ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1979, 598-604 Vol. 16, No. 5
0066-4804/79/11-0598/07$02.00/0

Amikacin Resistance Associated with a Plasmid-Borne Aminoglycoside Phosphotransferase in *Escherichia coli*

MICHAEL H. PERLIN AND STEPHEN A. LERNER*
Departments of Medicine and Microbiology, University of Chicago, Chicago, Illinois 60637

Received for publication 15 August 1979

Enzymatic phosphorylation of amikacin has not been reported previously in gram-negative bacteria. We found that extracts of MP1, a mutant of *Escherichia coli* JR66/W677 that is resistant to amikacin, were able to phosphorylate this aminoglycoside more rapidly than were extracts of the parental strain. Conjugal transfer of resistance from MP1 to a recipient strain was accompanied by acquisition in the transconjugants of amikacin phosphotransferase activity and of a 57-megadalton plasmid present in the donor. Partial purification of the phosphotransferase activity on amikacin-Sepharose 4B yielded an enzyme with a substrate spectrum similar to that of the 3′-neomycin-kanamycin phosphotransferase II found in *E. coli*, except that it was also active against amikacin. A mutant of MP1, MP5, had increased susceptibility to amikacin and reduced phosphotransferase activity. MP9, a mutant of MP5, was more resistant to amikacin and had increased phosphotransferase activity. The mutations leading to these alterations of amikacin susceptibility and amikacin phosphotransferase activity were transferable with the same plasmid that was associated with amikacin resistance and phosphotransferase activity in MP1. These studies demonstrate that resistance to amikacin in a laboratory strain of *E. coli* is due to an aminoglycoside phosphotransferase coded by a transferable plasmid-borne gene.

Amikacin is a synthetic derivative of kanamycin A with a hydroxyaminobutyric acid side chain substituted at the 1-amino position. This substitution renders amikacin free from attack by known enzymes of gram-negative bacteria at all of the susceptible sites on kanamycin, except the 6′-amino group, which is acetylated (6). As a consequence of its limited susceptibility to enzymatic modification, amikacin is effective against strains that modify kanamycin at the other sites (13).

Amikacin has been employed successfully in the treatment of serious infections caused by gram-negative bacteria, especially strains resistant to other aminoglycosides (1, 10, 16). Development of novel enzymatic modification of amikacin might extend resistance to amikacin and thereby decrease its effectiveness in the treatment of serious gram-negative infections.

An enzyme produced by a mutant laboratory strain of *Escherichia coli* with modest resistance to amikacin was found to phosphorylate amikacin, as well as its parent compound, kanamycin A. We sought to determine whether this enzymatic activity contributes to the amikacin resistance and whether resistance to amikacin could be affected by mutational alteration of this phosphorylating activity.

MATERIALS AND METHODS

Bacterial strains. MP1, a derivative of *E. coli* JR66/W677 (Thr^- Leu^- Thi^-), isolated from a culture mutagenized with nitrosoguanidine for increased resistance to chloramphenicol, was provided by L. Sands. *E. coli* JR66/W677 (containing plasmids pJR66a and pJR66b) (5) was supplied by K. Price of Bristol Laboratories, Syracuse, N.Y. *E. coli* JSRO (Pro^- Met^-, sensitive to aminoglycoside antibiotics and to chloramphenicol) (K-12 strain J53) and *E. coli* RP1/MRPO were from J. Shapiro. *E. coli* JSRO-N was a spontaneous nalidixic acid-resistant mutant of JSRO. Strains were kept at −20°C in Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy broth-heat-inactivated horse serum (1:1).

Media. Rich media were Mueller-Hinton (M-H) broth and agar (BBL Microbiology Systems). The PA minimal agar medium (20) included 1.2% agar (Difco Laboratories, Detroit, Mich.). Glucose was added to 0.8%, and proline and methionine were each supplemented at a concentration of 50 μg/ml.

Chemicals and reagents. Kanamycin, amikacin, lividomycin A, and dideoxykanamycin B were gifts from Bristol Laboratories; gentamicin complex and the C_1, C_{1a}, and C_2 components were from Schering Laboratories, Bloomfield, N.J.; gentamicin A and paromomycin were from S. Kabins; neomycin was from J. Shapiro; butirosin and chloramphenicol were from Parke, Davis & Co., Detroit, Mich.; ribostamycin was from Meiji Seika Keisha, Ltd., Tokyo, Japan; and nalidixic acid was from Winthrop Laboratories, New

York, N.Y. Streptomycin was purchased from Pfizer Inc., New York, N.Y.; proline and methionine were from Calbiochem, La Jolla, Calif.; adenosine 5'-triphosphate (ATP) was from P-L Biochemicals, Milwaukee, Wis.; [^{14}C]acetyl coenzyme A was from Amersham Corp., Arlington Heights, Ill.; and [8-^{14}C]ATP was from New England Nuclear Corp., Boston, Mass. [γ-^{32}P]ATP, synthesized by the method of Glynn and Chappell (8), was kindly provided by N. Cozzarelli and K. Agarwal.

Determination of antibiotic susceptibility. Antibiotic disk susceptibility testing was performed by the Kirby-Bauer method (2). Minimal inhibitory concentrations (MICs) of antibiotics were determined by an agar dilution method. Suspensions of test organisms at around 10^8/ml were applied with a pronged replicator (22) to M-H agar containing antibiotics in twofold dilutions. After the plates were incubated at 37°C for 18 h, they were scored for growth or no growth. The lowest antibiotic concentration at which no growth appeared was the MIC.

Conjugal transfer of kanamycin, amikacin, or gentamicin resistance. Aminoglycoside-resistant transconjugants of JSRO-N were selected by spreading mating cultures of JSRO-N and resistant donors onto M-H agar containing 3 μg of nalidixic acid per ml and 25 μg of kanamycin, 12.5 μg of amikacin, or 6.25 μg of gentamicin per ml. Alternatively, in crosses with JSRO as the recipient, counterselection against the donors was accomplished by plating mating cultures onto PA agar containing proline and methionine along with kanamycin or amikacin. With either method of counterselection, there was no growth on control plates spread with samples of cultures lacking a donor or recipient. All transconjugants grew on PA agar supplemented with proline and methionine but not in their absence.

Isolation of mutants with decreased resistance to amikacin. Two cultures of MP1 were mutagenized with ethyl methane sulfonate (Eastman Organic Chemicals, Rochester, N.Y.) (14) and then diluted and cultivated overnight in M-H broth at 37°C. Diluted samples from each culture were spread onto M-H agar plates, and colonies which grew up were replica plated onto M-H agar containing amikacin (12.5 μg/ml), kanamycin (200 μg/ml), gentamicin (25 μg/ml), or no antibiotic. After the plates were incubated overnight at 37°C, colonies which grew on all plates except those containing amikacin were picked.

Selection of mutants with increased resistance to amikacin. A sample of an overnight culture of MP5, a mutant of MP1 with a decreased level of resistance to amikacin, was spread onto M-H agar containing 25 μg of amikacin per ml. Mutants which grew spontaneously after 24 h at 37°C were purified on M-H agar containing amikacin.

Preparation of sonic extracts. Sonic extracts were prepared as described previously (21). After sonication, the suspensions were centrifuged at 12,000 × *g* for 15 min at 2°C, and the supernatants (sonic extracts) were aspirated and kept on ice. Extracts which were to be assayed for aminoglycoside phosphorylation alone were prepared in 0.6 M sodium cacodylate, pH 7.0.

Assay of aminoglycoside-modifying activity. In each case, enzymatic modification of an aminoglycoside was assayed by adsorption of the radioactive product onto phosphocellulose filter disks (Whatman P 81) by the method of Ozanne et al. (19). A unit of activity was 1 μmol of aminoglycoside modified per min at the assay temperature.

(i) Phosphorylation. Reaction mixtures of 0.120 ml included 0.1 M cacodylate (pH 7.0), 7.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.0825 mM [γ-^{32}P]ATP (5×10^{12} to 10×10^{12} cpm/mol), and 0.167 mM aminoglycoside. Enzyme was added to the reaction mixtures on ice, and the reactions were started by transfer to 25°C. Samples of 0.100 ml were spotted onto phosphocellulose filter disks at 3 or 20 min, or samples of 0.035 ml were spotted at 1, 2, and 3 min, and the disks were dropped into water at 80°C to stop the reaction.

(ii) Adenylylation. Reactions were carried out as for phosphorylation, except 0.033 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.8) and 0.33 mM [^{14}C]ATP (4 Ci/mol) replaced cacodylate buffer and [γ-^{32}P]ATP, and incubation was at 37°C for 3 min, for 20 min, or for 1, 2, and 3 min.

(iii) Acetylation. Reaction mixtures of 0.120 ml included 0.1 M Tris-maleate (pH 5.7), 0.01 M $MgCl_2$, 2.5 mM dithiothreitol, 0.0825 mM [^{14}C]acetyl coenzyme A (4 Ci/mol), and 0.167 mM aminoglycoside. Enzyme extracts were added to reaction mixtures on ice, and the reactions were started by transfer to 37°C. Samples were withdrawn at 20 min.

Assay for the presence of chloramphenicol acetyltransferase (CAT) activity was carried out by the radioenzymatic method of Robison et al. (21).

Preparation of amikacin-agarose. Agarose (Sepharose 4B; Pharmacia Fine Chemicals Inc., Piscataway, N.J.) was activated with cyanogen bromide and coupled with amikacin by the method of Kawabe et al. (12).

Purification of amikacin phosphorylating activity. A culture of an amikacin-resistant transconjugant from a cross of MP1 with JSRO was subjected to osmotic shock (18). After centrifugation at 27,000 × *g* for 15 min at 2°C, the supernatant was purified on amikacin-agarose by the method of Kawabe et al. (12). The column was eluted with 250 ml of a linear gradient from 0.05 to 0.75 M NaCl in the column buffer. Fractions with phosphorylating activity for amikacin were pooled, dialyzed against column buffer without salt, and kept on ice.

Protein determination. Protein concentrations were determined by the method of Lowry et al. (15).

Demonstration of plasmid content. Extracts of bacterial plasmid deoxyribonucleic acid were prepared by the method of Meyers et al. (17) from cultures grown overnight in Trypticase soy broth at 37°C. Samples were analyzed for the presence of plasmids by electrophoresis on agarose gels (17).

RESULTS

Antibiotic susceptibilities of MP1, JR66/W677, and JSRO-N. As shown in Table 1, whereas MP1 was resistant to amikacin, its antecedent strain, JR66/W677, was susceptible to amikacin and had lower MICs of gentamicin and tobramycin but greater resistance to tetracycline

TABLE 1. *Antibiotic resistance of strains*

Strain	Antibiotic resistance[a]									
	Ami[b]	Kan[b]	Liv[b]	Ddk[b]	Gen[b]	Tob[b]	Str[b]	Chl[c]	Te[c]	Su[c]
JR66/W677	3.12	>100	12.5	100	25	12.5	100	6	6	6
MP1	50	>200	25	>100	>50	>50	>100	6	15	6
MP5	12.5	>200	12.5	>100	>50	50	>100	6	17	6
MP9	50	>200	100	>100	>50	>50	>100	6	6	6
JSRO-N	3.12	6.25	12.5	6.25	0.78	1.56	0.78	20	20	22
JSRO-N(pJR66a)	3.12	>100	12.5	6.25	3.12	3.12	100	24	21	27
MP2	50	>200	25	12.5	3.12	1.56	50	20	23	24
MP2g	50	>100	ND[d]	ND[d]	25	50	>100	6	12	6
MP6	3.12	>200	12.5	6.25	0.78	0.78	50	22	23	27
MP10	50	>200	100	12.5	0.78	3.12	>100	22	22	27

[a] Ami, Amikacin; Kan, kanamycin; Liv, lividomycin A; Ddk, dideoxykanamycin B; Gen, gentamicin; Tob, tobramycin; Str, streptomycin; Chl, chloramphenicol; Te, tetracycline; Su, sulfisoxazole.
[b] MIC (micrograms per milliliter).
[c] Kirby-Bauer disk diameter (millimeters).
[d] ND, Not done.

than did MP1. The MIC of lividomycin A for MP1 was twice that for JR66/W677. Strain JSRO-N was susceptible to all antibiotics tested.

Conjugal transfer of amikacin resistance from MP1 to JSRO-N. In a cross between MP1 and JSRO-N, colonies appeared on amikacin and kanamycin plates at a frequency of 5×10^{-6} per donor. In the same cross, selection on gentamicin yielded 10^{-5} colonies per donor. Putative transconjugant colonies resistant to amikacin, kanamycin, or gentamicin were then replica plated onto M-H agar containing 3.12 μg of amikacin, 25 μg of kanamycin, 3.12 μg of gentamicin, or 3.12 μg of tobramycin per ml. All strains selected for resistance to amikacin were also resistant to kanamycin; all kanamycin-resistant transconjugants grew on amikacin. None of the transconjugants had acquired a clinically significant level of resistance to gentamicin or tobramycin. One transconjugant selected for resistance to amikacin (MP2) and another selected for resistance to kanamycin were picked for further study. As shown in Table 1, MP2 acquired resistance to streptomycin as well as to amikacin and kanamycin, but had only a modest rise in the MIC of gentamicin and no change in susceptibility to dideoxykanamycin, tobramycin, chloramphenicol, tetracycline, or sulfisoxazole. The transconjugant selected for its resistance to kanamycin had a profile of resistance to these antibiotics similar to that of MP2 (data not shown). A transconjugant strain similar to MP2 (MP2a) was selected in a cross between MP1 and JSRO by growth on PA agar containing amikacin, proline, and methionine. All of the transconjugants selected for resistance to gentamicin also grew on tobramycin, and 98 of 100 also grew on amikacin and kanamycin. One of these picked for further study (MP2g) acquired resistance to the same spectrum of antibiotics to which MP1 was resistant (Table 1).

To compare the transfer of aminoglycoside resistance from MP1 with that from JR66/W677, we crossed the latter strain with JSRO-N and selected kanamycin-resistant transconjugants with a frequency of 4×10^{-5} per donor. None of 75 tested grew on amikacin, gentamicin, or tobramycin. Transconjugant strain JSRO-N(pJR66a), picked for further study, had acquired clinically significant resistance to only streptomycin in addition to its kanamycin resistance (Table 1).

Isolation of a mutant of MP1 with enhanced susceptibility to amikacin. From a mutagenized culture of MP1, one strain (MP5) had an MIC of amikacin one-fourth that of the parental MP1 (Table 1). MP5 was still resistant to kanamycin, dideoxykanamycin B, and gentamicin, but the actual limits were not determined. A kanamycin-resistant transconjugant (MP6) from a cross of MP1 with JSRO-N remained as susceptible to amikacin as did the recipient (Table 1).

Selection of a mutant of MP5 with greater resistance to amikacin. Spontaneous amikacin-resistant mutants of MP5 appeared at a frequency of 10^{-6}. One of these, MP9, was chosen for further study. Its increased level of resistance to amikacin (MIC, 50 μg/ml) is shown in Table 1. MP9 also showed an increase in MIC of lividomycin A. MP10, an amikacin-resistant transconjugant, was selected in a cross between MP9 and JSRO-N. The resistance to lividomycin A was also transferable to MP10.

Aminoglycoside-modifying activities. Specific activities for phosphorylation of amikacin and kanamycin by sonic extracts of all strains in this study are listed in Table 2. Ex-

TABLE 2. *Phosphotransferase activity in crude extracts of strains*

Strain	Sp act[a]	
	Ami[b]	Kan[b]
JR66/W677	1.35	4.15
MP1	10.2	24.4
MP5	1.7	4.1
MP9	16.7	63.3
JSRO-N	0.1	0.1
JSRO-N(pJR66a)	0.59	3.8
MP2	2.0	5.6
MP2g	2.6	6.0
MP6	0.87	3.6
MP10	5.1	12.5

[a] Specific activity is stated in milliunits per milligram of protein at 25°C.
[b] Activity for each substrate: Ami, amikacin; Kan, kanamycin.

TABLE 3. *Phosphotransferase activity of MP2a purified on amikacin-agarose*

Substrate	Phosphotransferase activity[a]
Amikacin	32
Kanamycin	70
Gentamicin A	240
Neomycin	180
Butirosin	240
Paromomycin	220
Streptomycin	<0.1

[a] Specific activities, in milliunits per milligram of protein at 25°C, reflect curves obtained by sampling at 1, 2, and 3 min.

tracts of MP1 phosphorylated amikacin and kanamycin and also gentamicin A, neomycin, butirosin, ribostamycin, paromomycin, and streptomycin. Lividomycin A was phosphorylated weakly, at about 7% of the rate of kanamycin. An extract of MP1 failed to phosphorylate gentamicins C_1, C_{1a}, and C_2, tobramycin, sisomicin, netilmicin, spectinomycin, and dideoxykanamycin B. The phosphotransferase activity for each aminoglycoside was less for JR66/W677 than for MP1. In fact, activity for lividomycin A was hardly detectable in an extract of JR66/W677. JSRO-N had no phosphotransferase activity for any of the antibiotics tested. There was no acetylation by extracts of MP1, JR66/W677, and JSRO-N of any of the aminoglycosides tested for phosphorylation, but CAT activity was demonstrated in MP1. Of these aminoglycosides, only gentamicin C complex, tobramycin, dideoxykanamycin B, and kanamycin were adenylylated by MP1 and JR66/W677.

A crude extract of MP2 phosphorylated the same aminoglycoside spectrum as did extracts of MP1. Similarly, JSRO-N(pJR66a) acquired phosphotransferase activity against all of the aminoglycoside substrates of phosphorylation by JR66/W677. MP2 failed to acquire from MP1 its adenylylating activity for dideoxykanamicin B, gentamicin, or tobramycin or its CAT activity. On the other hand, MP2g, the transconjugant selected for gentamicin resistance, did acquire the adenylylating and CAT activities of MP1 and also its phosphorylating activities. MP2a, the amikacin-resistant transconjugant of MP1 obtained in a cross with JSRO, acquired the same phosphorylating activity as MP2 (data not shown). As shown in Table 3, amikacin phosphorylating activity partially purified from MP2a by affinity chromatography on amikacin-agarose was active against all of the substances phosphorylated by a crude extract except streptomycin (lividomycin A not tested).

MP5, the mutant of MP1 with enhanced amikacin susceptibility, had reduced phosphorylating activity for amikacin and kanamycin (Table 2). Its kanamycin-resistant transconjugant, MP6, acquired the same phosphotransferase activities. MP9, the amikacin-resistant mutant of MP5, had an increase in amikacin and kanamycin phosphotransferase activities over those of MP5 and even MP1. MP9 also had increased phosphotransferase activity for lividomycin A. Extracts of MP10 phosphorylated the same aminoglycosides as did extracts of MP9.

Plasmid content of strains. The agarose gel in Fig. 1a shows that MP1 and MP2g have two plasmids of the same size as those of JR66/W677: a larger plasmid, pJR66a, which is 57 megadaltons in size (7), and a smaller plasmid, pJR66b (5), which appears intermediate in size between pJR66a and RP1 (40 megadaltons) (9). JSRO-N has no detectable plasmid. MP2 (Fig. 1b) and JSRO-N(pJR66a) (Fig. 1a) have each acquired a plasmid equal in size to the larger plasmid of MP1. Figure 1a also shows that MP6 and MP10 acquired a plasmid of the same size as the larger plasmid present in MP1, MP5, and MP9.

DISCUSSION

We have confirmed the presence of two plasmids in the progenitor strain JR66/W677 (5). The larger (57 megadaltons) is able to transfer resistance to kanamycin and streptomycin and is presumably equivalent to pJR66a, a self-transmissible plasmid of 57 megadaltons (7) which confers resistance to kanamycin and streptomycin and determines I pili (5). The smaller plasmid is presumably equivalent to pJR66b, a non-self-transmissible plasmid which is a member of compatibility group FII, confers resistance to kanamycin, gentamicin, streptomycin, chlor-

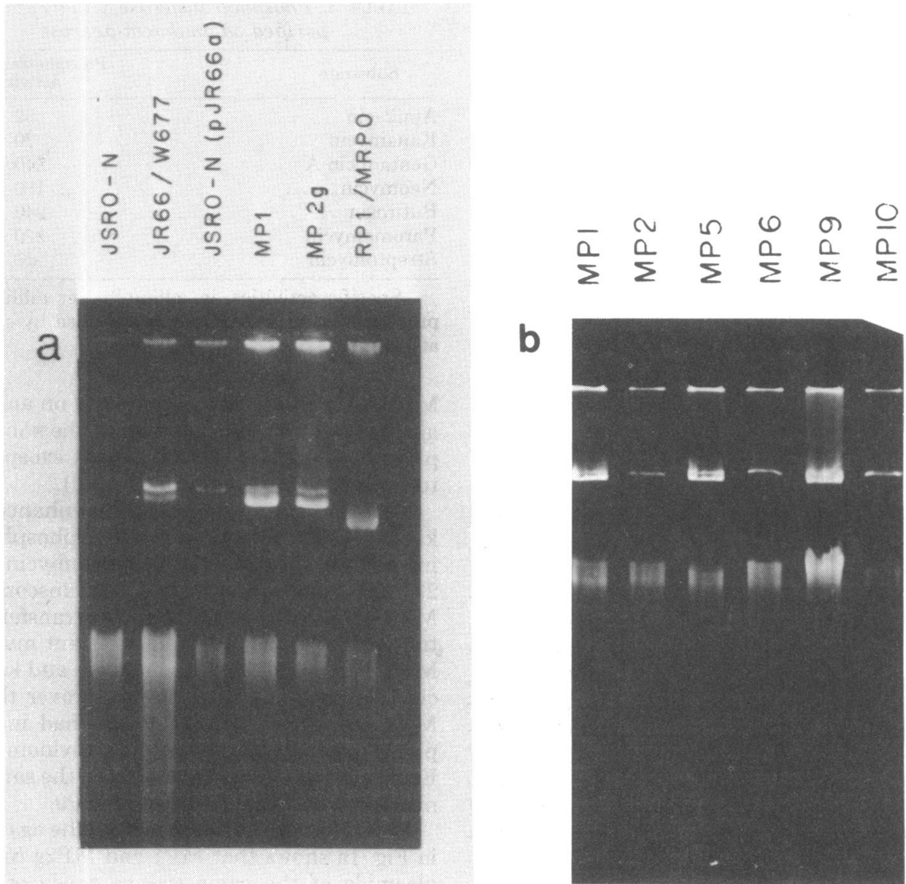

FIG. 1. *(a) Electrophoresis of deoxyribonucleic acid preparations in 0.6% agarose gel after 6 h at 150 V. E. coli JR66/W677 has a plasmid of 57 megadaltons in size (pJR66a) (7) and a smaller plasmid (5). RP1/MRPO contains RP1, a plasmid of 40 megadaltons (9). (b) Electrophoresis of deoxyribonucleic acid preparations in 0.8% agarose gel after 3 h at 150 V.*

amphenicol, tetracycline, and sulfisoxazole, and could be mobilized by pJR66a (5).

JR66/W677 has weak phosphorylating activity for amikacin and is susceptible to it. The origin of MP1 from this strain is somewhat obscure. The greater phosphotransferase activity for amikacin found in MP1 and its increased resistance to amikacin resulted either from the nitrosoguanidine-induced mutation that led to increased activity of CAT and to higher resistance to chloramphenicol or from some other mutational alteration.

The concomitant transfer from MP1 to JSRO-N of resistance to amikacin, phosphotransferase activity for this drug, and the 57-megadalton plasmid suggests that amikacin resistance in MP1 and MP2 is due at least in part to a gene borne on this plasmid, likely the structural gene for the phosphotransferase with activity for amikacin. MP2 acquired phosphorylating activity for the full substrate spectrum of MP1, but failed to gain high-level resistance to gentamicin, tobramycin, dideoxykanamycin B, chloramphenicol, tetracycline, or sulfisoxazole. MP2 also failed to acquire adenylylating activity for gentamicin, tobramycin, or dideoxykanamycin B (3) or CAT (21) known in JR66/W677. These results suggest that genes for these antibiotic-modifying enzymes and for resistance to tetracycline and sulfisoxazole lie on the smaller plasmid of MP1. In fact, selection for transfer of gentamicin resistance from MP1 into MP2g resulted in the acquisition of the other resistance markers of pJR66b from MP1, as well as those of pJR66a, corresponding to the transfer of both plasmids.

The partial purification of the phosphotransferase activity of MP2a by affinity chromatography with amikacin as the bound ligand showed that the 57-megadalton plasmid codes for at least two aminoglycoside phosphotransferases,

one that acts on streptomycin and another with activity for a series of other aminoglycosides including amikacin. Except for its activity on amikacin and on lividomycin A, this latter phosphotransferase has a spectrum of aminoglycoside substrates similar to that of the 3′-neomycin-kanamycin phosphotransferase II (3, 11). Amikacin phosphorylation by this enzyme has not been reported in gram-negative bacteria. Previous work on it either failed to detect amikacin phosphorylation (3, 11) or to test for it (19). In assays carried out in Tris-maleate at pH 7.1, diminished phosphorylation of amikacin may have been ignored as insignificant. Under our assay conditions in cacodylate buffer at pH 7.0, the ratio of specific activities for kanamycin and amikacin was generally in the range of 2:1 to 3:1 in extracts of JR66/W677, MP1, MP2, and MP2a and in the partially purified phosphotransferase preparation from MP2a. In similar assays of an extract of MP1 carried out in Tris-maleate at pH 7.0, the activity against amikacin was markedly diminished, so that the ratio of activities against kanamycin and amikacin increased to 15:1.

The role of the amikacin phosphotransferase in resistance to amikacin was supported by the demonstration that mutations in a plasmid-borne gene can lower and raise the level of resistance to amikacin along with the phosphotransferase activity for this drug. The actual level of activity in the transconjugant from each mutant was less than that in the corresponding donor. This may be due to a decreased level of expression in the genetic background of the recipient, JSRO-N. It is unlikely that the lower activity in the transconjugants was due to the absence of additional amikacin phosphorylating activity for which the untransferred plasmid pJR66b might be responsible, since transconjugant MP2g had both plasmids and yet its level of phosphotransferase activity for amikacin was less than that of MP1.

The aminoglycoside phosphotransferase activity, as usually assayed with excess aminoglycoside substrate, does not necessarily correlate well with the levels of resistance to different aminoglycosides. Our transconjugants were always much more resistant to kanamycin as compared with amikacin than would be predicted by the levels of phosphotransferase activity against these substrates. Crude kinetic studies of initial rates of phosphorylation (1, 2, and 3 min) of amikacin and kanamycin by extracts of MP1 indicated that the K_m of kanamycin was 1/10 or less than that of amikacin. Such differences in affinity were proposed to explain the susceptibility to amikacin of various strains of *Staphylococcus aureus* recently reported to possess a 3′-phosphotransferase activity for amikacin and other aminoglycosides (4). Although rates of phosphorylation are indicative of phosphotransferase activity, our assays were plagued by problems with poor linearity over time. A similar decline in the rate of phosphorylation with time was reported for the phosphotransferase in *S. aureus* (4).

The mutations of JR66/W677 which led to strains MP1 and MP9 resulted in a phosphotransferase activity with a broadened substrate spectrum. The mutant enzyme would appear to fall into a different class from the phosphotransferase reported for JR66/W677 (3′-neomycin-kanamycin phosphotransferase II), since it phosphorylates amikacin and lividomycin A more actively. Perhaps this type of mutation is responsible in part for the diversity of aminoglycoside-modifying enzymes.

The ability of spontaneous mutation to enhance the level of transferable resistance to amikacin is of concern. A strain such as MP5 with clinical susceptibility to amikacin (MIC, 12.5 μg/ml) mutated to MP9 with clinically significant resistance (MIC, 50 μg/ml). Whether similar mutants of pathogenic strains will be selected under conditions of increasing amikacin usage remains to be seen.

ACKNOWLEDGMENTS

We thank Ishan Bhattacharya for discussion and technical assistance; Edward Dudek, Hanna Avni, and Gary Boch for technical assistance; Diana Johnson-Zornow for editorial assistance; and Sherwin Kabins, James Shapiro, Stephen Rashbaum, Barbara Zehnbauer, and Craig Peebles for helpful suggestions.

This work was supported by the Infectious Disease Research Fund of the University of Chicago and Predoctorial Training Grant in Genetics and Regulation GM07107 from the National Institutes of Health.

LITERATURE CITED

1. **Bartlett, J. G.** 1977. Amikacin treatment of pulmonary infections involving gentamicin-resistant gram-negative bacilli. Am. J. Med. **62**:945–948.
2. **Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antimicrobial susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. **6**: 493–496.
3. **Benveniste, R., and J. Davies.** 1973. Mechanisms of antibiotic resistance in bacteria. Annu. Rev. Biochem. **43**:471–506.
4. **Courvalin, P., and J. Davies.** 1977. Plasmid-mediated aminoglycoside phosphotransferase of broad substrate range that phosphorylates amikacin. Antimicrob. Agents Chemother. **11**:619–624.
5. **Datta, N., and R. W. Hedges.** 1973. An I pilus-determining R factor with anomalous compatibility properties, mobilizing a gentamicin-resistance plasmid. J. Gen. Microbiol. **77**:11–17.
6. **Davies, J., and P. Courvalin.** 1977. Mechanisms of resistance to aminoglycosides. Am. J. Med. **62**:868–872.
7. **Falkow, S., P. Guerry, R. W. Hedges, and N. Datta.** 1974. Polynucleotide sequence relationships among plasmids of the I compatibility complex. J. Gen. Microbiol. **85**:65–76.

8 **Glynn, I. M., and J. B. Chappell.** 1964. A simple method for the preparation of ^{32}P-labelled adenosine triphosphate of high specific activity. Biochemistry **90:**147-149.
9. **Grinsted, J., J. R. Saunders, L. C. Ingram, R. B. Sykes, and M. H. Richmond.** 1972. Properties of an R factor which originated in *Pseudomonas aeruginosa* 1822. J. Bacteriol. **110:**529-537.
10. **Guerrant, R. L., L. J. Strasbaugh, R. P. Wenzel, B. A. Hamory, and M. A. Sande.** 1977. Nosocomial bloodstream infections caused by gentamicin-resistant gram-negative bacilli. Am. J. Med. **62:**894-901.
11. **Haas, M. J., and J. E. Dowding.** 1975. Aminoglycoside-modifying enzymes. Methods Enzymol. **43:**611-628.
12. **Kawabe, H., S. Kondo, H. Umezawa, and S. Mitsuhashi.** 1975. R factor-mediated aminoglycoside antibiotic resistance in *Pseudomonas aeruginosa*: a new aminoglycoside 6′-*N*-acetyltransferase. Antimicrob. Agents Chemother. **7:**494-499.
13. **Kelly, M. T., and J. M. Matsen.** 1976. In vitro activity, synergism, and testing parameters of amikacin, with comparisons to other aminoglycoside antibiotics. Antimicrob. Agents Chemother. **9:**440-447.
14. **Lin, E. C. C., S. A. Lerner, and S. E. Jorgensen.** 1962. A method for isolating constitutive mutants for carbohydrate-catabolizing enzymes. Biochim. Biophys. Acta **60:**422-424.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265-275.
16. **Meyer, R. D., R. P. Lewis, and S. M. Finegold.** 1977. Amikacin therapy for gram-negative septicemia. Am. J. Med. **62:**930-935.
17. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127:**1529-1537.
18. **Nossal, N. G., and L. A. Heppel.** 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241:**3055-3062.
19. **Ozanne, B., R. Benveniste, D. Tipper, and J. Davies.** 1969. Aminoglycoside antibiotics: inactivation by phosphorylation in *Escherichia coli* carrying R factors. J. Bacteriol. **100:**1144-1146.
20. **Robinson, D. S.** 1964. Oxidation of selected alkanes and related compounds by a *Pseudomonas* strain. Ned. Tijdschr. Hyg. Microbiol. Serol. **30:**303-316.
21. **Robison, L. R., R. Seligsohn, and S. A. Lerner.** 1978. Simplified radioenzymatic assay for chloramphenicol. Antimicrob. Agents Chemother. **13:**25-29.
22. **Steers, E., E. L. Foltz, and B. S. Graves.** 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. (Washington, D.C.) **9:**307-311.