyaminobutyric acid at the 1-amino site of kanamycin A to produce amikacin has profound effects on the susceptibility of the kanamycin molecule to bacterial resistance mechanisms. Whereas resistance to kanamycin A is conferred enzymatically by adenylylation at its 4′-hydroxyl and 2″-hydroxyl groups, acetylation at its 3-amino and 6′-amino groups, and phosphorylation at its 3′-hydroxyl group (7), the substitution at the 1-amino group, yielding amikacin, abolishes resistance produced by enzymatic modification at most sites on the kanamycin molecule (7, 8, 16). Thus, resistance to amikacin by enzymatic modification in gram-negative bacteria has been reported to be limited to strains that produce aminoglycoside 6′-acetyltransferase (7, 16). In addition, aminoglycoside 4′-adenylyltransferase activity confers resistance to amikacin in *Staphylococcus aureus* (11). 4′-Deoxy-6′-*N*-methylamikacin (BB-K311) is a derivative of amikacin designed to render it free from enzymatic attack at these sites by removal of the 4′-hydroxyl group and methylation of the 6′-amino group. Therefore, BB-K311 is expected to be refractory to enzymatic modification and active against those strains whose resistance to amikacin is due to enzymatic mechanisms (9, 14).

An aminoglycoside 3′-phosphotransferase III [APH(3′)-III] produced by some strains of *Streptococcus faecalis* phosphorylates amikacin well, but its rate of phosphorylation of BB-K311 is exceedingly low. Thus, it has been concluded that removal of the 4′-hydroxyl group from amikacin effectively blocks 3′-phosphorylation by the *S. faecalis* enzyme (3). The production of the enzyme by strains of *S. faecalis* does not substantially impair the synergy of the combination of penicillin and BB-K311 against these strains. In contrast, this enzyme confers not merely resistance to the synergy of penicillin and amikacin (10), but actual antagonism (3). Therefore, despite preservation of the 3′-hydroxyl group in BB-K311, attack at this site by the APH(3′)-III is sufficiently reduced so that BB-K311 retains its synergistic activity with penicillin against strains that produce this enzyme.

Even in gram-negative bacteria, enzymatic modification of amikacin is not limited to acetylation of the 6′-amino group. We have previously shown that a plasmid-borne mutation can increase the activity of aminoglycoside 3′-phosphotransferase II [APH(3′)-II] against its aminoglycoside substrates, including minor ones such as amikacin, sufficiently to produce amikacin resistance in *Escherichia coli* (15). Recently, Coombe and George have reported on the activity of an aminoglycoside 2″-adenylyltransferase against amikacin in clinical isolates of four genera of *Enterobacteriaceae* (5). In the present study, we examined whether the alterations at

TABLE 1. Bacterial strains

E. coli strain	Phenotype[a]	Comments
MP6	Pro$^-$ Met$^-$ Nal^r Kan^r Neo^r Str^r	Contains pMP6, a mutant plasmid derived from pJR66a (15)
MP61	Pro$^-$ Met$^-$ Nal^r Kan^r Neo^r Carb^r	Contains pBR322 into which a *Hind*III fragment of pMP6 bearing the APH gene has been cloned
MP10	Pro$^-$ Met$^-$ Nal^r Kan^r Ami^r Neo^r Str^r	Contains pMP10, a mutant of pMP6 that confers resistance to amikacin (15)
MP101	Pro$^-$ Met$^-$ Nal^r Kan^r Ami^r Neo^r Carb^r	Contains pBR322 into which a *Hind*III fragment of pMP10 bearing the APH gene has been cloned
JSR0-N	Pro$^-$ Met$^-$ Nal^r	Plasmid-free genetic background (15) for all of the above strains

[a] Nal, Nalidixic acid; Kan, kanamycin; Ami, amikacin; Neo, neomycin; Str, streptomycin; Carb, carbenicillin.

the 4′ and 6′ sites in BB-K311 affect its 3′-phosphorylation by the APH(3′)-II encoded by our mutant plasmid and whether the enzyme confers resistance to BB-K311 as well as to amikacin.

MATERIALS AND METHODS

Antibiotics. Antibiotics were provided as follows: amikacin, kanamycin, and BB-K311 by Bristol Laboratories, Syracuse, N.Y.; butirosin by Parke, Davis & Co., Detroit, Mich.; ribostamycin by Meiji Seika Keisha, Ltd., Tokyo, Japan; carbenicillin by Beecham Laboratories, Bristol, Tenn.; and nalidixic acid by Sterling-Winthrop Laboratories, Rensselaer, N.Y. Streptomycin was purchased from Calbiochem, La Jolla, Calif.

Bacterial strains. Table 1 lists the relevant characteristics of the *E. coli* strains used in this study. MP6 contains pMP6, a mutant plasmid derived from pJR66a that confers resistance to kanamycin but not to amikacin, as in the original *E. coli* W677/JR66 (6). MP10 contains pMP10, a plasmid that arose by spontaneous mutation of pMP6 to produce amikacin resistance. JSR0-N is the plasmid-free, susceptible *E. coli* strain which is the genetic background for each of the strains in Table 1.

Enzymes. *Hind*III was purchased from Boehringer Mannheim Corp., New York, N.Y., and T4 DNA ligase was purchased from New England Biolabs, Beverly, Mass.

Cloning of the APH gene. Methods for digestion with restriction endonucleases and ligation of fragments were as described by Chang and Cohen (4). The transformation of susceptible *E. coli* was by the method of Mandel and Higa (13). Electrophoresis was performed in 0.6% agarose at 40 V for 25 h. The buffer system contained 89 mM Tris-hydrochloride, 8.9 mM boric acid, and 2.5 mM EDTA (pH 8.3).

Preparation of sonic extracts and assay of APH activity. Sonic extracts prepared from all strains (15) were assayed for APH activity by a radioenzymatic phosphocellulose filter binding assay as described previously (15). Assay mixtures were incubated at 25°C and sampled at 30, 60, and 90 s or at 1, 2, and 3 min. The protein concentrations in the sonic extracts were determined by the method of Lowry et al. (12).

Determination of drug susceptibility. Strains were tested for drug susceptibility by an agar dilution method (15).

RESULTS

Cloning of the APH gene. To localize the mutation from pMP6 to pMP10 that produces resistance to amikacin, we cloned a fragment containing the APH gene from each of these plasmids. MP61 and MP101 bear the APH(3′) genes of pMP6 and pMP10 cloned on their respective *Hind*III fragments into pBR322. After transformation with each recombinant plasmid, colonies grew on 200 μg of carbenicillin per ml at a frequency of about 2.5×10^{-4} per recipient. MP61 was 1 of 14 transformants with recombinant DNA from pMP6 that subsequently grew on 200 μg of kanamycin per ml, but not on 6.25 μg of tetracycline per ml or on 25 μg of amikacin per ml. MP101 was 1 of 19 transformants with recombinant DNA from pMP10 that subsequently grew on 25 μg of amikacin per ml and on 200 μg of kanamycin per ml, but not on 6.25 μg of tetracycline per ml. These resistance patterns suggested that the transformants had received a fragment inserted into the single *Hind*III site within the tetracycline resistance gene of pBR322. This was confirmed by agarose gel electrophoresis of *Hind*III digests of plasmid DNA purified from the two clones (Fig. 1), which showed a 3.7-megadalton fragment in addition to pBR322. The phenotypes of MP61 and MP101 are listed in Table 1.

Phosphotransferase activity. Table 2 lists the phosphotransferase activity of each strain for various aminoglycoside substrates. JSR0-N had no detectable phosphotransferase activity

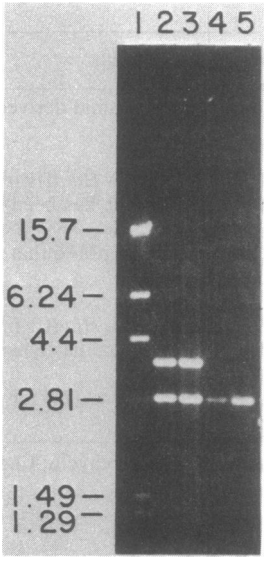

FIG. 1. Agarose gel electrophoresis of restriction endonuclease digests of plasmid DNAs. Electrophoresis was in 0.6% agarose at 40 V for 25 h. Lane 1, λ DNA digested with *Hin*dIII for size standards; lane 2, pMP61 digested with *Hin*dIII; lane 3, pMP101 digested with *Hin*dIII; lane 4, pBR322 digested with *Sph*I; lane 5, pBR322 digested with *Hin*dIII.

against any of the aminoglycosides tested. MP6 had detectable but low activity against amikacin and BB-K311 and higher activity against other substrates of APH(3')-II (kanamycin, neomycin, butirosin, and ribostamycin). Activity against streptomycin, a substrate of an APH(3") encoded by the plasmid, was also detectable. The insertion of a fragment of DNA bearing the APH(3')-II gene into pBR322 (to yield MP61) enhanced the activity against all the substrates of that enzyme, but phosphorylation of streptomycin was lost. MP10 had greater activity than MP6 against each substrate, although phosphorylation of butirosin was not much higher. The activity of MP101 for amikacin, BB-K311, and the other substrates of APH(3')-II was greater than that of MP10, but streptomycin phosphotransferase activity was no longer present.

Aminoglycoside susceptibility. Table 3 lists the susceptibility of each strain to various antibiotics. JSR0-N was susceptible to all of the drugs tested. MP6 was susceptible to amikacin, but resistant to kanamycin and streptomycin. It was also susceptible to BB-K311. Cloning of a DNA fragment that includes the APH(3')-II gene (to yield MP61) removed resistance to streptomycin along with the APH(3") gene present on pMP6. The resistance of MP61 to carbenicillin was from the β-lactamase encoded by pBR322. MP10 had a dramatic increase in the minimum inhibitory concentration (MIC) of amikacin from that for MP6, as reported previously (15). MP10 also showed resistance to BB-K311. The MICs of kanamycin and streptomycin for MP10 appeared to be even higher than the high levels for MP6. Cloning of the APH(3')-II gene from MP10 (to yield MP101) transferred resistance to BB-K311 (albeit at a lower level), as well as to amikacin and kanamycin, and eliminated streptomycin resistance.

DISCUSSION

The mutation in MP10 that was selected by resistance to amikacin resulted in an increase in APH activity against BB-K311, as well as against other substrates of APH(3')-II. This rise in phosphotransferase activity against amikacin and BB-K311 was associated with the development of resistance to both of these substrates. Thus, the alterations at the 4' and 6' sites in BB-K311 do not protect it from attack at the 3' site by APH(3')-II in our resistant mutant of *E. coli*. This result is in contrast to the protection of BB-K311 from phosphorylation by the APH(3')-III of *S. faecalis* and the consequent susceptibility of these strains to penicillin–BB-K311 synergy, as shown by Calderwood et al. (3).

Bongaerts and Kaptijn (2) recently reported on a mutant variant selected on amikacin from a derivative of *E. coli* W677/JR66. They claimed that the increased APH(3')-II activity that accompanied the rise in the MIC of amikacin in their variant and in our mutant results from chromosomal mutations that probably increase the copy number of the plasmid bearing the APH

TABLE 2. Aminoglycoside phosphotransferase activity of strains

Strain	Sp act[a] (×10^{-4} U) for the following substrate[b]:						
	Ami	BB-K311	Kan	Neo	But	Rib	Str
JSR0-N	≤0.02	≤0.02	≤0.02	≤0.02	≤0.02	≤0.02	≤0.02
MP6	0.11	0.07	0.81	1.0	4.8	0.31	0.05
MP61	2.47	0.142	5.96	10.4	20.6	4.3	≤0.02
MP10	0.834	0.153	2.27	4.5	4.96	3.3	0.184
MP101	3.99	0.3	12.2	30.7	41.4	3.76	≤0.02

[a] One unit = 1 μmol of substrate phosphorylated per min per mg of protein at 25°C.
[b] Ami, Amikacin; BB-K311, 4'-deoxy-6'-*N*-methylamikacin; Kan, kanamycin; Neo, neomycin; But, butirosin; Rib, ribostamycin; Str, streptomycin.

TABLE 3. Antibiotic susceptibility of strains

Strain	MIC (μg/ml) of the following drug[a]:								
	Ami	BB-K311	Kan	Neo	But	Rib	Gen	Str	Carb
JSR0-N	0.78	1.56	≤1.56	≤1.56	≤1.56	≤1.56	1.56	3.12	≤12.5
MP6	0.78	1.56	200	50	>400	>400	1.56	50	≤12.5
MP61	0.78	1.56	>400	200	>400	>400	3.12	1.56	>200
MP10	25–50	50	>400	>400	>400	>400	6.25	>400	≤12.5
MP101	25	12.5–25	>400	>400	>400	>400	3.12	3.12	>200

[a] Ami, Amikacin; BB-K311, 4′-deoxy-6′-*N*-methylamikacin; Kan, kanamycin; Neo, neomycin; But, butirosin; Rib, ribostamycin; Gen, gentamicin; Str, streptomycin; Carb, carbenicillin.

gene. The concomitant increase in APH (3″) activity in MP10 suggests that there might be an increase in copy number of the plasmid to account for the increased activity of the two enzymes. However, the mutation responsible for the amikacin resistance and the increase in APH(3′)-II activity in our mutant is not chromosomal but is in fact transferable with the plasmid (15). Furthermore, the present study indicates that the mutation responsible for resistance to BB-K311 and amikacin and for increased APH activity against these and other substrates is located on an approximately 3.7-megadalton *Hin*dIII fragment of pMP10 that includes the structural gene of APH(3′)-II but not that of APH(3″). This suggests a mutation in the structural gene or its promoter, although we have not ruled out a mutation in a closely linked gene governing another function, such as control of plasmid replication or copy number, or regulation of expression of the two APH genes in the intact plasmid.

One cannot necessarily correlate quantitatively the level of activity of an aminoglycoside-modifying enzyme, as determined by in vitro assay, with the level of resistance for which it is responsible. MP101, which differs from MP61 by a mutation in the cloned fragment containing the APH(3′)-II structural gene, is much more resistant than MP61 to amikacin and BB-K311. Despite the greater levels of resistance to these aminoglycosides exhibited by MP101, extracts of this strain have only about twice the APH activity of MP61 extracts against these two substrates. If the K_ms of minor substrates such as amikacin and BB-K311 for APH(3′)-II of a susceptible strain are much higher than the concentrations confronting the enzyme in vivo, then assays carried out with excess, saturating concentrations of these substrates in vitro will not reflect the effective activity at the lower substrate concentrations. A reduction in the K_m for a substrate by mutational alteration of the enzyme might thus improve the activity against it at therapeutic concentrations without raising appreciably the activity observed in the presence of excess substrate (V_{max}). The possibility that the mutation in MP10 is in the structural gene of APH(3′)-II, reducing the K_ms of minor substrates such as amikacin and BB-K311, is being investigated.

The introduction of the APH(3′)-II gene from pMP6 or pMP10 into the multicopy plasmid pBR322 (1) raises the activity against BB-K311 only twofold. In each case, there is no increase in the MIC of BB-K311; in fact, the MIC in MP101 is somewhat lower than that in MP10. It is of interest that cloning results in greater rises in activity against other substrates, with the exception of ribostamycin phosphorylation in MP10. The reason for the lack of equivalence in increases of activity produced by cloning of the APH(3′)-II gene is not clear. Despite the large increase in activity against amikacin resulting from the production of MP61 and MP101 by cloning, there was no increase in the MIC of amikacin in these strains relative to MP6 and MP10, the strains from which the APH(3′)-II gene was cloned.

Although the exact mechanism remains to be elucidated, it is clear that a spontaneous, plasmid-borne mutation occurring in or near the structural gene of APH(3′)-II has broadened the spectrum of resistance conferred by the enzyme to include two minor substrates, amikacin and BB-K311. This mutation thus overcomes chemical manipulation of kanamycin designed to eliminate bacterial resistance to it that is due to enzymatic inactivation. Similar mutations may play a role in the natural evolution of aminoglycoside-modifying enzymes and resistance conferred by them.

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