GENTAMICIN RESISTANCE IN CLINICAL-ISOLATES OF *PSEUDOMONAS AERUGINOSA* ASSOCIATED WITH DIMINISHED GENTAMICIN ACCUMULATION AND NO DETECTABLE ENZYMATIC MODIFICATION[1]

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Three strains of *Pseudomonas aeruginosa* resistant to gentamicin obtained as representative gentamicin-resistant clinical isolates from the University of Alberta Hospital (UAH) in Edmonton, Canada were characterized to determine their mechanism of resistance. All strains showed wide aminoglycoside resistance (tobramycin, sisomicin, amikacin, streptomycin, kanamycin, SCH 20569) but contained no evidence of gentamicin-acetylating, adenylylating or phosphorylating activity. Gentamicin inhibited amino-acid incorporation in cell-free systems equally well with either ribosomes or soluble cell fractions obtained from either resistant or sensitive strains. Plasmid DNA was detected in two strains but resistance could not be transferred by conjugation to either *P. aeruginosa* or *Escherichia coli* recipients. The resistant strains showed a marked reduction in energy-dependent accumulation of gentamicin compared to a sensitive strain. These strains which are common at UAH are most likely resistant due to a failure of gentamicin to be transported across the cytoplasmic membrane to ribosomal sites until relatively high external gentamicin concentrations.

Resistance of certain strains of *Pseudomonas aeruginosa* to gentamicin has been shown to be due to enzymatic adenylylation or acetylation of gentamicin (for review see (1)). Most of the strains possessing these enzymes that we have examined were able to transfer increased gentamicin resistance and the enzymatic activity by conjugation to recipient strains of *P. aeruginosa*. The nature of these strains and their R-factors have been previously detailed.[5] In carrying out surveillance of clinical-isolates of *P. aeruginosa* we have found at the University of Alberta Hospital in Edmonton, Canada a large number of strains resistant to gentamicin which do not contain detectable enzymatic gentamicin modification. We present in this study a characterization of these strains and an examination of their significance in mediating clinically important gentamicin resistance.

Material and Methods

Bacterial strains. The source and characteristics of strains are given in Table 1. Strains 8803, 1136 and 13934 were selected as representative of strains showing different levels of gentamicin resistance in the clinical laboratories of the University of Alberta Hospital (UAH), Edmonton, Canada. Strains were characterized as *P. aeruginosa* by previously described methods.[7]

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Table 1. Table of bacterial strains.

Strain	Source	Characteristics
P. aeruginosa 280	Our laboratory	Aminoglycoside sensitive strain. Used as rifampicin resistant, methionine auxotroph recipient in conjugation
P. aeruginosa 3503, 211	Our laboratory	Used as rifampicin-resistant recipients in conjugation
P. aeruginosa 8803, 1136, 13934	Clinical Laboratory UAH	Gentamicin resistant clinical isolates
E. coli K12 SA 1306	K.E. SANDERSON, Univ. of Calgary, Calgary	pro^-, met^-, Nal^r. Used as rifampicin resistant recipient in conjugation
P. aeruginosa PS130	J. DAVIES, Univ. of Wisconsin, U.S.	Acetylation of gentamicin, contains R-factor R130
P. aeruginosa POW 151	S.A. KABINS, Michael Reese, Medical Center, Chicago, U.S.	Adenylylation of gentamicin, contains R-factor R151
P. aeruginosa $280GSK^r$	Strain serially subcultured in increasing concentrations of gentamicin	Resistant at high levels to all aminoglycosides tested (see also text).

Media. The media used were MUELLER-HINTON agar (BBL), trypticase soy broth (TSB) (BBL), nutrient broth (NB) (BBL), or iso-sensitest agar (Oxoid).

Determination of minimal inhibitory concentration (MIC) values of antibiotics. A multi-inoculator agar-dilution method was used. The inoculum was $2\times10^4\pm1\times10^4$ bacteria per spot on plates of MUELLER-HINTON agar containing 75 mg/liter calcium and 20 mg/liter magnesium and an appropriate concentration of antibiotic. Incubation was at 35°C for 18 hours with 5 or more colonies scored as growth.

Antibiotic inactivation assays. Aminoglycoside-acetylating, adenylylating and phosphorylating radioactive assays were carried out by described methods.[2,8] Cell-free extract was prepared as a $30,000\times g$ supernatant (S-30) of sonically disrupted cells. The buffer used throughout was 10 mM Tris HCl, 50 mM NH_4Cl, 10 mM $MgCl_2$ and 2 mM dithiothreitol pH 7.8 (buffer A). Microbiological assays contained 0.8 ml of S-30 fraction of cell extract, 40 μg gentamicin sulfate (Schering Corp.) and 400 μg ATP or Acetyl Coenzyme A (total volume; 1 ml). The test strain was *P. aeruginosa* 280 (MIC for gentamicin 0.008 μg/ml in NB). Control *P. aeruginosa* strains containing acetylating (PS-130) or adenylylating activity (POW 151) were used with each microbiological assay. Assays were considered negative if the test strain was unable to grow at the normally inhibitory concentration of antibiotic. Thus a change of 0.004 μg/ml gentamicin would be detected.

Cell-free amino-acid incorporation. The poly-uridylic acid (poly-U) system used was that described by NIRENBERG[12] (specific activity of ^{14}C-phenylalanine-55 mCi/mmole, New England Nuclear). R17 RNA was prepared and used for amino-acid incorporation as previously described.[13] Preincubation of complete amino-acid incorporation mixtures for 30 minutes at 37°C was carried out prior to the addition of R17 RNA and ^{14}C-labelled valine (specific activity-50 mCi/mmole, Schwarz/Mann). The S-100 ($100,000\times g$ supernatant) fraction and ribosomes were prepared as described.[13] Endogenous protein synthesis was carried out using a non-preincubated deoxyribonuclease-treated S-30 fraction ($30,000\times g$ supernatant) of alumina ground cells.[18] Concentrations of reagents were the same as used for R17 RNA directed synthesis with a magnesium concentration of 9 mmolar. The S-30 fraction served as a source of mRNA, tRNA, ribosomes and other non-ribosomal components required in protein synthesis (see Table 6 for amino-acid concentrations).

Whole cell incorporation of ^{3}H-gentamicin. Gentamicin uptake was measured as described[4] using gentamicin tritiated by catalytic exchange (Amersham-Searle) and subsequently repurified

by Sephadex G-10 and paper chromatography.[4]

R-Factor transfer and detection of extra-chromosomal DNA. Methods used for broth and filter conjugations were as previously described.[6,7] Detection of DNA either by analytical CsCl gradient centrifugation or by labelling bacterial DNA with ^{32}P and subsequent ethidium bromide-CsCl density gradient centrifugation were as described.[6]

Results

Susceptibility of Clinical-isolates of *Pseudomonas aeruginosa* from the University of Alberta Hospital (UAH), Edmonton, Canada

Estimation of aminoglycoside susceptibility of bacteria is influenced by many factors most of which influence permeation of the aminoglycoside into bacterial cells (for a review see (3)). Cation concentration of the testing medium is particularly important for strains of *P. aeruginosa*. The concentration of magnesium and calcium varies considerably among different human tissues and fluids. As a result we have selected cation concentrations of serum as a guide. Most magnesium is present in a free form in serum but about 40 % of calcium is protein-bound. Frequently quoted serum values for magnesium are 20 mg/liter and for free (ionized) calcium, 50~60 mg/liter. In using medium containing agar some of the cations are bound to agar. We have found that total values of calcium and magnesium of 75~90 mg and 20~25 per mg/liter respectively in agar give minimal inhibitory concentration (MIC) values of aminoglycosides similar to the equivalent broth containing about 50 mg/liter calcium and 20 mg/liter magnesium. For routine surveillance of aminoglycoside susceptibility we perform agar-dilution MICs using either MUELLER-HINTON (BBL) or iso-sensitest (Oxoid) agar supplemented to contain 20 mg/liter magnesium and 75 mg/liter calcium. MIC values for 1,500 strains of *P. aeruginosa* for several antimicrobial agents are given in Table 2. It can be noted that 23 % of strains would be regarded as resistant to gentamicin. We have examined many of the gentamicin resistant

Table 2. Susceptibility of 1,500 strains of *Pseudomonas aeruginosa* to various antibiotics*

Antibiotics	Cumulative per cent of strains susceptible to antibiotics at various concentrations.							
	Concentrations of aminoglycosides (μg/ml)							
Aminoglycosides	1.0	2	4	8	16	32	64	≥128
Gentamicin	1	2	45	77	92	96	98	100
Tobramycin	43	78	90	98	99	100	—	—
Amikacin	0	4	14	54	82	92	95	100
	Concentrations of carbenicillin (μg/ml)							
	25	50	100	200	400	800	>800	
Carbenicillin	46	74	91	97	99	99.5	100	
	Concentration of colistimethate sodium (μg/ml)							
	10							
Colistimethate sulfonate	100							

* See methods for details of MIC determination.

strains in detail to characterize the prevalent mechanisms of resistance to that drug. The results of three resistant strains and a comparative sensitive strain are presented as representative of the group.

Many different pyocine types of *P. aeruginosa* were represented in the group of resistant strains with no single pyocine type clearly predominating. The most common type was a Govan and Gillies[10] type 16 strain which represented about 15 % of the total.

Minimal Inhibitory Concentrations of Aminoglycoside for Selected Strains

Table 3 illustrates that the three strains (13934, 1136 and 8803) selected for study have MIC values of gentamicin of 32, 64 and 128 μg/ml respectively. Most strains in the group have MIC values of 25~50 μg/ml. Many of these strains when tested by disc susceptibility had zone sizes of 13 mm or more and would, according to most criteria in use, be called susceptible, especially using agar with low cation concentrations. However, it is clear that these strains do not act as susceptible to gentamicin. Strains 8803 and 13934 for example were isolated from a burn-patient on whom topical gentamicin was being used. These strains were isolated throughout the time period gentamicin was in use. Strain 1136 was isolated from the sputum of a patient with bronchopneumonia receiving gentamicin and who had blood levels of 3~6 μg/ml of gentamicin during that time.

Table 3. MIC Values of aminoglycoside antibiotics for *P. aeruginosa* gentamicin-resistant strains.

Strain	MIC* (μg/ml)				
	Gentamicin**		Tobramycin	Amikacin	Sisomicin
	Untreated	Pretreated			
8803	128	256	32	128	128
1136	64	128	16	64	64
13934	32	128	16	64	32
280GSK^r	100	>400	>100	>100	>100
280***	0.06	—	0.03	0.12	0.06

* Determined with Mueller-Hinton broth containing calcium 50 mg/liter and magnesium 20 mg/liter. The inoculum was 10^5 cells/ml and broths were incubated for 18 hours at 35°C.

** Untreated: Tested without prior growth in gentamicin.
Pretreated: Serially subcultured 7 times in TBS containing 5 μg/ml gentamicin prior to determination of MIC.

*** Sensitive strain.

Strains 8803, 1136 and 13934 showed a characteristic resistance to all aminoglycosides against which they were tested (Table 3). Most strains in the group were susceptible to carbenicillin but usually resistant to tetracyclines, chloramphenicol and sulfonamides.

Strains 8803, 1136 and 13934 when cultured for 7 serial transfers in 5 μg/ml gentamicin (sub-inhibitory) in trypticase soy broth (TSB) show an increase in resistance to gentamicin as well as the other aminoglycosides. The change varied from two to four-fold for individual strains. This level of resistance is then maintained in the absence of further growth in gentamicin. The increase in resistance in the presence of a sub-inhibitory concentration of gentamicin could be of significance as *in vivo* concentrations of gentamicin are often of this magnitude.

Growth Characteristics of Resistant Strains

The three resistant strains had generation times in TSB of 30~40 minutes the same as sensitive strain 280. Each strain grew on minimal medium and thus was not auxotrophic. Strain 280 GSK^r is a gentamicin-resistant strain (Table 3) developed within the laboratory by exposing strain 280 to step-wise increases in gentamicin concentration. It has an MIC of gentamicin of greater 100 μg/ml and is highly resistant to streptomycin, kanamycin, sisomicin, amikacin, tobramycin and several other aminoglycosides. However it differs markedly from the naturally occurring resistant strains in that its generation time is about 150 minutes in

Table 4. Assay for gentamicin acetylation or adenylylation by strains of *P. aeruginosa*.

Strains	Assay			
	Microbiological		Radioactive	
	Adenylylation*	Acetylation*	Adenylylation**	Acetylation**
PS130	−	+	−	+***
8803****	−	−	−	−
1136****	−	−	−	−
13934	−	−	−	−
POW151	+	−	+***	−
280	−	−	−	−

* −, less than 0.004 μg/ml reduction in gentamicin concentration; + greater 0.008 μg/ml reduction in gentamicin concentration.

** − no increase or +, an increase in dpm bound to phosphocellulose paper after 15, 30 or 60 minutes of incubation of assay mixture. Each test strain was re-assayed three or more times using different preparations with and without added mercaptoethanol or magnesium.

*** representative activity for acetylation 4.8×10^5 dpm/mg protein; for adenylylation 2×10^3 to 1×10^4 dpm/mg protein.

**** also assayed for streptomycin-phosphotransferase, gentamicin-phosphorylation, kanamycin-acetyltransferase and kanamycin phosphotransferase by radioactive assay.

TSB and it does not grow on minimal medium. Strains similar to 280 GSK^r have been described by WEINSTEIN *et al.*[15] which have reduced animal virulence. Clearly the naturally occurring resistant strains are quite different from the laboratory strains.

Antibiotic Inactivating Enzymes

Microbiological and radioactive assays were used to examine cell extracts from each of the 3 resistant strains as well as control strains containing either gentamicin-acetylating or adenylylating activity and a known susceptible strain (Table 4). No evidence of gentamicin-acetylating or adenylylating activity was detected in any of 3 resistant strains although control strains were positive and we have detected both these activities in strains of *P. aeruginosa* from other sources.[5] Strains 8803, 13934 and 1136 were examined and found to be negative for gentamicin phosphorylation. This form of modification has been previously detected although it does not cause strains to be gentamicin-resistant.[9] Strains 8803, 1136 and 13934 were examined for streptomycin phosphotransferase and kanamycin and amikacin acetylating activity but none was detected. Thus known forms of enzymatic activity which could account for the resistance to gentamicin, tobramycin or amikacin were not detected in any strains examined.

Strains 8803, 1136 and 13934 were incubated with 5 μg/ml gentamicin in TSB for 24 hours

at 35°C. Strain 280 was similarly treated except the gentamicin concentration was 0.125 μg/ml. The cultures were centrifuged to pellet bacterial cells. Samples of the cell-free supernatant were assayed for gentamicin content using strain 280 in nutrient broth with an inoculum of 10^4 cells/ml. Under these conditions strain 280 is inhibited by 0.008 μg/ml and not by 0.004 μg/ml gentamicin. Each broth supernatant was diluted, based on the original gentamicin concentrations, to obtain concentrations of 0.012~0.001 μg/ml each varying by 0.001 μg/ml gentamicin. Under these conditions the loss of activity for each of the resistant strains never exceeded that obtained with the sensitive strain (1.5 %, average of three experiments). Thus no inactivated gentamicin was detectable in extracellular broth.

Strains 8803 and 280 (A_{600}=0.5) were exposed to 100 μg/ml and 1 μg/ml ^{3}H-gentamicin (100 dpm/ng) respectively for 2 hours at 35°C. The treated culture was centrifuged at 5,000 *g* for 15 minutes at 4°C and the supernatant and pellet were separated. Pelleted cells were washed twice with 10 times the original broth volume of 3 % NaCl and then extracted with 0.1 times the original volume of 10 % TCA for 15 minutes at 4°C followed by centrifugation at 10,000 *g* for 15 minutes. Samples of the supernatant (5 times concentrated) and the extract were chromatographed as described by WAGMAN *et al.*[14] Extract, supernatant and control preparations of ^{3}H-gentamicin all demonstrated 3 peaks consistent with gentamicin C_{1a}, C_2 and C_1. No evidence of any additional products such as a modified form of gentamicin could be detected.

Cell-free Amino-acid Incorporation Studies

In order to determine if ribosomes and other cellular components obtained from the three resistant strains were susceptible to the inhibitory action of gentamicin on protein synthesis a variety of cell-free amino-acid incorporating systems were examined for the effect of gentamicin

Table 5. Effect of gentamicin and tobramycin on amino acid incorporation in cell-free systems directed by poly U or R17 RNA.

Strain source of		System	^{14}C-Amino acid	pmoles incorporated /250 μl mixture		% Inhibition	pmoles incorporated /250 μl mixture		% Inhibition
a) Ribosomes	b) S-100			−Gent*	+Gent		−Tob*	−Tob	
8803	280	Poly U	phe**	267	108	60	58	25	58
1136	280	〃	〃	74	24	68	74	30	60
13934	280	〃	〃	67	21	69	70	18	75
280	280	〃	〃	502	192	62	502	108	64
8803	8803	〃	〃	250	99	60	250	98	61
1136	1136	〃	〃	122	45	63	ND***	ND	—
8803	280	R17 RNA	val**	20	6.5	66	ND	ND	—
1136	280	〃	〃	73	29	60	ND	ND	—
13934	280	〃	〃	28	10.5	63	ND	ND	—
280	280	〃	〃	36	16	58	ND	ND	—
8803	8803	〃	〃	75	30	60	ND	ND	—

* Gent: gentamicin 1 μg/ml in poly U system, 10 μg/ml R17 RNA system;
Tob: tobramycin 1 μg/ml.
** phe: phenylalanine,
val: valine.
*** ND: not done.

compared to that using components from a known sensitive strain. Table 5 illustrates that gentamicin inhibits amino-acid incorporation equally either using ribosomes from gentamicin resistant strains 8803, 1136 and 13934 or from the sensitive strain 280 with poly U or R17 RNA as messenger RNA. The soluble components for these systems were provided by the S-100 (see methods) fraction from strain 280. In order to ensure that some portion of the S-100 fraction was not insensitive to gentamicin, amino-acid incorporation in mixtures using ribosomes and S-100 fractions from strains 8803 and 1136 were also shown to be inhibited by gentamicin. Table 6 shows that using endogenous mRNA, ribosomes, transfer RNA and other non-ribosomal components of protein synthesis entirely from either strain 8803 or 280, gentamicin or streptomycin inhibited amino-acid incorporation into the acid-insoluble product. From these studies it seems safe to conclude that the cellular components required for protein synthesis are equally sensitive to gentamicin in the resistant or sensitive strain of *P. aeruginosa*.

Table 6. Effect of gentamicin and streptomycin on incorporation of amino acids in a cell-free system directed by endogenous messenger RNA.

Strain	MIC of gentamicin (μg/ml)	Experiment number	dpm* of ^{14}C Amino acids incorporated**				
			No antibiotic	+ Gentamicin	% Inhibition	+ Streptomycin	% Inhibition
8803	100	1	596	84	84	67	89
		2	714	285	60	128	75
		3	688	146	79	111	84
3503	0.6	1	786	196	75	102	87
		2	1,096	428	61	340	69
		3	542	195	64	189	65

* dpm-disintegrations per minute, counting efficiency-60 %.

** Each assay (0.7 ml) contains 0.5 ml of deoxyribonuclease-treated extract from alumina ground cells as a source of mRNA, tRNA, ribosomes and other non-ribosomal components of protein synthesis; 0.7 nmoles of all amino acids (12 of the 20 amino acids were labelled with ^{14}C each with a specific activity of 50 μCi/μmole; Schwarz/Mann); ATP, ATP-generating system, and other reagents were added as described. Magnesium concentration was 9 mmolar. Concentrations of gentamicin were 10 μg/ml and streptomycin 20 μg/ml. Sample size was 50 μl and incubation was at 34°C for 30 min. Control tubes (zero time) dpm were 100~150 and have been subtracted from the values given above.

Gentamicin Accumulation by Gentamicin Resistant and Sensitive Strains

Gentamicin is accumulated by strains of *P. aeruginosa* by multi-phasic kinetics. The initial very rapid phase has been shown to be energy-independent and inadequate to produce bacterial death.[4] Strains 8803, 1136 and 13934 and 280 show no clear differences in this initial accumulation phase. The amount of gentamicin accumulated at 4°C or in the presence of sodium azide (0.1 %) or potassium cyanide (1 mM) is concentration dependent in all of the strains. Later phases of gentamicin accumulation require energy and are necessary for bacterial death.[4] Strain 280 shows energy-dependent gentamicin accumulation at very low gentamicin concentrations. The exact concentration at which this accumulation begins is dependent on the growth medium. Several factors enhance accumulation including pH above 7, the presence of a peptone or yeast extract in the medium, and good oxygenation. Phosphates and cations in

any type of medium reduce accumulation. Gentamicin accumulation using nutrient broth (NB) (BBL) is particularly active and is reflected by lowered MIC values of gentamicin for all strains tested with that medium. Accumulation of gentamicin in this medium has been used to study the relative energy-dependent accumulation by strains 8803, 1136, 13934 and 280. Other media such as trypticase soy broth (TSB) (BBL) cause much reduced gentamicin accumulation associated at least in part with higher cation concentrations than in NB. MIC values of gentamicin are correspondingly much higher in TSB than NB.

Fig. 1 illustrates relative energy-dependent accumulation of gentamicin by the two most sensitive strains at 0.5 μg/ml gentamicin. Clearly only strain 280 shows significant uptake of gentamicin. Fig. 2 illustrates gentamicin accumulation at 20 μg/ml gentamicin for the three resistant strains and strain 280. Note that neither strain 1136 or 8803 have exceeded the levels of gentamicin accumulated by strain 280 at 0.5 μg/ml and the accumulation by strain 13934 at 20 μg/ml is about the same as that accumulated by strain 280 at 0.5 μg/ml. It may also be observed that the most sensitive strain 13934 accumulated the most gentamicin and strain 8803 the least of the three resistant strains. These results quite clearly show that there is a marked difference in the capability of these strains to accumulate gentamicin and that this difference is related to their susceptibility to gentamicin. As the components required for protein synthesis appear equally sensitive

Fig. 1. Accumulation of ^{3}H-gentamicin in nutrient broth by *P. aeruginosa* strains 280 and 13934 at 37°C using a concentration of 0.5 μg/ml gentamicin. Zero time gentamicin accumulation (1.25 ng/mg for each strain) which is taken to represent ionic binding of gentamicin has been subtracted from all values.

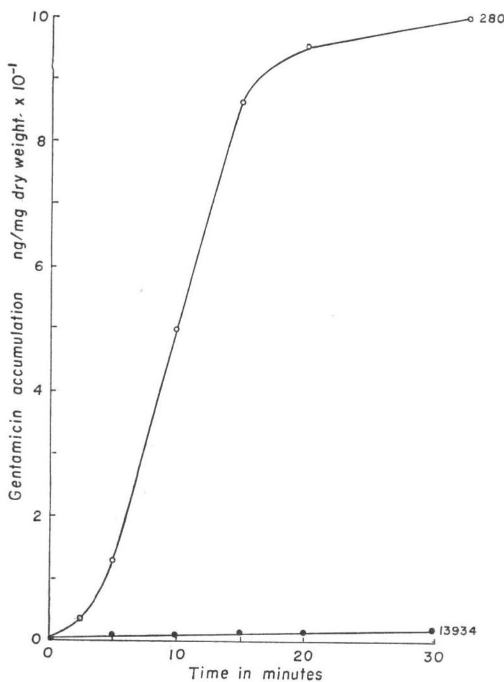
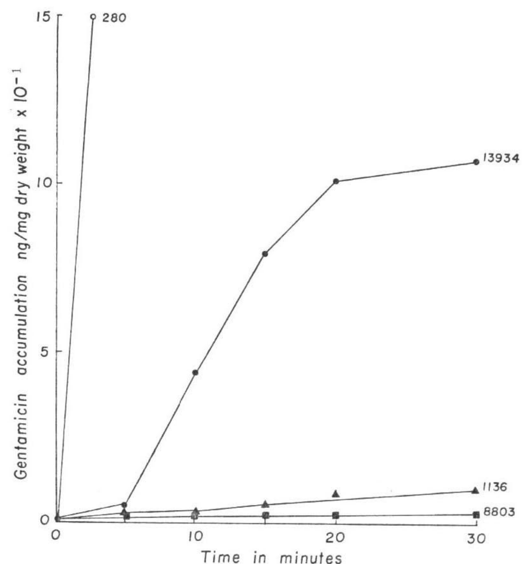


Fig. 2. Accumulation of ^{3}H-gentamicin in nutrient broth by *P. aeruginosa* strains 280, 13934, 1136 and 8803 at 37°C using a concentration of 20 μg/ml gentamicin. Zero time gentamicin accumulation (about 50 ng/mg for each of the four strains) which is taken to represent ionic binding of gentamicin has been subtracted from all values.



to gentamicin in all strains, this accumulation does not reflect differential ribosomal binding. Although we have observed that enzymatic inactivation reduced energy-dependent gentamicin accumulation,[3] there is no evidence for enzymatic activity in these strains. Thus it seems reasonable to conclude that the resistant strains are resistant because of a relative impermeability for gentamicin and most likely for other aminoglycosides. It is possible that the reason for this impermeability may be either in the cell wall or in the membrane accumulation mechanism. Reagents which modify the cell wall such as carbenicillin or lysozyme and 3 % LiCl do enhance the accumulation of gentamicin (BRYAN, L.E. and H.M. VAN DEN ELZEN, Microbiology 1976 (In press)). Thus the cell wall appears to act as a penetration barrier. However this effect is obsrved in both sensitive and resistant strains and in fact these reagents appear to enhance gentamicin accumulation to a slightly greater extent in sensitive strain 280 (not shown).

Extrachromosomal DNA in Enzyme-negative Strains

Extrachromosomal DNA can be detected in some enzyme-negative strains such as 8803 and 13934 by either CsCl density gradient or ethidium bromide-CsCl density gradient centrifugation. However other strains like 1136 do not demonstrate detectable plasmid DNA. Repeated attempts to transfer aminoglycoside resistance from strain 8803, 1136 and 13934 to *P. aeruginosa* strains 280 met$^-$ Rif^r, 3503 Rif^r, 211 Rif^r and *Escherichia coli* K12 SA 1306 Rif^r by conjugation in broth or on membrane filters for 2 or 18 hours at 35°C[7] have not been successful. We have used R-factor RP4 in an attempt to mobilize resistance from strain 8803 or 1136 to *P. aeruginosa* 280 or *E. coli* K12 SA 1306 but have been unable to transfer any aminoglycoside resistance to those strains. These results suggest the genetic information for such resistance is not plasmid mediated.

Discussion

The three gentamicin resistant strains characterized in this study are representative of a large number of similar isolates obtained from UAH in Edmonton over the past four years all of which contain no evidence of enzymatic inactivation. Strains like 8803 have plasmid DNA but we have been unable to transfer resistance from any such strain to a variety of *P. aeruginosa* recipients. Transfer of broad aminoglycoside resistance from an enzyme-negative strain of *P. aeruginosa* to a second strain of *P. aeruginosa* has been reported.[11] Although this report is strongly suggestive of transfer of resistance of the type described here, no evidence for plasmid DNA nor of subsequent transfer from the recipient was presented so that the role of an R-factor remains in doubt for the strain reported.[11]

The broad aminoglycoside resistance patterns shown by the strains reported in this study would require multiple mechanisms of enzymatic inactivation be present to explain such wide resistance. In addition these strains are resistant to amikacin and to SCH 20569, a new aminoglycoside from Schering Corporation, New Brunswick, New Jersey. Amikacin is known to be inactivated only by kanamycin acetyltransferase. We have found that a sensitive strain of *P. aeruginosa* which has received various R-factors specifying a variety of aminoglycoside modifying enzymes do not show any increase in resistance to SCH 20569. However 8803, 1136 and 13934 are resistant to SCH 20569, strongly supporting our proposal that resistance in these strains is not due to inactivating enzymes.

There is no evidence that any component of protein synthesis specifies the resistance to gentamicin with natural endogenous mRNA, R17 RNA or poly U as messenger RNA. Thus,

the most likely explanation of resistance is a reduction in the amount of the aminoglycosides entering the bacterial cell. Such could be due to a permeability barrier provided by the cell wall. The cell wall has been repeatedly shown to influence antibiotic penetration including aminoglycoside penetration. Cell wall active reagents do enhance gentamicin accumulation but to a similar extent in both resistant and sensitive strains. Thus although the cell wall contributes to gentamicin resistance in general it does not seem the explanation of the differential resistance of strains like 8803, 1136 and 13934. We have recently shown that gentamicin cellular entry is an active process composed of at least three kinetic components.[4] The first or ionic binding phase is similar in each of the resistant strains and sensitive strain 280. However second phase accumulation does not begin in strain 8803 until a much higher concentration than that necessary for second phase accumulation in strain 280. Thus the major explanation for resistance seems to be a requirement for an increased gentamicin concentration to initiate second phase transport. This transport phase is dependent on aerobically generated energy and is necessary for cell death.[4] The actual mode of transport is unknown but we propose that the transport mechanism for gentamicin has reduced affinity for or access to gentamicin in the resistant strains. Thus gentamicin is not transported to the ribosomal site until much higher concentrations of gentamicin are present in the medium. This interpretation is supported by the observation that mutants with reduced cytochrome content and thus reduced aerobically generated energy show increased resistance to aminoglycosides. It is important to emphasize that strains 8803, 1136 and 13934 do not appear to be defective in cytochromes by visual spectrometry and have normal growth patterns. Also they were isolated from clinical circumstances (burns and pneumonia) and thus are capable of contributing to a pathological state. However, strain 8803 in particular may have some reduction in virulence. In spite of high gentamicin resistance and delayed healing of a burn associated with heavy colonization with 8803 in a 8-year-old boy the child did not suffer from septicemia and his skin graft was eventually successful.

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