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Isolation, Characterization, and Cloning of a Plasmid-Borne Gene Encoding a Phosphotransferase That Confers High-Level Amikacin Resistance in Enteric Bacilli

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Clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens* at a hospital that had used amikacin as its principal aminoglycoside for the preceding 42 months demonstrated high-level resistance to amikacin (≥256 μg/ml), kanamycin (≥256 μg/ml), gentamicin (≥64 μg/ml), netilmicin (64 μg/ml), and tobramycin (≥16 μg/ml). The resistant strains contained an identical 6.8-kilobase plasmid, pRPG101. Transformation of pRPG101 into *Escherichia coli* produced high-level resistance to amikacin (≥256 μg/ml) and kanamycin (≥256 μg/ml) but unchanged susceptibilities to gentamicin, netilmicin, and tobramycin. The clinical isolates and transformants produced a novel 3′-phosphotransferase, APH(3′), that modified amikacin and kanamycin in vitro. The location and orientation of the *amk* gene encoding this APH(3′) were determined by analysis of insertions in pRPG101 of the defective gene fusion phage Mu dII1681 (mini-Mu*lac*). Cells containing plasmids with insertions into *amk* that had the *lac* operon fused to the *amk* promoter were selected as Lac$^+$ and amikacin susceptible. A collection of these mini-Mu*lac* insertions was mapped by restriction enzyme analysis. This characterization of *amk* facilitated its cloning as a 1.8-kilobase *Eco*RI-*Bgl*I fragment of pRPG101 into the pUC19 vector. *E. coli* strains containing this recombinant plasmid had APH(3′) activity and demonstrated high-level resistance to amikacin and kanamycin (≥256 μg/ml) but were as susceptible to gentamicin, tobramycin, and netilmicin (≤1.0 μg/ml) as the strains harboring the original pRPG101 plasmid.

The development of bacterial resistance to aminoglycosides has compromised the effectiveness of antibacterial therapy with these drugs. In clinical isolates, the principal mechanism of high-level resistance to aminoglycosides is enzymatic modification (4, 7). The enzymes involved are generally plasmid encoded and modify the aminoglycoside before its access to the ribosome.

The semisynthetic aminoglycoside amikacin was developed to circumvent enzymatic mechanisms of resistance (4, 13). Although 3′-phosphotransferase enzymes [APH(3′)-I through -V] have not conferred resistance to amikacin in vivo (4, 16, 20, 21, 23), APH(3′)-II and -III enzymes have been shown to modify amikacin in vitro. This discrepancy has been attributed to the poor affinity of the enzymes for the substrate (8, 17). At the low concentrations of amikacin encountered in vivo, these enzymes modify the drug inefficiently and do not confer amikacin resistance (17). APH(3′)-II can confer high-level amikacin resistance only if the rate of aminoglycoside uptake in a cell is simultaneously decreased (17). If strains with a new phosphotransferase that modified amikacin efficiently appeared, proliferated, and disseminated, the utility of amikacin, which has served as the ultimate backup aminoglycoside for use against multiresistant gram-negative bacilli, might be seriously compromised.

Since January 1982, the San Juan Veterans Administration Medical Center has employed amikacin as its primary aminoglycoside (>85% of aminoglycoside usage) (19). From a prevalence in 1981 of 0.2%, amikacin resistance among aerobic gram-negative bacilli rose to 3.6% after 4 years of predominant amikacin usage. Although most hospitals have not experienced an increase in amikacin resistance with primary amikacin use, several other hospitals have reported an increase in amikacin resistance related to amikacin use (11, 14).

To investigate the mechanism(s) of amikacin resistance, we obtained a *Klebsiella pneumoniae* strain and a *Serratia marcescens* strain isolated from patients in the San Juan Veterans Administration Hospital in 1984. We believe that these isolates have a novel phosphotransferase which is encoded on a 6.8-kilobase (kb) plasmid (pRPG101) that modifies and confers resistance only to amikacin and its parent compound, kanamycin, among the clinically available aminoglycosides. Knowledge of the location of the phosphotransferase gene on pRPG101 would be helpful in establishing a successful strategy for cloning. Cloning would help determine whether the expression of this phosphotransferase alone is sufficient to confer high-level resistance to amikacin. In this report, we describe the isolation, cloning, and characterization of this amikacin resistance gene that includes the use of a defective gene fusion bacteriophage Mu dII1681 (mini-Mu*lac*).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and their plasmids and phage are listed in Table 1. *Escherichia coli* JM83 and MC1065 are susceptible to all currently available 2-deoxystreptamine aminoglycosides. JM83 has a deletion in the *lac* operon, and it contains phage 80d, which has the *lacI* gene but not the *lacZ* gene (24). The plasmid vector pUC19

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TABLE 1. Bacterial strains

Strain (plasmid)	Enzyme present[a]	Reference or source
E. coli JM83	None	24
E. coli MC1065	None	5
E. coli JM83(pRPG101)	APH(3′)	This study
E. coli MC1065(pRPG101)	APH(3′)	This study
E. coli PRC930(pAO43::Tn*903*)	APH(3′)-I	18
E. coli 83-75	ANT(2″)	18
E. coli 83-125	AAC(6′)-I	18
E. coli POII1681	APH(3′)-II	6
E. coli M8820Mu	None	(See text)
K. pneumoniae	APH(3′), AAC(6′), ANT(2″)	This study
S. marcescens	APH(3′), AAC(6′)	This study

[a] AAC(6′), Aminoglycoside 6′-acetyltransferase; ANT(2″), aminoglycoside 2″-nucleotidyl transferase.

includes the alpha-complementing region of the *lacZ* gene, which contains a synthetic polylinker, and the *amp* gene. The addition of the pUC19 plasmid vector to JM83 results in lactose-fermenting colonies. An insertion into the polylinker of pUC19 alters the *lacZ* gene and results in loss of lactose fermentation (24).

E. coli POII1681 contains the defective *lac* gene fusion bacteriophage Mu dII1681 (mini-Mu*lac*) and a helper Mu, both on the chromosome (6). *E. coli* M8820Mu is a lysogenic strain that has a deletion of the entire *lac* operon (6). Bacteriophage Mu is a temperate phage that undergoes transposition hundreds of times when it replicates. The defective gene fusion bacteriophage Mu dII1681 lacks the promoter for the *lac* operon and the DNA sequence corresponding to the translation initiation signals and the first eight amino acid codons of *lacZ* (6). When mini-Mu*lac* transposes and fuses to the gene of interest in the appropriate transcriptional orientation and reading frame, the Lac^+ phenotype is expressed (5, 6). The location of genes on a plasmid can be determined rapidly by mapping the location of the mini-Mu*lac* insertions. By analysis of insertions which fuse mini-Mu*lac* to the gene of interest, the transcriptional orientation of a gene on a replicating plasmid can also be determined (6).

Phenotypic characterization of isolates. Testing of the MICs of aminoglycosides (kanamycin, amikacin, tobramycin, netilmicin, and gentamicin) were carried out by either broth microdilution with cation-supplemented Mueller-Hinton (MH) broth or agar dilution with MH agar (BBL Microbiology Systems) (15). Aminoglycoside-modifying enzyme (AME) assays were performed by the phosophocellulose paper assay (9, 18) with the following aminoglycoside substrates at final concentrations of 167 μM: kanamycin A, amikacin, dibekacin, and lividomycin A (Bristol Laboratories, Syracuse, N.Y.); netilmicin, sisomicin, Sch 21561, Sch 21562, and gentamicins C_1, C_{1a}, C_2, and B (Schering Corp., Bloomfield, N.J.); tobramycin (Eli Lilly & Co., Indianapolis, Ind.); butirosin and paromomycin (Parke, Davis & Co., Ann Arbor, Mich.); and ribostamycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan). Isolates *E. coli* PRC93 (pAO43::Tn*903*), 83-75, and 83-125 containing known AMEs were used as controls (Table 1). Cell-free extracts of the isolates were prepared by a series of three 30-s sonic disruptions in phosphate buffer (17, 18). All radioactive cofactors used in the AME assay were acquired from Amersham Corp.

DNA biochemistry. Plasmid preparations from the *K. pneumoniae* and *S. marcescens* clinical isolates were obtained with techniques of Birnboim and Doly (3). Other plasmid preparations were purified by ultracentrifugation and cesium chloride gradients with ethidium bromide as described previously (12). Separation of all plasmids was by electrophoresis in agarose or polyacrylamide gels (12). Transformation of laboratory strains in *E. coli* was performed with $CaCl_2$ as described previously (12). Restriction enzymes, T4 DNA ligase, and the Klenow fragment were acquired from Boehringer Mannheim Biochemicals or New England BioLabs, Inc. Restriction enzyme analysis was performed with a variety of enzymes and their appropriate buffers according to the suppliers' specifications. Bacteriophage lambda (New England BioLabs) digested with *Hin*dIII or the plasmid pBR322 digested with *Hin*fI was used for size standards in restriction enzyme analyses.

Bacterial genetic techniques. pRPG101 was transformed into $CaCl_2$-treated *E. coli* POII1681 as previously described (12). Selection for pRPG101 occurred on lactose-MacConkey agar containing 25 μg of amikacin per ml. POII1681 contains not only the mini-Mu*lac* genome but also a kanamycin resistance gene encoding APH(3′)-II, a phosphotransferase that mediates resistance to kanamycin but not to amikacin (17). POII1681 did not give rise to amikacin-resistant colonies in the absence of pRPG101 (frequency, $<2.28 \times 10^{-8}$).

A phage lysate of strain POII1681(pRPG101) was prepared as previously described (6). The resulting lysate containing mini-Mu*lac* with cointegrates of pRPG101 was then used to transduce a Mu lysogen, M8820Mu. The Mu lysogen was used as a recipient to maintain the defective phage as a prophage. M8820 Mu is $recA^+$ and capable of homologous recombination, so a packaged cointegrate can form a plasmid with an insertion (6). Transductants were selected on lactose-MacConkey agar containing 25 μg of kanamycin per ml. Kanamycin-resistant transductants were expected to include those with plasmid pRPG101 containing Mu dII1681 mini-Mu*lac*. All transductants were scored for amikacin susceptibility and lactose fermentation by replica plating onto lactose-MacConkey agar containing 25 μg of amikacin per ml. Cells scored as kanamycin resistant but amikacin susceptible were examined for plasmids containing mini-Mu*lac* insertions into *amk*. The cells containing plasmids that had the *lac* operon fused to the *amk* promotor were Lac^+ and amikacin susceptible. The resulting plasmids containing mini-Mu*lac* insertions into pRPG101 were purified by ultracentrifugation in cesium chloride gradients with ethidium bromide as described previously (12). The purified plasmids underwent restriction enzyme analysis as described above.

To clone *amk*, we digested pRPG101 with appropriate enzymes and buffers. When needed, blunt ends were formed with nucleotides and the Klenow fragment of DNA polymerase I or T4 polymerase (12). Fragments of pRPG101 were ligated with T4 ligase to pUC19 that had been digested with appropriate enzymes (12). The resulting constructs were used to transform JM83. Transformants containing *amp* of pUC19 were selected on lactose-MacConkey agar with 25 μg of ampicillin per ml and replica plated onto lactose-MacConkey agar with 25 μg of amikacin per ml. The replicants were scored for lactose utilization and amikacin resistance.

RESULTS

Amikacin-resistant enteric strains. The *K. pneumoniae* and *S. marcescens* clinical isolates both demonstrated high-level resistance to kanamycin, amikacin, and gentamicin; tobra-

TABLE 2. Characteristics of aminoglycoside-resistant clinical isolates and their transformants

Strain	MIC (μg/ml)					AME activity[a]
	Kanamycin A	Amikacin	Gentamicin	Tobramycin	Netilmicin	
K. pneumoniae	≥256	≥256	≥256	≥256	64	AAC(6′) ANT(2″) APH(3′)
S. marcescens	≥256	≥256	64	16	64	AAC(6′) APH(3′)
E. coli						
MC1065(pRPG101)	≥256	≥256	≤0.25	≤0.25	≤0.25	APH(3′)
MC1065(pRPG107)	≥256	≥256	≤0.25	≤0.25	≤0.25	APH(3′)
MC1065	≤0.25	≤0.5	≤0.25	≤0.25	≤0.25	ND[b]

[a] See footnote *a* of Table 1.
[b] ND, None detected.

mycin resistance was high level in *K. pneumonia* but modest in *S. marcescens* (Table 2). Various AME activities were also detected in crude extracts of the isolates, including APH(3′) in each. *K. pneumoniae* contained plasmids of 2.2, 6.8, and 50 kb; in *S. marcescens* there were plasmids of 2.1, 6.8, and 60 kb. Only the 6.8-kb plasmid appeared common to both isolates.

Amikacin-resistant transformants. After transformation of *E. coli* MC1065 with plasmid preparations from the *K. pneumoniae* and *S. marcescens* clinical isolates, transformants were selected on MH agar containing kanamycin at 25 μg/ml. Amikacin resistance was confirmed in all of the 105 transformants by growth on MH agar with 25 μg of amikacin per ml. Transformants MC1065(pRPG101) from *K. pneumoniae* and MC1065(pRPG107) from *S. marcescens* were tested for antibiotic susceptibility and the presence of AMEs (Tables 2 and 3). Both transformants had acquired APH activity and high-level resistance to amikacin and kanamycin but were susceptible to gentamicin, tobramycin, and netilmicin. The site of phosphorylation appears to be the 3′-hydroxyl group, since kanamycin A was a substrate but tobramycin (3′-deoxykanamycin) was not. Other substrates for this APH(3′) were amikacin, gentamicin B, butirosin, paromomycin, and ribostamycin. Unfortunately, susceptibility could be tested only for amikacin, since only limited quantities of the other substrates were available. Of the AME activities present in the clinical isolates, only APH(3′) was detected in the transformants. Although we did not determine initial short-term rates of activity, the results reflected remarkably consistent patterns of activity in each strain against the various substrates. In all strains the activity against amikacin was substantially higher than against kanamycin. MC1065(pRPG101) and MC1065(pRPG107) each contained a single 6.8-kb plasmid. To determine whether pRPG101 and pRPG107 were identical plasmids, we incubated each of these plasmids with *Hin*fI and *Sau*3a restriction enzymes separately with the appropriate buffers and analyzed the resulting patterns of DNA fragments on polyacrylamide gels (12). The patterns for the two plasmids cut with each enzyme were identical (Fig. 1). By this analysis,

TABLE 3. Assays of aminoglycoside phosphotransferase activity in crude sonic extracts of amikacin-resistant clinical isolates and their amikacin-resistant *E. coli* transformants

Substrate	Aminoglycoside phosphotransferase activity[a]			
	K. pneumoniae	*E. coli* MC1065 (pRPG101)	*S. marcescens*	*E. coli* MC1065 (pRPG107)
None	8	19	3	20
Kanamycin A	100	100	100	100
Amikacin	199	173	127	149
Tobramycin	9	27	4	20
Gentamicin B	91	90	96	88
Gentamicin C_1	7	21	3	18
Gentamicin C_{1a}	7	24	5	19
Gentamicin C_2	7	21	4	19
Sisomicin	7	24	5	21
Netilmicin	6	24	6	20
Sch 21561	6	27	7	24
Sch 21562	6	27	6	20
Butirosin	200	210	126	188
Ribostamycin	203	195	297	163
Paromomycin	228	261	217	249
Lividomycin A	18	22	11	22
Dibekacin	9	24	5	21

[a] Phosphorylation after 20 min at 35°C, normalized to 100 as the activity against kanamycin for each strain.

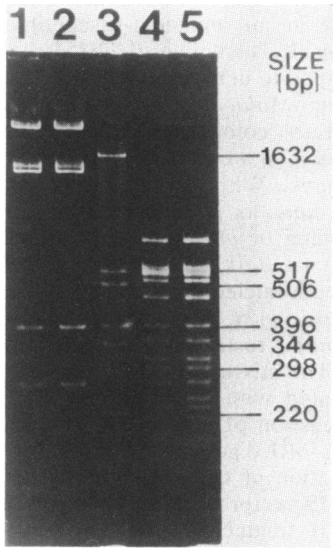

FIG. 1. Polyacrylamide (7%) gel of pRPG101 and pRPG107. Lanes: 1, pRPG101 digested with *Hin*fI; 2, pRPG107 digested with *Hin*fI; 3, pBR322 digested with *Hin*fI; 4, pRPG101 digested with *Sau*3a; 5, pRPG107 digested with *Sau*3a.

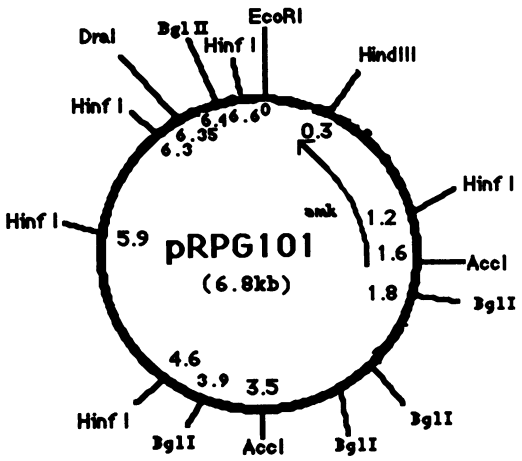

FIG. 2. Restriction map of pRPG101.

pRPG101 and pRPG107 appeared identical, although minor differences in base sequence may exist.

Restriction analysis of pRPG101. A restriction map of pRPG101 is shown in Fig. 2. Unique sites were found for *Bgl*II, *Dra*I, *Eco*RI, and *Hin*dIII. There were four sites for *Bgl*I, but the precise location of the two sites between 1.8 and 3.5 kb on pRPG101 could not be determined precisely. No sites were found for *Aat*II, *Ava*I, *Bam*HI, *Cla*I, *Hpa*I, *Kpn*I, *Nar*I, *Nde*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Sph*I, *Xba*I, *Xma*I, and *Xho*I.

Gene localization of *amk*. A bank of pRPG101 plasmids with Mu dII1681 (mini-Mu*lac*) insertions was isolated as described in Materials and Methods. Of 810 transductants, 8% were Lac$^+$; this is consistent with previously reported frequencies for this phage (6). Lac$^+$ transductants arose by the fusion of plasmid-borne transcriptional-translational signals of pRPG101 to the *lac* operon DNA of Mu dII1681. Kanamycin-resistant, lactose-fermenting, amikacin-susceptible transductants were expected to have the right end of bacteriophage Mu dII1681 near the promoter of the *amk* gene. All transductants which were examined contained pRPG101 with mini-Mu*lac* insertions. A map of insertions which resulted in an amikacin-susceptible phenotype is presented in Fig. 3. The unique *Eco*RI sites in pRPG101 and mini-Mu*lac* helped to determine the relative positions of the insertions of mini-Mu*lac* and the location of *amk*. Thus, the expression of Lac$^+$ colonies determined the transcriptional orientation of *amk* in a counterclockwise fashion (Fig. 2).

Cloning of *amk*. With knowledge of the location and orientation of *amk* in pRPG101 (Fig. 2), we attempted directional cloning of *amk* by treating pRPG101 with the restriction enzyme *Bgl*I; protruding ends of the fragments were filled in with nucleotides and the Klenow fragment of DNA polymerase I (12). The 4.7-kb fragment containing the *Eco*RI and *Hin*dIII restriction sites presumably contained the *amk* gene. The mixture of fragments was restricted with *Eco*RI and ligated with pUC19 digested with *Eco*RI and *Sma*I; after digestion pUC19 had one end with a cohesive end from the *Eco*RI digest and a blunt end from the *Sma*I digest. The ligation of these pRPG101 fragments with the restricted pUC19 vector thus allowed for directional cloning of the pRPG101 fragment expected to contain *amk*. The resulting constructs were used to transform JM83. The transformants were selected on lactose-MacConkey agar containing 25 μg of ampicillin per ml and replica plated onto agar containing 25 μg of amikacin per ml. All of the colonies

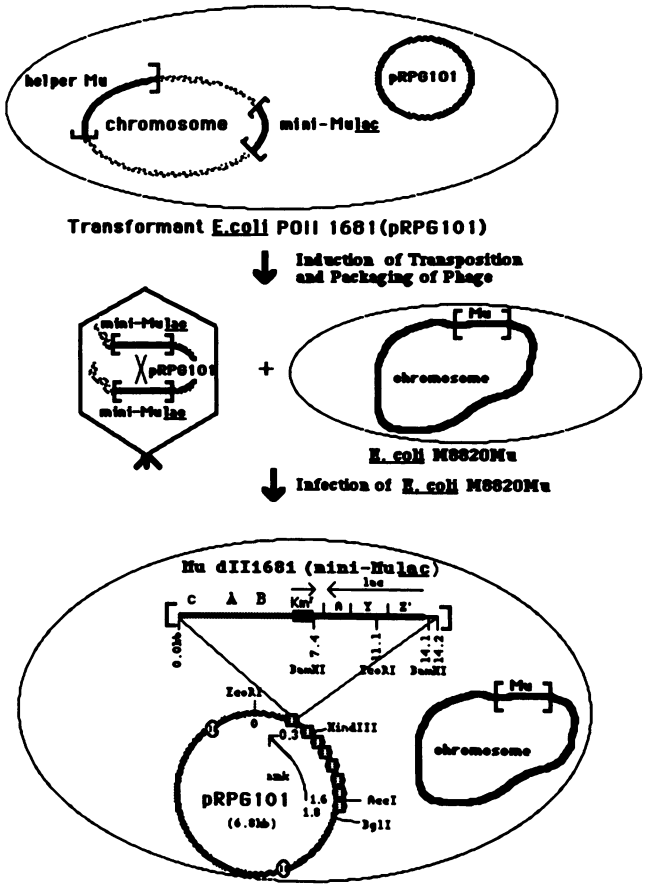

FIG. 3. Scheme for selecting insertions of bacteriophage Mu dII1681 (mini-Mu*lac*) into pRPG101. The locations of mini-Mu*lac* insertions into the amikacin resistance determinant, *amk*, are shown (*) in the orientation indicated. Cells containing such constructs were Lac$^+$, kanamycin resistant, and amikacin susceptible. The locations of mini-Mu*lac* insertions in two isolates that were Lac$^+$, kanamycin resistant, and amikacin resistant were also evaluated and are indicated ( ).

tested were Lac$^-$, ampicillin resistant, and amikacin resistant. Plasmid DNA extracted from these strains, restricted with *Eco*RI and *Pst*I, and analyzed on agarose gel electrophoresis revealed a plasmid, pRPG205, with a 1.8-kb fragment containing *amk* and a 2.7-kb vector fragment. A restriction map of pRPG205 is shown in Fig. 4. The MICs of both amikacin and kanamicin for *E. coli* JM83 containing pRPG205 were ≥256 μg/ml, and the MICs of netilmicin, gentamicin, and tobramycin were <1 μg/ml. With this information and data from the insertion of mini-Mu*lac* in pRPG101 in Fig. 3, we determined that the upstream boundary of the structural gene and possibly the promoter of *amk* is located in one 200-base-pair *Bgl*I-*Acc*I fragment and that the downstream boundary of *amk* lies in the 360-base-pair *Eco*RI-*Hin*dIII fragment of pRPG101. Whether the 1.8-kb fragment in pRPG205 contains the promoter for *amk* is unknown, since the *lacZ* promoter of pUC19 may permit the expression of *amk*.

DISCUSSION

Our *K. pneumoniae* and *S. marcescens* strains were isolated in a hospital that had been using amikacin as its primary aminoglycoside for 42 months (19). Both isolates

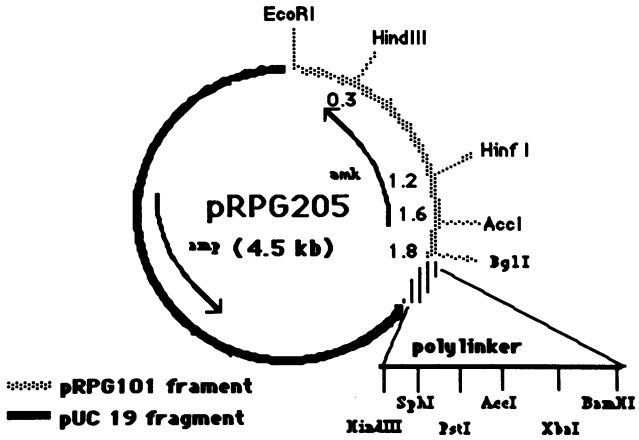

FIG. 4. Restriction map of recombinant plasmid pRPG205.

contain a 6.8-kb plasmid, pRPG101, that confers high-level amikacin resistance. The transformation of *E. coli* with pRPG101 produced a strain that was resistant to amikacin (and its parent compound, kanamycin) but not to tobramycin, gentamicin, or netilmicin.

These enteric isolates contain a novel 3′-phosphotransferase that modifies amikacin in vitro and confers amikacin resistance. The APH(3′) enzymes described in other pathogenic gram-negative bacteria differ in their substrate profiles, and their corresponding genes are not closely related (22). APH(3′)-I enzymes modify lividomycin A but not butirosin or amikacin in vitro. APH(3′)-II enzymes modify butirosin but not lividomycin A. Amikacin is generally not considered a substrate for APH(3′)-II; however, in vitro activity has been described, although the enzyme still does not confer resistance to amikacin (17). APH(3′)-III modifies butirosin, lividomycin A, and amikacin in vitro. The APH(3′) in this study modified butirosin and amikacin but not lividomycin A. This substrate profile suggests that the APH(3′) in this study is either entirely new or evolved from one of the other forms. A recent paper reported an APH(3′) in *Acinetobacter baumannii* that conferred amikacin resistance and had a substrate profile similar to that of the APH(3′) presented here (10). Insufficient data are available to determine whether the APH(3′) from *A. baumannii* is identical to the APH(3′) in this study, although the isolation of the strains appears to be independent. Finally, preliminary evidence suggests that our APH(3′) enzyme has a low K_m for amikacin; this may account for its ability to confer resistance (17; C. Torres, D. Lerner, M. Perlin, R. Gaynes, and S. A. Lerner, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 945, 1986). These results suggest that resistance in gram-negative bacilli to amikacin (and its parent compound, kanamycin) can occur without resistance to other clinically available aminoglycosides, in contrast to previous claims (18).

We have expanded the use of mini-Mu*lac* gene fusion technology for the rapid characterization of the *amk* gene (2, 5, 6). The techniques not only helped localize the gene on its plasmid but also determined the transcriptional orientation. This knowledge allowed us to clone *amk* directionally into the polylinker of pUC19. The insertional mutagenesis with mini-Mu*lac* into *amk* selectively altered the amikacin resistance of the cell containing the construct. This suggested that the gene product of *amk* was responsible for amikacin resistance. The generation of a bank of mini-Mu*lac* insertions in pRPG101 could also be used to detect the origin of replication of the plasmid, regions involved in mobilization of the plasmid, or the location of other genes on the plasmid.

The cloning of *amk* into pUC19 facilitated the expression of this novel APH(3′) in *E. coli* and the clone will be a convenient vehicle for in vitro manipulation of this gene, including DNA sequencing (1). After *amk* was cloned, the expression of this APH(3′) in *E. coli* JM83 produced resistance to amikacin and kanamycin but not to gentamicin, tobramycin, or netilmicin. Insertional mutagenesis with mini-Mu*lac* selectively altered the amikacin resistance. These observations indicate that the APH(3′) enzyme alone confers resistance to amikacin. The possible homology of *amk* and APH(3′) to other phosphotransferase genes and enzymes is currently under investigation. We are also determining the DNA sequence of *amk* to compare the sequence (and its inferred amino acid sequence) to genes encoding other aminoglycoside 3′-phosphotransferases.

The presence of pRPG101 in two genera of aerobic gram-negative bacilli suggests that dissemination of the plasmid within the hospital has already occurred. We plan to study the transfer properties of the plasmid. In conjugation experiments, we hope to determine whether pRPG101 is conjugative or mobilizable from our *K. pneumoniae* or *S. marcescens* strain. If so, we plan to use different insertions of mini-Mu*lac* to determine the location on pRPG101 of the regions involved in plasmid mobilization.

In summary, we have reported amikacin resistance mediated by a novel phosphotransferase in enteric gram-negative bacilli isolated from a hospital in which amikacin is used as the principal aminoglycoside. Expression of this APH(3′) in *E. coli* produced a high-level amikacin resistance not previously described among enteric gram-negative bacilli. We have also expanded the use of gene fusion technology with mini-Mu*lac* to characterize *amk*, the gene for APH(3′) enzyme, and to define its location and transcriptional orientation on pRPG101. We used this information to clone *amk* in vitro.

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