R-FACTOR MEDIATED GENTAMICIN RESISTANCE: A NEW ENZYME WHICH MODIFIES AMINOGLYCOSIDE ANTIBIOTICS

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1. Introduction

Several of the aminoglycoside antibiotics can be inactivated by enzymes which occur in gram-negative bacteria carrying R-factors. These enzymes are listed in table 1. Until recently, R-factor-mediated resistance and concomitant inactivation of the gentamicin C complex had not been reported. A gentamicin-resistant strain of *Klebsiella pneumoniae* [10] has now been shown to contain an R-factor which mediates resistance to the antibiotics of the gentamicin C complex. The gentamicins and several related amino-glycoside antibiotics are apparently inactivated by enzymatic adenylylation.

2. Materials and methods

2.1. *Strains*

K. pneumoniae strain type 22 #3038 was isolated by Dr. C.M.Martin and provided by Dr. J.A.Waitz.

Table 1
Aminoglycoside and aminocyclitol inactivating enzymes.

Antibiotic	Inactivating mechanism	Reference
streptomycin, spectinomycin	adenylylation.	1, 2, 3, 4
streptomycin	phosphorylation	5
neomycin, kanamycin, gentamicin C_{1a}	acetylation	6, 7, 8
neomycin, kanamycin	phosphorylation	5, 9

Escherichia coli W2985N is K12, F^-, *thi*$^-$, *arg*$_G^-$, *nal*^{r}; *E. coli* W677 is K12, F^-, *thi*$^-$, *thr*$^-$, *leu*$^-$, *lac*$^-$, *mal*$^-$, *gal*$^-$, *xyl*$^-$, *ara*$^-$, *mtl*$^-$, *tonA*$^-$.

2.2. *R-factor transfer*

Transfer of R-factors was carried out by mixing 0.5 ml of the R-factor strain (5×10^8 cells/ml) and 0.5 ml of the recipient strain (5×10^8 cells/ml) in 4 ml of tryptone broth. The mixed culture was allowed to stand at 37° for 1 hr and then incubated, with shaking, at 37° for 10 hr. The culture was then streaked on a selective plate. For the *K. pneumoniae* × *E. coli* W2985N cross, the selective medium was minimal glucose medium containing arginine (20 μg/ml), gentamicin (10 μg/ml) and nalidixic acid (30 μg/ml). In crosses where *E. coli* W677 was the recipient, the selective plates contained threonine (20 μg/ml), leucine (20 μg/ml), and gentamicin (10 μg/ml). The R-factor thus transferred was called JR66.

2.3. *Preparation of enzyme extracts*

E. coli JR66/W677 was grown in 40 ml of ML broth, harvested in late logarithmic phase of growth, and osmotically shocked using a modification of the procedure described by Nossal and Heppel [11]. The cells were washed twice at room temperature with 10 ml 0.01 M tris and 0.03 M NaCl, pH 7.3. The pellet was suspended in 10 ml 20% sucrose, 3×10^{-3} M EDTA, and 0.033 M tris (pH 7.3), stirred for 10 min at room temperature, and centrifuged at 16,000 *g* for 5 min. The pellet was then suspended in 2 ml of cold 5×10^{-4} M $MgCl_2$ and stirred for 10 min at 2°.

The supernatant fluid of a 10 min, 26,000 *g* centrifugation is referred to as the osmotic shockate.

2.4. *Inactivation of antibiotics*

A small scale inactivation of gentamicin C_1 was performed in a 50 μl reaction mixture containing 22 nmoles of gentamicin C_1, 120 nmoles of ATP, 2.5 μmoles tris pH 8.1 (at 30°), 0.4 μmole $MgCl_2$, 50 nmoles dithiothreitol, and 25 μl of an osmotic shockate of JR66/W677 (containing 1 mg/ml protein). This mixture was incubated for 3 hr at 30°. To monitor the extent of inactivation of gentamicin C_1, 10 μl of the reaction mixture were spotted on a filter paper disc which was then placed on a lawn of *E. coli* W677. In the absence of the osmotic shockate or of ATP a growth inhibition zone extending 5 mm from the edge of the disc was observed.

2.5. *Assay for adenylylating activity*

The adenylation of gentamicin was detected by a phosphocellulose paper binding assay that had previously been used to monitor the phosphorylation [5], adenylylation [3], or acetylation [8] of the aminoglycoside antibiotics. The reaction mixture contained osmotic shockate, 20 nmoles of ^{14}C-ATP or α-^{32}P-ATP (specific activity, 4 μCi/μmole), 10 nmoles of aminoglycoside antibiotic, 2.5 μmoles of tris (pH 8.1 at 30°), 0.4 μmole of $MgCl_2$, and 50 nmoles of dithiothreitol in a total volume of 55 μl. Incubation was at 30° for various periods of time, after which 10 μl was pipetted onto a 0.75 cm square of phosphocellulose paper (Whatman P-81). The squares were allowed to stand for 15 sec to absorb the basic antibiotic, and were then immersed in hot, distilled water (70 to 80°) for 2 min to stop the reaction and remove any radioactivity that is not bound to the antibiotic. They were then washed several times with large volumes of distilled water, dried, and counted in a Packard Tri-Carb scintillation spectrometer. Control reactions for non-specific binding of ^{14}C-ATP to the paper were run in the absence of either enzyme or antibiotic.

3. Results

The presence of an R-factor which mediates gentamicin resistance in a strain of *K. pneumoniae* was shown by transfer of this resistance to an *E. coli* recipient (W2985N) and subsequent transfer to *E. coli* W677. Simultaneous transfer of resistance to neomycin, kanamycin, streptomycin, chloramphenicol, tetracycline, ampicillin and sulfonamide occurred. The resistance patterns of these strains is shown in table 2.

Since the known aminoglycoside inactivating enzymes are released from R-factor strains by cold water shocking of sucrose-tris-EDTA-treated cells [2, 3], an 'osmotic shockate' of the gentamicin-resistant strain JR66/W677 was prepared and incubated with gentamicin C_1 as outlined in Materials and methods. The antibiotic was inactivated only when magnesium and ATP were present in the incubation mixture.

Table 2
Resistance characters of strains.

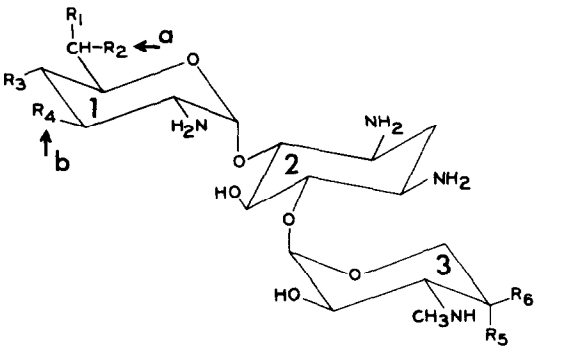
Strain	Resistance characters[a]									
	genC_1	*kan*	*neo*	*str*	*spc*	*cam*	*tet*	*sul*	*amp*	*nal*
Klebsiella pneumoniae 3038[b]	+	+	+	−	+	+	+	+	+	+
Escherichia coli W677	−	−	−	−	−	−	−	−	−	−
Escherichia coli W2985N	−	−	−	−	−	−	−	−	−	+
JR66/W2985N[c]	+	+	+	+	−	+	+	+	+	+
JR66/W677[d]	+	+	+	+	−	+	+	+	+	−

[a] *gen*C_1, gentamicin C_1 (10 μg disc); *kan*, kanamycin A (20 μg disc); *neo*, neomycin B (20 μg disc); *str*, streptomycin (20 μg disc); *spc*, spectinomycin (50 μg disc); *cam*, chloramphenicol (10 μg disc); *tet*, tetracycline (10 μg disc); *sul*, gantrisin (2 mg disc); *amp*, ampicillin (30 μg disc); *nal*, nalidixic acid (20 μg disc).
[b] This strain carries R-factor JR66.
[c] W2985N containing R-factor JR66.
[d] W677 containing R-factor JR66.

Table 3
Enzymatic adenylylation of aminoglycosides.

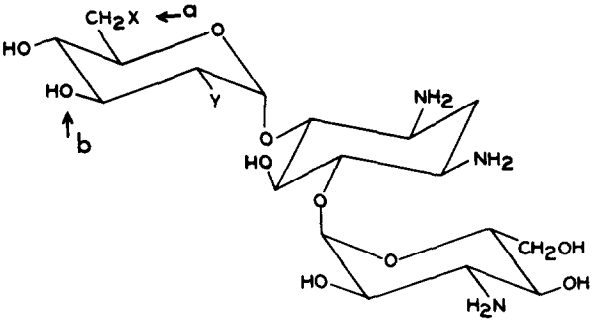
Antibiotic	cpm incorporated in assay[a]	
	10 min	20 min
None	40	60
Gentamicin C_1	7350	14000
Gentamicin C_{1a}	3000	4500
Gentamicin C_2	2750	5000
Gentamicin A	9650	18000
Kanamycin A	5900	11700
Neomycin B	64	72
Neamine	60	65
Streptomycin	53	82
Spectinomycin	32	40

[a] Total amount of radioactivity in a 55 μl reaction, present as ^{14}C-AMP bound to the antibiotic. For details of incubation conditions, see Materials and methods. 1000 cpm corresponds to 0.18 nmole of antibiotic-AMP produced in this reaction.



		R_1	R_2	R_3	R_4	R_5	R_6
GENTAMICINS	A	H	OH	OH	OH	H	OH
	C_{1a}	H	NH_2	H	H	OH	CH_3
	C_2	CH_3	NH_2	H	H	OH	CH_3
	C_1	CH_3	$NHCH_3$	H	H	OH	CH_3

Fig. 1. The structures of gentamicin A and of the components of the gentamicin C complex. *a* indicates the amino group in gentamicin C_{1a} and C_2 that has been shown to be enzymatically acetylated [8], and *b* the hydroxyl group in gentamicin A that is phosphorylated [5]. In the gentamicin C complex, ring 1 is purpurosamine, 2 is 2-deoxystreptamine, and 3 is garosamine.



		X	Y
KANAMYCINS	A	NH_2	OH
	B	NH_2	NH_2
	C	OH	NH_2

Fig. 2. The structures of the kanamycins. *a* indicates the amino group in kanamycins A and B that can be enzymatically acetylated [7, 8], and *b* the hydroxyl group that is enzymatically phosphorylated [9].

O-Phosphorylation [10] or *N*-acetylation (unpublished results) of gentamicin do not occur in extracts of the resistant strain of *K. pneumoniae* 3038 or in *E. coli* JR66/W677. Since ATP was an absolute requirement for the inactivation, we reasoned that the modification of gentamicin occurred by adenylylation, as has previously been shown for streptomycin [3].

The enzymatic formation of an adenylylated derivative of gentamicin was strongly supported by the fact that the transfer of radioactivity from ^{14}C-ATP or α-^{32}P-ATP to cation exchange paper occurred only in the presence of gentamicin and the 'osmotic shockate' from strain JR66/W677. Several other aminoglycoside antibiotics were also substrates for the enzyme, as is shown in table 3. It can be seen that the adenylylating enzyme which modifies gentamicin, kanamycin, and related antibiotics is different from the enzyme which has been shown to adenylylate streptomycin, since neither streptomycin nor spectinomycin are substrates. Conversely, gentamicin is not a substrate for the enzyme which adenylylates streptomycin and spectinomycin.

4. Discussion

We have demonstrated that a new R-factor, originally present in strains of *K. pneumoniae*, and subsequently transferred to *E. coli*, is capable of mediating the enzym-

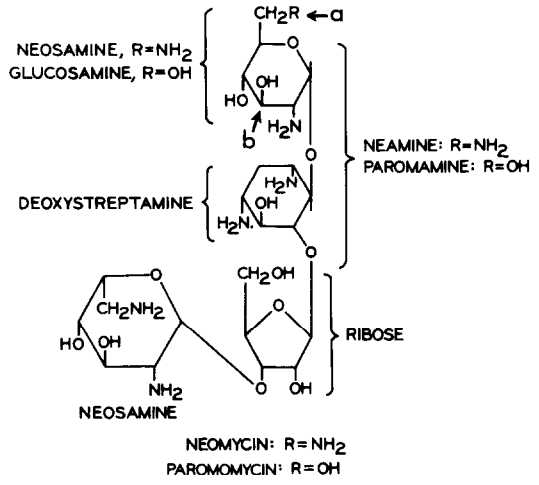


Fig. 3. The structure of neomycin. *a* indicates the amino group in the neamine portion of the antibiotic which can be enzymatically acetylated [8], and *b* the hydroxyl group which is enzymatically phosphorylated [9].

atic adenylylation of gentamycin. The same enzyme probably inactivates kanamycin (fig. 2) and gentamicin A since these drugs are also adenylylated by an osmotic shockate of JR66/W677. Although the site of inactivation is not known, the absence of hydroxyl groups in the purpurosamine ring of the gentamicin C components would indicate that the inactivating modification occurs on the deoxystreptamine or garosamine rings (see fig. 1). The fact that both neomycin B and neamine (see fig. 3) are not substrates for this adenylylating enzyme further suggests that the modification might occur on the garosamine ring of gentamicin C. Alternatively, it is possible that the enzyme does modify the deoxystreptamine ring of the gentamicin antibiotics but fails to recognize neomycin and neamine as substrates. The exact location of the adenylylation is currently being determined.

Strain JR66/W677 also contains the neomycin-kanamycin phosphorylating enzyme, and the streptomycin-phosphorylating enzyme. This strain is therefore resistant to all the aminoglycoside antibiotics in current clinical use. The new adenylylating enzyme modifies the aminoglycoside antibiotics of the kanamycin-gentamicin group; thus there are three separate mechanisms for the inactivation of the kanamycins: *N*-acetylation [6–8] *O*-phosphorylation [9], and now, probably, *O*-adenylylation.

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