Characterization of *Pseudomonas aeruginosa* Adaptive Resistance to Aminoglycoside Antimicrobial Agents

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78

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  - European 0335
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  - Aerospace 0538
  - Agricultural 0539
  - Automotive 0540
  - Biomedical 0541
  - Chemical 0542
  - Civil 0543
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  - Heat and Thermodynamics 0348
  - Hydraulic 0545
  - Industrial 0546
  - Marine 0547
  - Materials Science 0794
  - Mechanical 0548
  - Metallurgy 0743
  - Mining 0551
  - Nuclear 0552
  - Packaging 0549
  - Petroleum 0765
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CHARACTERIZATION OF *Pseudomonas aeruginosa* ADAPTIVE RESISTANCE TO AMINOGLYCOSIDE ANTIMICROBIAL AGENTS

BY

JAMES ALLAN KARLOWSKY

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

anr	anaerobic regulation of arginine degradation and nitrate reduction
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cfu	colony forming unit
Cp_{max}	maximum plasma concentration
CSPD	disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate
DCCD	N,N'-dicyclohexylcarbodiimide
ddNTP	dideoxynucleotide triphosphate
denA	nitrite reductase gene
denB	cytochrome c-551 gene
denAB	*denA* and *denB* operon
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNP	2,4-dintrophenol
EDTA	ethylenediaminetetraacetic acid
H^+-ATPase	proton adenosine triphosphatase
KCN	potassium cyanide
MHB	Mueller-Hinton broth
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
MOPS	morpholinepropanesulfonic acid
MRT	MIC reversion time

NaCl	sodium chloride
NADH	nicotinamide-adenine dinucleotide reduced form
NaN_3	sodium azide
NEM	N-ethylmaleimide
PAGE	polyacrylamide gel electrophoresis
PAE	postantibiotic effect
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulfate
T_m	melting temperature
$T_{10}E_1$	aqueous solution containing 10 mM Tris and 1 mM EDTA, pH 8.0
UV	ultraviolet

ABSTRACT

Aminoglycosides are important clinical agents in the treatment of *Pseudomonas aeruginosa* infections. Occasionally however therapeutic failures do occur, some of which have been attributed to transient aminoglycoside resistance that dissipates with in vitro subculture. The reproducible pattern by which initially susceptible *P. aeruginosa* demonstrate transient resistance to aminoglycosides, which is lost with growth in antimicrobial free media, has been termed adaptive resistance.

The objective of this thesis was to characterize *P. aeruginosa* adaptive resistance to aminoglycoside antimicrobial agents. Adaptively resistant *P. aeruginosa* demonstrated resilience to aminoglycoside killing, increased aminoglycoside minimum inhibitory concentrations (MICs), decreased cytoplasmic aminoglycoside accumulation, logarithmic phase growth, normal morphology, and a small, but statistically significant ($P<0.05$) reduction in proton motive force (pmf). Some of these initial observations suggested that aminoglycoside adaptive resistance may arise via changes in the *P. aeruginosa* cell envelope. However, there were no differences in lipopolysaccharide (LPS) or outer membrane protein (OMP) profiles among wild-type control, adaptively resistant, and post-adaptively resistant forms of *P. aeruginosa.* In contrast, cytoplasmic membrane protein profiles of adaptively resistant *P. aeruginosa* demonstrated six discernable band changes in SDS-PAGE gels, three of which also occurred in anaerobically grown cells, when compared to wild-type control and post-adaptively resistant cells. Anaerobically maintained *P. aeruginosa*, which also accumulate aminoglycosides poorly, depend exclusively upon an anaerobic, oxidative respiratory pathway for energy generation. Nitrite reductase (*denA*) facilitates terminal electron acceptance in the anaerobic respiratory pathway of *P. aeruginosa* and is under ANR (anaerobic regulation of arginine

deiminase and nitrate reduction) positive transcriptional control. *P. aeruginosa* adaptively resistant to gentamicin demonstrated both increased *denA* and *anr* transcription, in the absence of promoter DNA sequence changes in either gene, suggesting that aminoglycoside adaptive resistance may result from, at least in part, increased cellular reliance upon the anaerobic respiratory pathway. This hypothesis was tested further by performing adaptive resistance determinations in an ANR deletion mutant (*P. aeruginosa* PAO6261) which demonstrated significantly ($P<0.05$) increased killing during the adaptive resistance interval compared with its adaptively resistant *anr*$^+$ parent (*P. aeruginosa* PAO1). Complementation of *P. aeruginosa* PAO6216 with *anr* (pME3580) reinstated the original pattern of adaptive resistance. However, aminoglycoside killing of *P. aeruginosa* PAO6261 was still significantly ($P<0.05$) less than that demonstrated in wild-type, control cultures of *P. aeruginosa* PAO1 indicating that additional, presently enigmatic factors remain to be elucidated to fully describe *P. aeruginosa* adaptive resistance to aminoglycoside antimicrobial agents.

Characterization of *Pseudomonas aeruginosa* Adaptive Resistance to Aminoglycoside Antimicrobial Agents

A. INTRODUCTION

1. Aminoglycosides

a. History

Aminoglycosides were first identified in the 1940s, by the systematic screening of soil actinomycetes for the presence of antimicrobial molecules (Davies, 1986; Gilbert, 1995). Since then approximately 150 aminoglycosides have been characterized. Of these, more than 10 have been marketed for therapeutic use in humans (Table 1) (Davies, 1986; Gilbert, 1995). The first medically relevant aminoglycoside isolated was streptomycin. It was introduced into clinical medicine in 1944 for the treatment of tuberculosis. The discovery of gentamicin (1963) and tobramycin (1968) led to a tremendous upsurge in the clinical use of aminoglycoside antimicrobials for nosocomial bacterial infections (Koch and Rhoades, 1971; Holtje, 1979). Increases in bacterial resistance, specifically to the later two agents, spawned the development of semi-synthetic aminoglycoside derivatives such as amikacin and netilmicin (Davies, 1986).

The majority of identified aminoglycosides are produced by *Streptomyces* species, although several are also produced by *Micromonospora* species and *Bacillus* species (Davies, 1986). The role of aminoglycosides in their native environment remains cryptic, as does the deliberate or coincidental nature of their protective, antibacterial function.

Table 1. Aminoglycosides in clinical use[a].

Generic Name	Proprietary Name	Source	Year Reported
Streptomycin	None	*Streptomyces griseus*	1944
Neomycin	Mycitradin, Neobiotic	*Streptomyces fradiae*	1949
Kanamycin	Kantrex	*Streptomyces kanamyceticus*	1957
Paromomycin	Humatin	*Streptomyces fradiae*	1959
Gentamicin	Garamycin	*Micromonospora purpurea* and *Micromonospora echinospora*	1963
Tobramycin	Nebcin	*Streptomyces tenebrarius*	1968
Amikacin[b]	Amikin	*Streptomyces kanamyceticus*	1972
Netilmicin[b]	Netromycin	*Micromonospora invoensis*	1975
Sisomicin[c]	Siseptin	*Micromonospora invoensis*	1970
Dibekacin[b,c]	-	*Streptomyces kanamyceticus*	1971
Isepamicin[b,c]	-	*Micromonospora purpurea*	1978

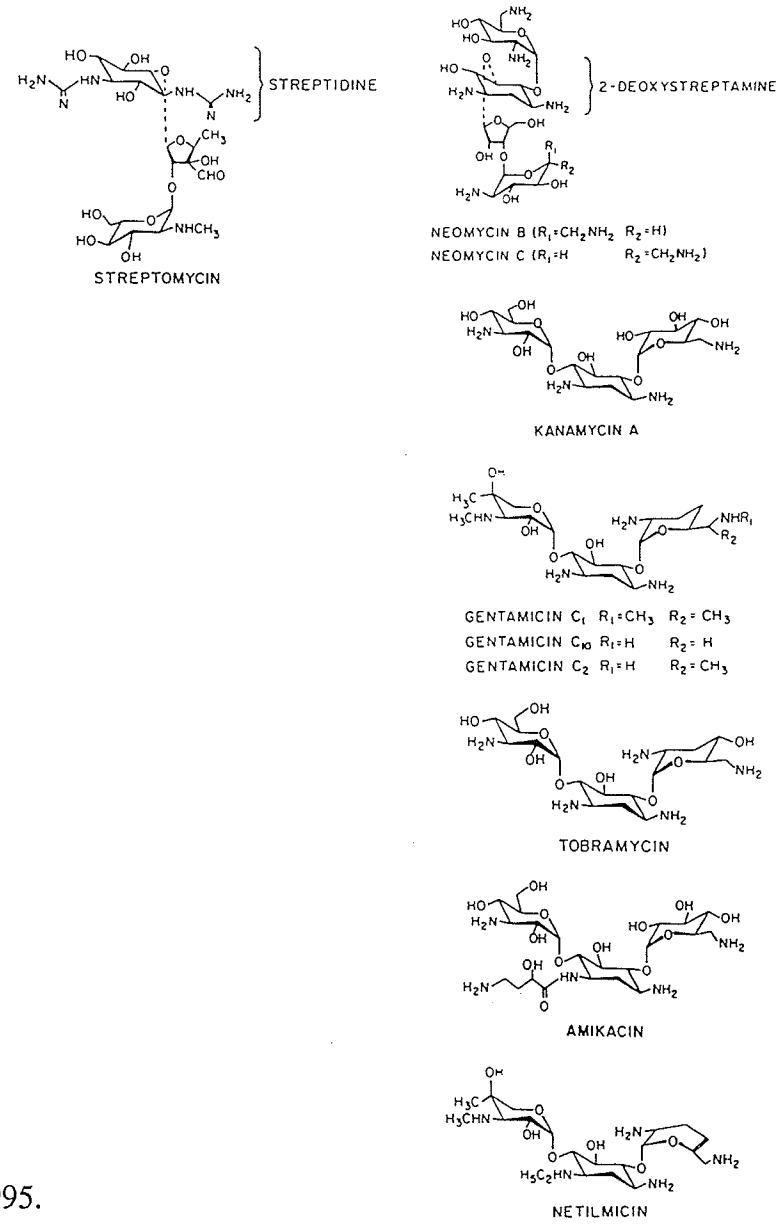
[a] Table adapted from Gilbert, 1995.
[b] Semi-syntheic aminoglycoside.
[c] Approved for human use in countries other than Canada and U.S.A.

b. Structure and Chemical Characteristics

All aminoglycosides possess a six-membered hydrocarbon ring, with amino-group constituents, known as an aminocyclitol. The aminocyclitol ring is either streptidine (streptomycin) or 2-deoxystreptamine (all other identified aminoglycosides) (Figure 1). Glycosidic bonds exist between the aminocyclitol ring and two or more amino-containing

or non-amino-containing sugars (Figure 1) (Gilbert, 1995).

Figure 1. Structures of selected aminoglycosides[a].



[a] Gilbert, 1995.

The aminoglycosides can be divided into four distinct chemical families (Table 2). Streptomycin is placed into its own group because of its chemically distinct aminocyclitol ring. The kanamycin family contains kanamycin A, kanamycin B, amikacin, tobramycin

and dibekacin. All kanamycin family members are products of *Streptomyces* species and join cyclic sugars at positions 4 and 6 of the aminocyclitol, 2-deoxystreptamine. Tobramycin is 3'-deoxykanamycin B (Gilbert, 1995). Amikacin is a semi-synthetic derivative of kanamycin A with a 2-hydroxy-4-aminobutyric acid moiety added at position 1 of 2-deoxystreptamine. Gentamicin is a mixture of three structurally similar, quantitatively inseparable, constituents, C_1, C_{1a}, and C_2, produced by *Micromonospora purpurea.* Gentamicin possesses glycosidic linkages at positions 4 and 6 of the aminocyclitol, 2-deoxystreptamine. Sisomicin is the dehydro analog of gentamicin C_{1a}. Netilmicin is derived from sisomycin by the addition of an ethyl group to the amino group at position 1 of 2-deoxystreptamine (Gilbert, 1995).

Table 2. Aminoglycoside chemical families[a].

Family	Member(s)
Streptomycin	Streptomycin
Kanamycin	Kanamycin A, Kanamycin B, Amikacin,
	Tobramycin, Dibekacin
Gentamicin	Gentamicin C_1, C_{1a}, C_2
	Sisomicin, Netilmicin, Isepamicin
Neomycin	Neomycin B, C
	Paromomycin

[a] Table adapted from Gilbert, 1995.

The aminoglycosides have molecular masses in the range of 445 to 600 daltons and are highly water soluble. The ionization constants for individual amino groups are difficult to determine, but the overall pK_a of gentamicin, for example, has been estimated at 8.4 (Gilbert, 1995). The aminoglycosides, as represented by gentamicin, are thus

strongly basic compounds that exist as polycations at pH 7.4. Aminoglycoside antimicrobial activity has been repeatedly demonstrated to be enhanced in media with an alkaline pH and reduced in media with an acidic pH (Gilbert, 1995).

c. Antimicrobial Activity

The in vitro spectrum of activity of selected aminoglycosides is presented in Table 3. Aminoglycosides demonstrate concentration dependent bactericidal activity against a broad spectrum of aerobic and facultative gram-negative bacilli and *Staphylococcus aureus*. Aminoglycosides do not have activity against rickettsia, fungi, *Mycoplasma*, viruses, *Streptococcus pneumoniae*, *Xanthomonas maltophilia*, *Pseudomonas cepacia*, *Bacteroides* species, *Clostridium* species, and other anaerobic organisms (Gilbert, 1995).

Aminoglycosides do not demonstrate an inoculum effect, that is, the minimum inhibitory concentrations (MICs) of test strains generally remain unchanged at inocula of 10^5 bacteria/ml and 10^7 bacteria/ml (Brook, 1989). In contrast, an increase in test inoculum often results in a substantial increase in the MIC of ß-lactams (Brook, 1989). Cation content (Ca^{2+}, Mg^{2+}, and to a lesser extent Na^{+}) of MIC test media greatly influences the aminoglycoside MIC measured. The higher the cation content, the higher the aminoglycoside MIC. As well, an alkaline medium will yield an erroneously low MIC and an acidic medium an erroneously high MIC (Gilbert, 1995).

d. Pharmacokinetic Properties

All aminoglycoside antibiotics are very poorly absorbed following oral administration (less than 1% under normal circumstances), and in fact, may be given via this route for the treatment of gastrointestinal infections. When administered to treat systemic infections, aminoglycosides must be given parenterally, either as a 30 to 60 minute intravasculature (IV) infusion or as an intramuscular (IM) injection (Gilbert 1995).

Table 3. In vitro spectrum of activity of aminoglycosides[a].

Organism	Aminoglycoside[b]					
	Streptomycin	Kanamycin	Gentamicin	Tobramycin	Amikacin	Netilmicin
Gram-negative						
Escherichia coli		+	+	+	+	+
Proteus mirabilis		+	+	+	+	+
Klebsiella sp.		+	+	+	+	+
Enterobacter sp.		-	+	+	+	+
Citrobacter sp.			+	+	+	+
Serratia sp.		+	+	+	+	+
Salmonella sp.						
Providencia sp.			+	+	+	+
Aeromonas sp.			+	+	+	+
Acinetobacter sp.			-	+	-	
Pseudomonas aeruginosa		-	+	+	+	+
Pseudomonas cepacia		-	-	-	-	-
Xanthomonas maltophilia		-	-	-	-	-
Neisseria gonorrhoeae		±	-	-	-	-
Haemophilus influenzae		+	+	+	+	+
Yersinia pestis	+		+			
Francisella tularensis	+	+	+			
Gram-positive						
Streptococcus pneumoniae		-	-	-	-	-
Staphylococcus aureus (MSSA)		+	+	+	+	+
Staphylococcus aureus (MRSA)		-	-	-	-	-
Miscellaneous						
Mycobacterium tuberculosis	+	-	-	-	+	-
Mycobacterium avium-intracellulare	-	-	-	-	+	-
Bacteroides fragilis	-	-	-	-	-	-

[a] Table adapted from Gilbert, 1995.

[b] + indicates sensitivity (relative degrees of sensitivity are not indicated); ± indicates variable sensitivity; - indicates resistance; blanks indicate data not available.

Aminoglycosides distribute well into most body fluids, but not into the central nervous system, bone, fat, or connective tissue. They tend to concentrate in the kidney and are

excreted via glomerular filtration. Metabolism of aminoglycosides in vivo does not occur (Gilbert 1995).

All aminoglycosides have similar pharmacokinetic properties which can be divided into three interrelated phases (Gilbert, 1995). Phase 1 concerns aminoglycoside distribution from the vasculature to the extravascular space. This occurs with a half-life ($t_{1/2}$) of 15 to 30 minutes (Gilbert, 1995). The second phase, is the phase of greatest concern in clinical dosage regimens, and involves the elimination of aminoglycoside from the plasma and the extravascular space. The second (elimination) phase is determined by glomerular filtration rate (Gilbert, 1995). The elimination $t_{1/2}$ of all aminoglycosides, in adults and infants older than 6 months with normal renal function, is 1.5 to 3.5 hours. The third phase is the prolonged and slow elimination of aminoglycoside that has accumulated in the kidney (Gilbert, 1995).

e. Pharmacodynamic Parameters

Pharmacodynamic parameters describe the relationship between drug concentrations, and the pharmacologic and toxicologic effects of drugs (Craig and Gudmundsson, 1991). In the specific case of antimicrobial agents, pharmacodynamic parameters concern the relationship between antibiotic concentrations achieved at one or more sites of infection, their fluctuation with time, and any therapeutic or toxicological effects observed (Rotschafer *et al.*, 1992). Minimum inhibitory and bactericidal concentrations (MICs and MBCs) were the original in vitro measures of antimicrobial activity. The limitations of these static measures of antimicrobial activity have given way to more dynamic methods of describing the time course of antimicrobial activity. These newer methods include kill curves, as well as postantibiotic effect (PAE), subinhibitory MIC effect, postantibiotic leukocyte enhancement, and adaptive resistance determinations

(Daikos *et al.*, 1990b; Craig and Gudmundsson, 1991; Gould *et al.*, 1991; Rotschafer *et al.*, 1992). The newer pharmacodynamic parameters, which can vary markedly for different classes of antimicrobial agents, give a more accurate description of the time course of antimicrobial activity than do the MIC and MBC. Knowledge of the pharmacodynamic characteristics of an antimicrobial agent can assist in determining optimal dose size and dosing interval (Rotschafer *et al.*, 1992). Application of pharmacodynamic parameters to antimicrobial dosing regimens can influence the rate of bacterial eradication at an infection site (Levison, 1995).

To maximize efficacy and limit toxicity, traditional aminoglycoside dosing regimens dictated that plasma levels be maintained within narrow limits. Recent pharmacodynamic research has demonstrated that aminoglycosides possess concentration dependent bactericidal activity, a prolonged and concentration dependent postantibiotic effect (PAE), antimicrobial activity at concentrations below the MIC, and an adaptive resistance phase in certain, susceptible gram-negative bacilli, most notably *P. aeruginosa* (Daikos *et al.*, 1990b; Craig and Gudmundsson, 1991). These findings suggest that the efficacy of aminoglycosides may be improved by using higher aminoglycoside doses with longer dosage intervals (Chan, 1989; Daikos *et al.*, 1990b; Gilbert, 1991; Karlowsky *et al.*, 1994). As well, the results of several recent animal and human studies using once daily aminoglycoside dosing suggest that this may be equally effective and possibly less nephrotoxic and ototoxic than traditional dosing schedules (Tulkens, 1990). However, the movement towards once daily aminoglycoside dosing as universally efficacious therapy must be approached with caution as pharmacokinetic limitations arise in some patients (Gilbert, 1991; Rotschafer *et al.*, 1992).

Experiments performed in this thesis concerned two principal pharmacodynamic

parameters, PAE and adaptive resistance. A discussion of the salient features of each follows. This thesis also introduces a novel pharmacodynamic parameter, MIC reversion time (MRT), which is described in detail in the Materials and Methods section (Karlowsky *et al.*, 1994b).

α. Postantibiotic Effect

The postantibiotic effect or PAE is defined as the persistent suppression of bacterial re-growth following a brief (generally 1 or 2 hour) exposure to, and subsequent extracellular removal of, an antimicrobial agent (Craig and Gudmundsson, 1991). The significance of the PAE pertains primarily to the impact that it may have on antimicrobial dosing regimens in clinical practice. The PAE appears to be a feature of virtually every antimicrobial agent that has been examined and has been documented with most common Gram-positive and Gram-negative bacterial pathogens (Craig and Gudmundsson, 1991). However, a PAE is not present for all antimicrobial agent/bacteria combinations. The presence and duration of the PAE for any specific antimicrobial agent/bacteria combination is a function of the bacterial genus, the chemical class and concentration of antimicrobial agent, and the duration of antimicrobial exposure. In general, inhibitors of protein and nucleic acid synthesis (aminoglycosides, chloramphenicol, tetracycline, clindamycin, rifampin, and fluoroquinolones) can induce prolonged PAEs of up to 2 to 6 hours against most Gram-positive cocci and Gram-negative bacilli within their spectrum of activity (Craig and Gudmundsson, 1991). In contrast, cell wall active agents (penicillins, cephalosporins, carbapenems, monobactams, vancomycin) and trimethoprim induce PAEs of up to approximately 2 hours in duration in Gram positive cocci, but very short or no PAE in Gram-negative bacilli (Craig and Gudmundsson, 1991). Imipenem, a cell wall active agent, is unique in that it can produce significant PAEs with some

strains of *P. aeruginosa.* In some instances Gram-negative bacilli exposed to penicillins and cephalosporins may produce what appears to be a negative PAE, that is, the antibiotic exposed culture appears to grow faster than the growth control. This phenomenon has been suggested to be the result of filamentous forms, induced by exposure to ß-lactam antibiotics, dividing faster than control organisms soon after drug removal (Kroeker *et al.*, 1995).

As mentioned above, two of the most important factors that determine the duration of the PAE for any antimicrobial agent/bacteria combination are the concentration of antimicrobial agent and the length of exposure. In general, persistent suppression of bacterial growth is only seen after bacteria have been exposed to drug concentrations that approach or exceed the MIC. Aminoglycosides are, however, one of the few antimicrobials that appear to induce a PAE even at concentrations below the MIC of the organism (Zhanel *et al.*, 1991). Maximum PAEs are demonstrated in Gram-positive cocci exposed to ß-lactams, and in both Gram-positive and Gram-negative organisms exposed to bacteriostatic agents such as erythromycin, tetracycline, and chloramphenicol, at 5 to 10 times the MIC. In contrast, PAEs with *E. coli* and *P. aeruginosa* continually increase with aminoglycoside concentrations between 1 and 64 mg/l (Isaksson *et al.*, 1988). As well, rifampin, a DNA dependent RNA polymerase inhibitor, does not appear to have a ceiling on its PAE. Studies with *S. aureus* reveal that rifampin PAEs continue to increase even at concentrations as high as 200 times the MIC (Isaksson *et al.*, 1988).

Increasing the duration of antimicrobial exposure has been shown to prolong the duration of the PAE (Craig and Gudmundsson, 1991). The duration of exposure has also been shown to have an upper limit for many antimicrobials, that is, a maximal PAE is obtained regardless of the duration of antimicrobial exposure (Craig and Gudmundsson,

1991). Subsequent experiments have demonstrated that the length of exposure and the concentration of antibiotic have equal influence on the PAE (Craig and Gudmundsson, 1991). Doubling the concentration of the antibiotic has the same approximate effect as doubling the time of exposure. It appears that the area under the concentration-time curve (AUC) is the major determinant of PAE duration for many antimicrobial agent/bacteria combinations (Drusano, 1991).

The precise mechanism(s) by which antimicrobials induce PAEs is/are not understood. The observed differences in PAEs of various antimicrobial agent/bacteria combinations suggest that multiple mechanisms are involved (Craig and Gudmundsson, 1991; MacKenzie and Gould, 1993). The two most commonly proposed mechanisms are drug induced nonlethal damage at the cellular site of action and/or limited persistence of the drug at its binding site (Craig and Gudmundsson, 1991; MacKenzie and Gould, 1993). For agents such as the macrolides, tetracycline, and chloramphenicol, which reversibly bind to specific subunits of susceptible bacterial ribosomes it has been suggested that the PAE represents the time required for the antibiotics to diffuse from the ribosome. The aminoglycosides, in contrast, exhibit their highly lethal effects by irreversibly binding to the 30S ribosomal subunit. It has been suggested that the prolonged PAE induced by these agents may represent the binding of sublethal amounts of aminoglycoside to 30S ribosomal subunits of surviving cells, with subsequent, non-lethal disruption of protein synthesis. A result of this disruption may be the depletion of functional proteins required by the cell for its intermediary metabolism and growth. The PAE may represent the time period required for the resynthesis of these proteins (Craig and Gudmundsson, 1991).

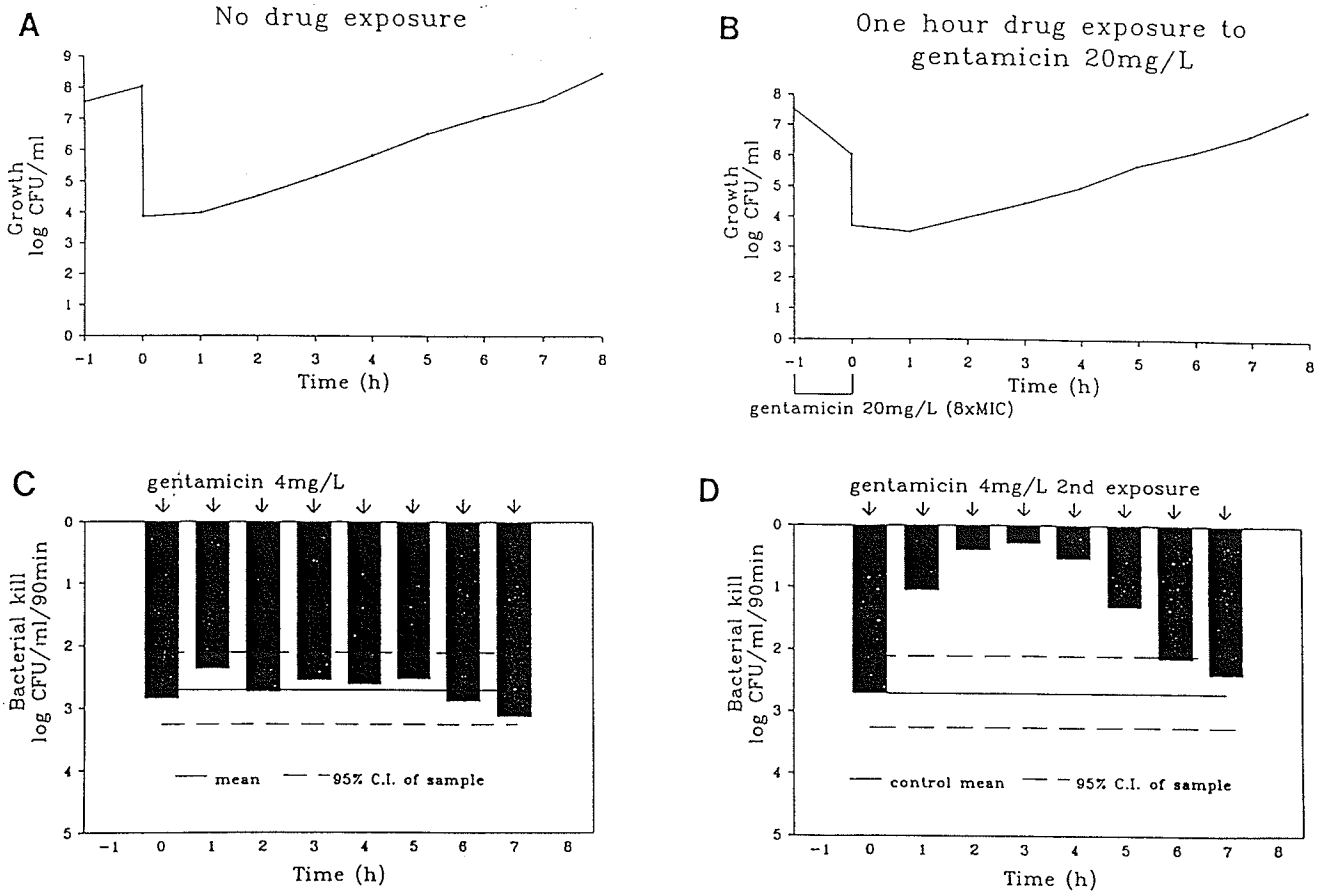
Recent work on defining the mechanism of aminoglycoside PAE has expanded on the aforementioned supposition of Craig and Gudmundsson (1991). A 30 minute

tobramycin exposure, at 2X MIC (2 $\log_{10}$ cfu/ml bacterial kill), was shown to inhibit DNA, RNA, and protein synthesis in *E. coli* (Barmada *et al.*, 1993). Following extracellular tobramycin removal, both DNA and RNA synthesis promptly resumed, while protein synthesis remained inhibited for an additional 4 hours. Protein synthesis inhibition correlated with the duration of the PAE (Barmada *et al.*, 1993). In regard to the prompt resumption of DNA and RNA synthesis the authors suggest that this indicates readily dissociable non-specific binding of tobramycin to DNA and RNA (Barmada *et al.*, 1993). However, this result appears dubious. It is widely accepted that cytoplasmic aminoglycoside accumulation is irreversible. Therefore once extracellular aminoglycoside is removed, aminoglycoside does not begin to exit cells down its concentration gradient (discussed in a later section). Therefore, why does removal of extracellular tobramycin suddenly trigger reactivation of DNA and RNA syntheses if the intracellular tobramycin concentration remains unchanged? A subsequent report has demonstrated that DNA synthesis is suppressed during the tobramycin induced PAE interval in *E. coli* and *P. aeruginosa* (Gottfredsson *et al*, 1995).

ß. Adaptive Resistance

Adaptive resistance is defined as reduced antimicrobial killing in bacterial populations pre-exposed to that agent (Daikos *et al.*, 1990b; Daikos *et al.*, 1991; Gould *et al.*, 1991). This observation has been made in aerobic and facultative Gram-negative bacilli, most often *P. aeruginosa*, that have been pre-exposed to fractions ($\geq$ 1/2X MIC) and multiples of the minimum inhibitory concentration (MIC) for 1 to 2 hours. In these experiments the extracellular antibiotic is removed by dilution or repeated centrifugation, and aliquots from the culture then re-exposed to the same agent at specific time intervals to look at changes in the bactericidal activity of antibiotics in pre-exposed cells. The

Figure 2. **Gentamicin adaptive resistance induction in *Pseudomonas aeruginosa* ATCC 27853[a].** (A) Bacterial growth of control culture with no prior gentamicin exposure. (B) Bacterial growth with 1 hour of pre-exposure to gentamicin (20 mg/l). Control (A and C) and test (B and D) cultures were diluted to 10^4 cfu/ml at time zero. (C and D) Bacterial killing 90 minutes after the addition of gentamicin (4 mg/l) to culture samples removed at successive hourly intervals. (C) Bactericidal effect of first drug exposure on control culture. (D) Bactericidal effect of second drug exposure on test culture. The 95% confidence interval (C.I.) for the control is superimposed on both control and test culture graphs.



[a] Barclay *et al.*, 1992.

reproducible pattern by which initially susceptible bacteria acquire, or appear to acquire, transient resistance to aminoglycosides, which is lost with growth in antimicrobial free media, has been termed adaptive resistance (Daikos *et al.*, 1990b). Figure 2 depicts a typical in vitro adaptive resistance determination result with *P. aeruginosa* ATCC 27853 and gentamicin (Barclay *et al.*, 1992).

i. In vitro Data

In vitro experiments have demonstrated reproducible changes in the extent and rate of aminoglycoside killing in pre-exposed *P. aeruginosa* cultures (Gilleland *et al.*, 1989; Daikos *et al.*, 1990b; Gould *et al.*, 1991; Barclay *et al.*, 1992; Karlowsky *et al.*, 1994). Adaptive resistance to aminoglycosides has been shown to occur most readily with *P. aeruginosa*, but it has also been reported, although much less frequently and consistently, with *E. coli*, *S. aureus* and other bacteria (Nilsson *et al.*, 1987; Daikos *et al.*, 1990b; Gould *et al.*, 1991). Adaptive resistance to ciprofloxacin has also been shown in a few isolates of *P. aeruginosa*, but it was of a very limited duration and demonstrated significant strain to strain variation (Gould *et al.*, 1991).

In vitro aminoglycoside adaptive resistance has been shown to persist for 6 to 8 hours during *P. aeruginosa* growth in a antimicrobial free medium following a 1 hour aminoglycoside exposure at 8X MIC (Daikos *et al.*, 1990b; Barclay *et al.*, 1992). The initial 1 hour aminoglycoside exposure produced approximately 1 to 2 $\log_{10}$ (90% to 99%) of bacterial killing. The duration of aminoglycoside adaptive resistance has been shown to be concentration dependent at concentrations above the MIC (Daikos *et al.*, 1990b; Barclay *et al.*, 1992).

Adaptive resistance is stable only in the continued presence of aminoglycoside (Daikos *et al.*, 1990b). Growth in the absence of aminoglycoside eventually gives rise

to progeny of a sensitive phenotype. Adaptive resistance induced by gentamicin in *P. aeruginosa* produced cross-resistance to netilmicin, tobramycin, amikacin and isepamicin (Gilleland *et al.*, 1989; Daikos *et al.*, 1990b) but not to polymyxin B, another, structurally unrelated, cationic antibiotic which is thought to share binding sites within the outer membrane with aminoglycosides (Gilleland *et al.*, 1989). The reproducibility and instability of adaptive resistance, the number of adaptively resistant cells found within a culture (cells which survive initial aminoglycoside exposure) and the cross-resistance among aminoglycosides suggest that aminoglycoside adaptive resistance is not due to specific enzymatic inactivation, or mutation (random mutation rate is 10^{-7} to 10^{-9}), be it ribosomal, or otherwise (Gilbert, 1995).

Aminoglycoside adaptive resistance appears greatest (i.e. bacterial killing was least upon re-exposure) when logarithmic growth of the pre-exposed culture had just resumed, generally considered the end of the PAE period (Daikos *et al.*, 1990b). Prior to its peak, adaptive resistance shows a gradual induction phase (i.e. a gradual reduction in aminoglycoside killing with a second aminoglycoside exposure) (Figure 2). Maximal resistance to aminoglycoside killing is not present immediately. The time required for the maximum induction of adaptive resistance is generally 4 to 6 hours following the initiation of aminoglycoside exposure (Daikos *et al.*, 1990b; Barclay *et al.*, 1992). Maximum adaptive resistance development is associated with bacterial replication (commonly 1 to 2 $\log_{10}$) (Gould *et al.*, 1991) and can occur in antimicrobial free media. The induction phase is not seen if initial aminoglycoside exposures are 6 to 8 hours in duration (Daikos *et al.*, 1990b). Following peak adaptive resistance, the bacterial culture, grown in antibiotic free media, gradually returns to its initial susceptibility. Cultures continuously incubated with aminoglycoside remain adaptively resistant indefinitely

(Daikos *et al.*, 1990b). Adaptive resistance lasts longer than the PAE suggesting that the two phenomena may occur by mutually exclusive mechanisms (Daikos *et al.*, 1990b; Gould *et al.*, 1991). The appearance of aminoglycoside adaptive resistance was shown to correlate with decreased intracellular aminoglycoside accumulation, especially during the period of accelerated energy-dependent drug transport (EDP II - discussed in a following section) (Daikos *et al.*, 1990b).

Polymyxin B is a cationic polypeptide antibiotic that is bactericidal through its insertion into, and disruption of, the cytoplasmic membrane. Adaptive resistance to polymyxin B has been shown to involve ultrastructural alterations of the outer membrane. These alterations excluded the antibiotic so that it could no longer reach the cytoplasmic membrane (Gilleland *et al.*, 1984). Polymyxin B appears to compete with Mg^{2+} and Ca^{2+} for binding sites within the outer membrane. Changes in the LPS and phospholipid composition of the outer membrane reduce the number of these binding sites and render the outer membrane less permeable to polymyxin B (Gilleland *et al.*, 1984).

It has been reported that the antipseudomonal ß-lactam ticarcillin and imipenem, may prevent and repair aminoglycoside induced adaptive resistance (Lolans *et al.*, 1988; Daikos *et al.*, 1990a). It was shown that inhibitory or subinhibitory (1/2X MIC) concentrations of ticarcillin or imipenem prevented the transient drug refractoriness resulting from a 1 hour aminoglycoside exposure at 1/2X MIC. In cultures previously exposed to an aminoglycoside, imipenem or ticarcillin at 1X MIC accelerated cytoplasmic ^{3}H-tobramycin accumulation. This observation, although interesting, has not been confirmed by other investigators.

Subsequent exposures to progressively increasing aminoglycoside concentrations allows the emergence of *P. aeruginosa* populations with higher and higher MICs (up to

128-256X MIC for the sensitive parent) (Gilleland *et al.*, 1989). A culture such as this can grow in the presence of an aminoglycoside concentration that would normally have sterilized it. However, cultures such as this demonstrate significantly slower replication and generally appear as small colony variants upon plating (Nilsson *et al.*, 1987). If incubated in antimicrobial free media for several days cultures generally return to their initial sensitivities and cells to their original size. Some small colony variants also arise following a single 1 to 2 hour aminoglycoside exposure (Daikos *et al.*, 1990b).

ii. In Vivo Data

Several reports of in vivo aminoglycoside adaptive resistance have been published (Gerber *et al.*, 1982b; Olson *et al.*, 1985; Bayer *et al.*, 1987; Daikos *et al.*, 1991). Gerber and colleagues (1982b) used leukopenic mice, experimentally infected with *P. aeruginosa*, to assess gentamicin bactericidal activity with time. The authors reported that slow growing aminoglycoside resistant subpopulations of *P. aeruginosa* were isolated from leukopenic mice following aminoglycoside treatment. Gentamicin alone could not eradicate "susceptible" *P. aeruginosa* from leukopenic mice even when multiple doses were given which produced very high peak plasma levels (known to be toxic in humans). The authors describe *P. aeruginosa* as demonstrating an aminoglycoside escape phenomenon, that is, the initial killing of the test organisms was followed by regrowth of more resistant variants despite ongoing treatment. The only significant effect of the ongoing treatment was that it prevented reversion to, and regrowth of, the most gentamicin susceptible cells. Breakthrough growth of aminoglycoside resistant *P. aeruginosa* subpopulations was not seen in immunocompetent mice suggesting that in these animals leukocytes successfully eradicated the gentamicin-resistant subpopulations. Resistance to gentamicin reversed in vivo when gentamicin treatment was discontinued

or when the resistant subpopulation was subcultured in vitro using antimicrobial free media. The emergence of gentamicin resistant subpopulations, in leukopenic mice, was successfully suppressed by combining gentamicin with the ß-lactam ticarcillin. Gentamicin-ticarcillin synergy in these infections may be due, at least in part, to ticarcillin suppression of aminoglycoside resistant subpopulations. Adaptive resistance may explain the disappointing results seen when aminoglycosides were used alone in the treatment of leukopenic patients (Daikos *et al.*, 1990b)

Olson and co-workers (1985) studied the epidemiology of aminoglycoside resistant *P. aeruginosa* in a hospital intensive care unit. Bacterial population analysis, performed by replica plating of primary isolation plates onto gentamicin containing agar, revealed the presence of resistant subpopulations in the initial isolates from 71% of assessable patients (nonleukopenic, critically ill patients with diverse medical and surgical problems). These isolates were stably resistant in the presence of gentamicin and proportionately cross-resistant to other aminoglycosides (tobramycin and amikacin). Decreased resistance followed discontinuation of the aminoglycoside. Most of the changes in aminoglycoside resistance were due to the appearance of new, more resistant subpopulations of *P. aeruginosa*, of the same serotype. All aminoglycoside susceptible and resistant subpopulations in initial and serial isolates from individual patients were of the same serotype. The authors felt that this pattern suggested the presence of a nonenzymatic mechanism of aminoglycoside resistance, most likely a reduction in membrane permeability.

Bayer *et al.* (1987) characterized five amikacin resistant variants of *P. aeruginosa* isolated from aortic valve vegetations during unsuccessful therapy of experimental endocarditis. These organisms were cross-resistant to other aminoglycosides and no

aminoglycoside modifying enzymes were identified. However, all five variants demonstrated significantly reduced intracellular accumulation of ^{3}H-amikacin when compared with their amikacin susceptible parental strain. The reductions in permeability were unstable in vitro, and normalized following serial passage in antibiotic free media. Variants also failed to produce pyocyanin at the time of initial isolation from cardiac vegetations. This phenotypic trait was labile, and pigment production was regained after serial passage of the variants on antibiotic free media. The variants often grew as smaller than normal colonies and demonstrated in vitro generation times approximately 1.5 to 2 times longer than their parental strain. Two impermeability variants were compared with their parental strain for the ability to induce experimental endocarditis in rabbits with aortic catheters. Both variants were virulent in vivo; however, mean bacterial densities in vegetations were approximately 2.5 $\log_{10}$ cfu/ml lower in animals challenged with the variants than in animals challenged with the parental strain, probably reflecting a slower in vivo growth rate.

Daikos *et al.* (1991), using a *P. aeruginosa*/neutropenic mouse thigh infection model, demonstrated that adaptive resistance occurred when aminoglycoside doses were given more than 2 hours after the start of treatment (initial dose). An aminoglycoside free interval of 8 hours (approximately 16 times the antibiotic half-life) renewed bacterial susceptibility to aminoglycoside action (Daikos *et al.*, 1991).

One important reason underlying the continued clinical use of aminoglycosides, despite their well documented toxicity and the development of new, highly active fluoroquinolone, carbapenem and monobactam antimicrobials, is their limited tendency towards detectable resistance development during therapy (Gilbert, 1995). However, epidemiological surveys suggest that unstable aminoglycoside resistant bacterial

subpopulations are commonly present at sites of colonization and infection in hospitalized patients receiving aminoglycoside therapy (Weinstein *et al.*, 1980; Price *et al.*, 1981; Young, 1984; Olson *et al.*, 1985). These variants appear to be of little concern in immunocompetent patients (Weinstein *et al.*, 1980; Price *et al.*, 1981; Young, 1984) and may very well be going undetected in the clinical microbiology laboratory as isolates may revert to susceptibility at initial isolation (Daikos *et al.*, 1990b; Gilbert, 1995). In contrast, aminoglycoside resistant subpopulations may have definite clinical relevance to cases of treatment failure in conditions requiring prolonged or repeated aminoglycoside therapy such as in immunocompromised, endocarditis and cystic fibrosis patients (Bryan, 1989; Gilbert, 1995).

iii. Clinical Significance of Adaptive Resistance

Clinically undetected aminoglycoside resistant subpopulations have been shown to be common in *P. aeruginosa* and to emerge readily during therapy (Gerber *et al.*, 1982b; Olson *et al.*, 1985; Bayer *et al.*, 1987). Routine clinical sensitivity testing is likely of limited value in demonstrating adaptively resistant organisms. Passage through drug free media is a part of routine diagnostic procedures and conventional MIC determinations. This may be sufficient for reversion to the susceptible phenotype. Consequently, the bacterial inoculum used for conventional MIC determinations would consist primarily of aminoglycoside susceptible revertants, rather than adaptively resistant populations. Thus, due to the instability of adaptively resistant organisms, selected during treatment in vivo, isolates appearing resistant to aminoglycosides in vivo may actually test as susceptible in vitro (Bayer *et al.*, 1987). In patients suffering relapse or failure, it may be suggested that the clinical laboratory plate the appropriate cultures onto media containing supra-MICs of aminoglycosides in parellel with standard susceptibility testing

schemes. Disparities in results between the two procedures (i.e. organisms testing as susceptible by routine methods, yet exhibiting growth on aminoglycoside containing media) would suggest the presence of an impermeable variant (Bayer *et al.*, 1987).

The emergence and selection of aminoglycoside resistant variants, of aminoglycoside susceptible bacteria such as *P. aeruginosa*, slows therapeutic response, facilitates colonization, and may produce outright therapeutic failure (Bayer *et al.*, 1987). Adaptive resistance should be regarded as a possible cause of clinical failure when there is a local or general deficiency of host defenses, at sites where drug entry is poor or where the local environment is antagonistic to the action of the drug. The supposition that adaptively resistant cells are killed equally well by a heathly immune system suggests no changes are occurring in surface characteristics that would make them less immunogenic (Bayer *et al.*, 1987). The excellent results obtained during more than 25 years of clinical use of aminoglycoside antimicrobial agents, without attention to adaptive resistance, may also be assigned to the almost routine use of these agents in combination with another antibiotic, generally a ß-lactam (Daikos *et al.*, 1990b).

2. Mechanism of Aminoglycoside Action

A unified, concise mechanism of aminoglycoside action remains enigmatic despite an extensive literature describing a remarkably pleiotropic set of effects. A detailed critique of this subject is spatially prohibitive but excellent recent reviews have been published (Hancock, 1981a; Hancock, 1981b; Davis, 1987; Taber *et al.*, 1987; Bryan, 1989a). Aminoglycosides are bactericidal protein synthesis inhibiors contrary to the bacteriostatic action of most ribosomally active antibiotics such as tetracycline, chloramphenicol. An outline of the important features of aminoglycoside cellular

accumulation and killing is presented to serve as a background against which adaptive resistance may be appreciated. A detailed critique focusing on the membrane transport of aminoglycosides is provided, as adaptively resistant cells fail to accumulate aminoglycosides. Loss of aminoglycoside transport would suggest that the mechanism(s) responsible for adaptive resistance may be associated with the cytoplasmic membrane.

a. Aminoglycoside Accumulation May be Divided into Three Phases

Aminoglycoside accumulation in bacterial cells has been divided into three phases; ionic binding to the cell surface, energy dependent phase I (EDP I) and energy dependent phase II (EDP II) (Bryan and van den Elzen, 1976). Aminoglycoside binding to phosphate residues of the lipopolysaccharide (LPS) core region, in the outer membrane of gram-negative bacteria, and to anionic moieties of proteins and phospholipids on the external surface of the cytoplasmic membrane is instantaneous, ionic (non-covalent) in nature, and energy independent (Hancock, 1981a; Hancock, 1981b; Bryan, 1989a). Aminoglycosides may traverse the outer membrane of gram-negative bacilli by passively diffusing through porins and/or by what has been described as the self-promoted pathway (Hancock, 1984; Hancock and Bell, 1988). The self promoted pathway has been described specifically for *P.aeruginosa* which has a less permeable outer membrane than other gram-negative bacilli, such as *E. coli* (Hancock, 1984; Hancock and Bell, 1988). The self promoted pathway involves aminoglycoside binding to LPS phosphate residues which displaces divalent cations (most notably Mg^{2+}) that stabilize LPS and assist in maintaining outer membrane integrity (Leive, 1974; Hancock and Bell, 1988). The disruption of the integrity of this barrier (aminoglycoside binding cannot be accommodated within the normal structure of LPS) is followed by aminoglycoside partitioning into the periplasm of the cell (Hancock and Bell, 1988). The self-promoted

pathway is much less active in other gram-negative bacilli, such as *E. coli* due to lower concentrations of phosphate residues in their LPS, as well as different porin structures and properties (Bryan, 1989a). The binding of these basic molecules (aminoglycosides) to bacterial cytoplasmic membranes is hypothesized to lead to impaired membrane functioning and to be responsible for some of the pleiotropic effects of these agents and may be a major contributor to cell death (Davies, 1986).

EDP I is thought to represent the initial transport of a limited number of aminoglycoside molecules across the cytoplasmic membrane, perhaps associated with a transporter molecule for another compound. Initial aminoglycoside entry might also depend on transient or permanent channels created by low levels of membrane associated, intrinsically mistranslated protein, or by membrane imperfections arising in the process of cell growth (Davis, 1987). It has been widely observed that the duration of the lag preceding the secondary, rapid uptake of aminoglycoside (EDP II) into *P. aeruginosa* and *E. coli*, decreases with increased streptomycin and gentamicin concentration until it appears non-existent (Bryan and van den Elzen, 1975; Bryan and van den Elzen, 1976b). This pattern suggests that the initial, nondetectable entry of aminoglycoside during the lag (EDP I) occurs by diffusion (or electrophoresis) through an aqueous channel or by low affinity for one or more transport systems (Davis, 1987). However, EDP I may also simply represent an expansion of aminoglycoside surface adsorption proportional to the continued growth of cells (Davis, 1987). EDP I precedes any of the intracellular events leading to cell death and appears to be the rate limiting step in accumulation. The number of aminoglycoside molecules required to trigger EDP II is not known (Davis, 1987). EDP I occurs by a mechanism that remains to be fully elucidated but which is driven by the transmembrane electrical potential, a component of the proton motive force

(pmf) (Bryan and van den Elzen, 1977) (discussed in the following section).

EDP I is followed by EDP II, a process which accelerates aminoglycoside movement across the cytoplasmic membrane. EDPII describes the rapid intracellular accumulation of aminoglycoside. EDP II is synchronous with irreversible aminoglycoside binding to 30S ribosome site(s), the inhibition of protein synthesis and inevitable cell death (Bryan and van den Elzen, 1977; Davis, 1987). Specifically, aminoglycoside binding to initiating ribosomes blocks further progress, while binding to ribosomes already engaged in chain elongation causes only slowing and misreading (Davis, 1987; Taber *et al.*, 1987). Bacteria possess cytoplasmic membrane bound ribosomes which facilitate protein translocation into and across the membrane (Davis, 1987). Misread protein, translated on membrane bound ribosomes, has been demonstrated to become incorporated into the cytoplasmic membrane (Davis, 1987; Taber *et al.*, 1987). Concurrent membrane leakage of small molecules from bacterial cells, as well as the unidirectional movement of aminoglycoside into bacterial cells, may be the result of abnormal protein folding creating non-specific channels (Davis, 1987; Taber *et al.*, 1987). Limited protein synthesis, in the presence of aminoglycoside, is a prerequisite of aminoglycoside killing (Davis, 1987; Taber *et al.*, 1987; Bryan 1989a). EDP II leads to changes in the permeability of the cell membrane, a rapid escalation in aminoglycoside accumulation, cell disorganization and, ultimately, cell death (Davis, 1987; Taber *et al.*, 1987). EDP II is coincident with the lethal event in aminoglycoside action (Hancock, 1981a).

The aminoglycoside literature describes additional effects such as alterations in DNA, RNA and polyamine synthesis, and alterations in levels of cAMP and guanosine tetraphosphate (Davis, 1987). Such effects may occur secondary to the inhibition of protein synthesis or as the result of interactions between the highly cationic

aminoglycoside and membrane components (Davis, 1987; Taber *et al.*, 1987). It has also been suggested, although never proven, that secondary effects are the basis of the bactericidal action (killing) of aminoglycosides (Davis, 1987). Thus, although protein synthesis is inhibited as an early event, the accumulation of strongly positively charged aminoglycoside antibiotics within the cell has been proposed to induce a number of lethal consequences. Most notably are effects on the membrane and its structural and functional integrity (Davis, 1987; Taber *et al.*, 1987).

b. Aminoglycoside Accumulation Depends on the Proton Motive Force

The proton motive force (pmf) is a measurement of the electrochemical gradient of protons across a biological membrane. In prokaryotic cells the pmf is generated by proton extrusion across the cytoplasmic membrane by components of electron transport chains and H^+-ATPases (Bryan and van den Elzen, 1977; Damper and Epstein, 1981; Mates *et al.*, 1983; Nichols, 1987). The pmf can be maintained by cellular respiration or ATP hydrolysis (Nichols, 1987). The electrochemical gradient of protons (pmf) is composed of both an electrical potential component ($\Delta\Psi$) and a chemical component (ΔpH). The size of $\Delta\Psi$ is inversely related to ΔpH (Felle *et al.*, 1980). Over a pH range of 5 to 7.5 the ΔpH is maximal when the external pH is 5 and minimal when the external pH is 7.5. An inverse relationship is true for $\Delta\Psi$. $\Delta\Psi$ is higher at pH 7.5 than 5 and is believed responsible for improved aminoglycoside uptake into bacterial cells. When pH of the culture medium is greater than 7.5, the pH gradient does not contribute effectively to the pmf (Nichols, 1987). Streptomycin and gentamicin are highly effective at medium pH values greater than 7.5 (Bryan and van den Elzen, 1977; Damper and Epstein, 1981; Mates *et al.*, 1983). This observation, considered with knowledge that aminoglycosides are highly cationic molecules, suggests that the electrical potential gradient (interior

negative) provides the driving force for aminoglycoside uptake across the cytoplasmic membrane (Bryan *et al.*, 1980). The transport of gentamicin and streptomycin declines with lowering pH, resulting in greater resistance (less bacterial killing) (Bryan, 1989a). The pH effect may be related to the ionization of groups either on the aminoglycoside molecule itself or on cell surface binding sites.

An essential element in the process of bacterial killing is the transport of aminoglycosides from the external milieu across the cytoplasmic membrane and into the interior of the bacterium (Bryan and van den Elzen, 1976; Hancock, 1981a; Hancock, 1981b). The extent and rate of aminoglycoside accumulation depends on the magnitude of the electrical potential gradient ($\Delta\Psi$) across the cytoplasmic membrane (Bryan *et al.*, 1980; Eisenberg *et al.*, 1984). A threshold (minimum) $\Delta\Psi$ appears necessary for aminoglycoside accumulation and killing in bacterial cells (i.e. aminoglycoside accumulation is electrically "gated") (Miller *et al.*, 1980; Mates *et al.*, 1982; Mates *et al.*, 1983). Once this threshold is exceeded, the initial rates of both aminoglycoside accumulation and killing are proportional to the magnitude of $\Delta\Psi$ (Bryan and van den Elzen, 1979; Damper and Epstein, 1981; Mates *et al.*, 1982; Bryan and Kwan, 1983; Mates *et al*, 1983; Eisenberg *et al.*, 1984; Fraimow *et al.*, 1991)

Aminoglycosides accumulate inside susceptible bacteria to concentrations far in excess of external concentrations (Bryan, 1989a). Solute transport against a concentration gradient requires energy. However, EDP II has been shown to be non-saturable, and instead demonstrate diffusion kinetics (Bryan and van den Elzen, 1977). The same conclusion can be drawn for EDP I accumulation, except that at extremely high concentrations (2000 mg/l streptomycin), in ribosomal resistant mutants, saturation is obtainable (Bryan and van den Elzen, 1977). Thus the process of aminoglycoside

cytoplasmic membrane transport acts like one of diffusion driven by an electrical potential (Bryan, 1989a).

The cytoplasmic accumulation of aminoglycosides is irreversible and independent of cellular ATP levels in vitro (Nichols and Young, 1985). However, aminoglycosides are not held within cells by covalent attachment to a cellular component, and are largely released when the permeability barrier is breached by organic solvents such as toluene (Nichols and Young, 1985). Aminoglycoside is recovered from lysed cells in an active, unmodified form (Schlessinger, 1988). Aminoglycoside accumulation does not compete with any of an extensive list of sugars, amino acids, or polyamines, including aminoglycosides themselves, that have been tested (Bryan, 1989a). Divalent cations and low pH inhibit aminoglycoside accumulation (Bryan, 1989a) (discussed in following section).

Aminoglycosides may themselves partially facilitate their own cytoplasmic membrane transport by transiently elevating pmf following cytoplasmic membrane association. Gentamicin adsorption to one side of a phosphatidylcholine bilayer results in a potential difference across the membrane (Butko *et al.*, 1990). Lipid bilayer adsorption was saturated at a gentamicin concentration of approximately 40 mg/l, which is equivalent to one positive charge per 50 phosphatidylcholine molecules, or one gentamicin molecule per 200 phosphatidylcholine molecules. A gentamicin concentration of 40 mg/l produced a potential difference across a phosphatidylcholine membrane of approximately 45 mV (interior negative), at pH 7.4 in a 2.5 mM KCl/125 mM sucrose bathing solution (Butko *et al.*, 1990). The relevance of this work however must be tempered by the fact that phospatidylcholine is not a common bacterial phospholipid.

c. Inhibition of Aminoglycoside Accumulation

Aminoglycoside cytoplasmic membrane transport has a wide range of antagonists. Several mechanisms contribute to reduced aminoglycoside accumulation in *P. aeruginosa*, *E. coli*, and *S. aureus*. Cation antagonism, acidic pH and protonophores such as 2,4-dinitrophenol (DNP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) dissipate pmf by uncoupling ATP synthesis from electron transport. Cyanide (KCN) and sodium azide (NaN_3) bind cytochrome active sites and block electron flow through respiratory pathways. Other contributing factors include carbon monoxide (CO) which specifically binds cytochrome oxidase, growth under anaerobic conditions, and mutational loss of respiratory pathway components (Bryan and van den Elzen, 1975; Bryan and van den Elzen, 1976; Bryan and van den Elzen, 1977; Miller *et al.*, 1980; Chopra and Ball, 1982). Both EDPI and EDPII are inhibited under these conditions or by these inhibitors. The primary, energy independent, ionic binding of aminoglycoside to cell wall components however, is not.

Aminoglycosides are highly positively charged (3-6 positive charges at neutral pH) compounds that bind to anionic groups within the outer membrane of Gram-negative bacteria and on the exterior surface of the cytoplasmic membrane of bacteria as described earlier (Bryan, 1989a). This binding can be antagonized by divalent cations (Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}) and at higher concentrations by monovalent cations (Na^+, K^+) (Bryan and van den Elzen, 1977). The divalent cations Mg^{2+} and Ca^{2+} have also been shown to inhibit aminoglycoside accumulation in *E. coli* spheroplasts (permeabilized outer membranes) (Hancock, 1981a). It is unlikely that cationic antagonism is merely an ionic strength effect, as the ionic strength of the $MgCl_2$ 50% inhibitory concentration (IC_{50}) has been demonstrated to be 35 fold less than the ionic strength of the NaCl IC_{50} (Hancock, 1981a).

As the concentration of divalent cations increases, there is competition with aminoglycosides for binding to phosphate residues (Bryan, 1989a). Divalent cations interfere with the initial binding phase and membrane transport of aminoglycosides, and contribute to higher MIC values (Bryan, 1989a). This suggests that at least one of the sites of divalent cation antagonism is required for aminoglycoside bactericidal activity. While aminoglycoside accumulation is antagonized by inhibitors of both electron transport chain components and the pmf, not all inhibitors of energetics are effective antagonists. N,N'-dicyclohexylcarbodiimide (DCCD), an inhibitor of H^+-ATPase, does not inhibit, and in fact enhances gentamicin accumulation in *S. aureus* and *E. coli*, provided cells are able to maintain a pmf via electron transport (Miller *et al.*, 1980).

Anaerobic growth conditions have been demonstrated to impair, although not completely eliminate, cytoplasmic aminoglycoside accumulation and bactericidal activity in *P. aeruginosa* (Bryan *et al.*, 1980; Bryan and Kwan, 1981). Aminoglycoside accumulation under anaerobic conditions can be prevented by CCCP treatment, indicating that it is pmf dependent (Muir *et al.*, 1985). Reduced aminoglycoside accumulation may be due to a reduction in the quantity of a component of the primary aerobic respiratory pathway (discussed in a later section), which functions as an aminoglycoside transporter or cotransporter. Information pertaining to the value of the pmf under anaerobic conditions has been conflicting and may be explained partially by growth circumstances or possibly by growth rates of the cells being studied (Muir *et al.*, 1985). Changes in the protein synthetic machinery function do not account for anaerobic aminoglycoside resistance, as indicated by the observation that protein synthesis in cell extracts of anaerobically grown *E. coli* are highly susceptible to streptomycin for many generations of *E. coli* growth (tested under anaerobic conditions) (Schlessinger, 1988).

The influence of catabolite repression on the inhibition of cytoplasmic aminoglycoside accumulation also requires mention. The phenomenon of catabolite repression concerns the inhibition of transcription of certain genes (e.g. the lactose operon in *E. coli*) in the presence of a readily metabolized substrate (e.g. glucose in *E. coli*). Uptake of the readily metabolized substrate down-regulates membrane-bound adenyl cyclase synthesis and function, and indirectly reduces the cytoplasmic cyclic AMP concentration. This then prevents transcription of a wide range of genes requiring cyclic AMP and the cyclic AMP receptor protein (CAP) for transcription. Catabolite repressible genes or phenomena are usually studied using adenyl cyclase deficient (*cya*) or cyclic AMP receptor protein (*crp*) deficient mutants. The fact that such strains are viable demonstrates that no indispensible processes in the cell absolutely require cyclic AMP. It has been demonstrated that *cya* and *crp* mutants of *E. coli* and *Salmonella typhimurium* show increased resistance to streptomycin (Artman and Werthamer, 1974; Alper and Ames, 1978; Holtje, 1978) and reduced streptomycin accumulation (Holtje, 1978). The defect, both in streptomycin accumulation and other catabolite repressible systems, is correctable in *cya* mutants but not *crp* mutants by the addition of exogenous cyclic AMP (Artman and Werthamer, 1974; Holtje, 1978). The role of cyclic AMP is further illustrated by the "glucose effect" on growth inhibition by streptomycin, where glucose addition to acetate grown *E. coli* reduces both streptomycin killing and cellular cyclic AMP levels (Artman *et al.*, 1972). Many different phenomena are affected by catabolite repression, and by *cya* and *crp* mutants, including oxidative phosphorylation (Hancock, 1981a), pmf (Dills and Dobrogosz, 1977), cell envelope proteins (Aono *et al.*, 1978), fatty acid composition, cytochrome content, various dehydrogenases and transport systems (Dallas *et al.*, 1976). One or more of these factors may explain the increased resistance

to streptomycin in *cya* and *crp* mutants. Knowledge that cyclic AMP levels are generally regulated at the level of the cytoplasmic membrane in bacteria (Saier, 1979) suggests that the observed resistance may also be related to cytoplasmic membrane changes reducing intracellular aminoglycoside transport. In view of the known stimulation of streptomycin action by cyclic AMP addition to *cya* mutants (Artman and Werthamer, 1974; Holtje, 1978), it is tempting to postulate a key role for catabolite regulation or a catabolite repressible product in streptomycin action. However, *cya* and *crp* mutants are unable to support transcription of catabolite repressible genes, and demonstrate only partial resistance to streptomycin (Alper and Ames, 1978). Despite this, one can conclude that the level of cyclic AMP in the cell may be involved in sensitivity to streptomycin, and other aminoglycosides, in a contributory but non-essential fashion. The role of cyclic AMP in *P. aeruginosa* adaptive resistance to aminoglycoside antimicrobial agents may be an area worthy of future study.

d. Bacterial Cytoplasmic Membrane Transport

Aminoglycosides accumulate against a concentration gradient (Bryan, 1989a). In bacterial cells, three basic mechanisms of transporting solutes against a concentration gradient are known (Higgins, 1990). Phosphotransferase systems (PTS) utilize the high energy phosphate group of phosphoenol pyruvate to drive transport (Postma and Lengeler, 1985). The phosphate group is transferred, via a series of phosphorylated protein intermediates, to the substrate as it crosses the membrane. The substrate never enters the cell in a chemically unmodified form. It has been demonstrated that intracellular aminoglycosides are unmodified (Bryan, 1989a). Therefore, this class of transporter is probably not important in the context of aminoglycoside resistance. Two groups of active transport systems have also been described. They are primary active transport systems

(ABC [ATP-binding cassette] transporters and ion-translocating ATPases), which are driven directly by the hydrolysis of ATP (or other high-energy compound), and secondary active transport systems, which rely on the generation of transmembrane gradients to energize substrate transfer (Higgins, 1990). Because under normal growth conditions ATP has no role in the transport of aminoglycosides, primary active transport systems will not be considered further (Fraimow *et al.*, 1991).

Secondary transport systems are driven by an electrochemical gradient which is generated by respiration or by ATP hydrolysis (Higgins, 1990). The most widely distributed systems, and the most important in the present context, are symporters in which the uptake of a molecule of solute is accompanied by an ion moving down its concentration gradient and providing the energy necessary to drive solute accumulation (Higgins, 1990). The cotransported ion is generally a proton in bacteria, but can be another monovalent cation such as sodium (Higgins, 1990). Alternatively, antiporters are those in which, as an ion passes into the cell down its concentration gradient while a molecule of substrate is translocated outwards (Higgins, 1990). All secondary transporters, both symporters and antiporters, share a very similar membrane organization and many share sequence similarities (Higgins, 1990). Each secondary system characterized to date consists of a single, highly hydrophobic integral membrane protein, the best characterized of which is the lactose permease of *E. coli* (Higgins, 1990). The sequences of many transporters in this class have been determined and all share similar structural characteristics. Each involves a single hydrophobic protein with twelve predicted membrane spanning helices, and small periplasmic or cytoplasmic hydrophilic loops (Higgins, 1990). Over a dozen transporters in this class are now known, and include transporters from both prokaryotic and eukaryotic species (Higgins, 1990).

Although most of these are sugar transporters, this probably reflects a bias in those selected for study, rather than a restriction in the type of substrate that can be handled by this class of transport system (Higgins, 1990). Secondary transporters can function in either direction, depending on the direction of the ion gradient (Higgins, 1990). This is in contrast to the ATP-dependent transporters which are unidirectional (Higgins, 1990).

e. Cytoplasmic Membrane Aminoglycoside Transporter

The characteristics of aminoglycoside accumulation appear to exclude all standard mechanisms of bacterial transport. Most antimicrobial agents, including aminoglycosides, are not lipid soluble and must gain entry into a cell via specific transport systems which have evolved to mediate the uptake of nutrients, or are involved in other cellular processes (Higgins, 1990). It seems unlikely that bacteria have evolved a specialized mechanism to irreversibly concentrate bactericidal antibiotics. Bacteria appear to transport aminoglycosides across their cytoplasmic membranes by a uni-directional (i.e. inwards), irreversible process energized by the pmf (Hancock, 1981a; Hancock, 1981b; Bryan 1989a).

No convincing evidence for an aminoglycoside carrier has ever been provided. It remains possible that a series of multiple, low affinity carriers, or other membrane components which are known to bind aminoglycosides such as respiratory quinones, could indirectly facilitate aminoglycoside transport across the cytoplasmic membrane as part of a transporter complex, but this remains speculative (Hancock, 1981a; Hancock, 1981b; Muir *et al.*, 1981; Bryan and Kwan, 1983; McEnroe *et al.*, 1984; Nichols, 1987; Bryan, 1989a). It has been demonstrated that the quinone dependency of aminoglycoside uptake is restricted to EDPI. The bulk of aminoglycoside uptake, represented by EDPII, is not absolutely dependent upon quinones (Bryan, 1989a).

Other energized transport systems have often been shown to be coupled to the pmf. However, the absolute requirement of a functional electron transport chain is somewhat unusual among known transport systems (Bryan, 1989a). None of the three classes of energized transport systems, as discussed above, is as exquisitely sensitive to cyanide as is the uptake of aminoglycosides (Bryan, 1989a). This suggests that a component of the electron transport chain may be directly required for aminoglycoside accumulation, either as a cytoplasmic membrane receptor or as a facilitator of transmembrane movement of aminoglycosides (Bryan, 1989a). If this is so, it would seem that the partly or fully oxidized state of such a component would be required for aminoglycoside binding or accumulation. For example, cyanide-treated cells would have electron transport components in the reduced state. The effect of anaerobic conditions, and of electron transport inhibitors such as sodium azide, is freely reversible upon O_2 addition or removal of sodium azide respectively (Bryan and van den Elzen, 1975; Bryan *et al.*, 1976; Bryan, 1989a). This would seem to eliminate the trivial explanation that irreversible damage of electron transport is responsible for the observed antagonism of aminoglycoside accumulation and killing by electron transport inhibitors.

It is also important to note that aminoglycoside accumulation cannot be demonstrated by the use of conventional membrane vesicles (no membrane potential [pmf]) from *E. coli* (Bryan and Kwan, 1983; Nichols and Young, 1985). However, some gentamicin accumulation could be demonstrated in vesicles which apparently contain a functional protein synthesis mechanism (Bryan and Kwan, 1983; Bryan, 1989a). The importance of the membrane potential, more specifically $\Delta\Psi$, in aminoglycoside accumulation may suggest entry of the antibiotic by electrophoresis through aqueous membrane channels (Davis, 1987). However, aminoglycoside is not washed out in buffer

(which would not sustain a potential), even though a nonspecific aqueous channel should be equally permeable to aminoglycoside in either direction (Davis, 1987). This difference between entry and exit could be explained if the channel is not fixed but is gated in response to the presence or absence of a potential (Davis, 1987). However, the known gated channels in bacteria are not open to molecules as large as aminoglycosides (e.g. streptomycin - 581 daltons) (Davis, 1987).

In summary, despite decades of investigation, a definitive mechanism of aminoglycoside action remains elusive, as does the mechanism of aminoglycoside transport into susceptible bacteria.

3. Mechanisms of Aminoglycoside Resistance

a. Aminoglycoside Resistance Occurs by Three Main Mechanisms

Stable aminoglycoside resistance in bacteria can arise by at least three distinct mechanisms (Gilbert, 1995). They are synthesis of aminoglycoside inactivating enzymes, changes in ribosomal binding of aminoglycoside, and reduction in cytoplasmic accumulation of aminoglycoside (Gilbert, 1995).

α. Aminoglycoside Inactivating Enzymes

The most publicized mechanism of aminoglycoside resistance in potentially susceptible clinical isolates is enzymatic modification (Gilbert, 1995). Aminoglycoside modifying enzymes chemically alter and inactivate aminoglycosides by N-acetylation, O-nucleotidylation, or O-phosphorylation. Most genes coding for these enzymes are plasmid mediated and many are known to be carried on transposons. More rarely, the enzymes are chromosomally specified. Aminoglycoside modifying enzymes are thought to be constitutively expressed (Gilbert, 1995). There is no evidence that aminoglycoside is

needed to induce resistance, as is the case with erythromycin and ß-lactam resistance in Gram-positive organisms (Gilbert, 1995). Enzymatically modified aminoglycosides are inefficiently transported across bacterial cytoplasmic membranes and bind poorly to ribosomes.

ß. Ribosomal Mutation

Mutations affecting aminoglycoside binding to the 30S ribosomal subunit have been described but do not appear to be of great clinical importance due to their very low frequency of appearance (Gilbert, 1995). Ribosomal mutants may be produced in the laboratory, as spontaneous or mutagen induced strains, by exposure to a high aminoglycoside concentration (>100 mg/l). EDP II is eliminated or reduced in bacterial mutants with altered ribosomes that fail to bind aminoglycoside (Hancock, 1981a; Hancock, 1981b). The frequency of appearance of spontaneous, ribosomal, aminoglycoside resistant mutants in the laboratory is on the order of one in 10^9 -10^{10} cells per generation. In clinical situations, it is unlikely that this number of colonies would be screened in the presence of such a high concentration (>100 mg/l) of aminoglycoside (Gilbert, 1995). Ribosomal alterations do not confer cross resistance to other aminoglycosides (Davis, 1987). A streptomycin ribosomal mutant can be eliminated by other aminoglycosides (Davis, 1987). Ribosomal mutations arise from single amino acid substitutions in ribosomal proteins or from single nucleotide changes in the ribosomal RNA (Davis, 1987). Ribosomes so altered have lowered binding affinity for a particular aminoglycoside. Resistance achieved by mutations which alter the target site and render it aminoglycoside insensitive, is often less than ideal from the cell's point of view, as the target may also be altered so as to be less effective in performing its normal cellular function (Higgins, 1990).

γ. Impermeability Resistance

In general, stable impermeability resistance affects a wide range of aminoglycosides and is usually of a low level (Bryan, 1989a; Bryan, 1989b). High concentrations of aminoglycoside may overcome this impermeability barrier and render the organism sensitive (Hancock, 1981a; Hancock, 1981b; Hancock and Bell, 1988). This type of resistance contrasts enzymatic modification and ribosomal mutation, as they tend to affect specific aminoglycosides and usually produce higher level resistance.

Impermeability resistance has arisen by porin (outer membrane protein) loss in *E. coli*. However, this does not provide a complete barrier to passage across the outer membrane and only confers low level resistance (Bryan, 1989a). In some clinical isolates of *P. aeruginosa* showing aminoglycoside resistance, an altered LPS structure in the cell membrane has been described (Bryan *et al.*, 1984). Such resistance is chromosomally specified and non-transferable. It is stable in vitro and independent of exposure to aminoglycoside.

It appears to be the cytoplasmic membrane that provides the principle barrier to aminoglycoside entry into cells (Higgins, 1990). Bacteria may be intrinsically resistant to aminoglycosides because they are unable to accumulate sufficient antibiotic. Strict anaerobes that respire without a functioning electron transport chain, for example, are unable to drive the energy requiring phases of accumulation and cannot maintain an adequate negative internal potential for accumulation to occur. Likewise some fermentative bacteria, such as streptococci, may exhibit low level resistance due to incomplete electron transport systems (Hancock, 1981b). Oxidative energy production is an absolute requirement for aminoglycoside transport across bacterial membranes (Hancock and Bell, 1988).

Several energetic mutants have been isolated which demonstrate impermeability to aminoglycosides. An inability to maintain the requisite electrical potential or a functional electron transport chain is characteristic of each of these mutants. These energetics mutants are blocked before EDP I. Any mutation affecting the establishment of the necessary proton gradient will block the accumulation of aminoglycoside antimicrobial agents (Hancock and Bell, 1988). Several examples of clinical or animal infection isolates have been reported (Annear, 1975; Musher *et al.*, 1977; Musher *et al.*, 1979; Pelltier *et al.*, 1979; Miller *et al.*, 1980; Gerber and Craig, 1982). These strains and others have been shown to have defects such as a deficiency in respiratory quinone content (Bryan and van den Elzen, 1976; Acar *et al.*, 1978; Miller *et al.*, 1980), a deficiency in terminal cytochromes (Bryan *et al.*, 1980; Bryan and Kwan, 1981), a hemin deficiency (Bryan and van den Elzen, 1976), increased proton leakiness (Alder and Rosen, 1976), and reduced NADH and D-lactate oxidase content (Muir *et al.*, 1981). These mutants generally produce 4 to 8 fold increases in resistance (Bryan, 1989a). Two interpretations of the cause of resistance in these mutants have been made. One is that there is a need for electron transport and that one or more components of electron transport act as transporters or facilitators of aminoglycoside transport (Bryan, 1989a). The second is that resistance arises because the cytoplasmic membrane potential is impaired such that the threshold value is not achieved or achieved at a much slower rate (Bryan, 1989a).

Stable permeability mutants regularly produce a small colony phenotype (Bryan, 1989a). The clinical significance of stable permeability mutants remains unknown. It has been suggested that their occurence should, generally, be considered clinically unimportant because their growth rates are significantly slower than those of normal size

colonies (Bryan, 1989a). Therefore permeability mutants in an aminoglycoside free environment are at a selective disadvantage and may be readily overgrown by revertants or sheltered susceptible cells.

4. *Pseudomonas aeruginosa*

a. Identification and Morphology

Most *P. aeruginosa* are identified on the basis of their characteristic grapelike odor (aminoacetophenone), colony morphology, growth at 42°C, and production of a water-soluble blue, non-fluorescent, phenazine pigment known as pyocyanin (Lory, 1990; Gilardi, 1991). *P. aeruginosa* also produce yellow fluorescent pigments known as pyoverdins which are Fe^{3+} chelating siderophores. The combination of yellow pyoverdins and blue pyocyanin give the green color associated with most *P. aeruginosa* strains. *P. aeruginosa* is classified as a nonfastidious, oxidase and catalase positive, indole negative, glucose nonfermenting, obligate aerobic gram-negative bacilli (Lory, 1990; Gilardi, 1991). *P. aeruginosa* cells are straight or slightly curved asporogenous rods (usually less than 1 μm in diameter and not more than 4-5 μm in length) with polar, usually monotrichous flagella for motility. *P. aeruginosa* form flat spreading colonies with irregular edges on blood agar plates. Strong hemolysis is noticeable on blood agar plates, especially with longer incubation times.

b. Pathogenic Profile

P. aeruginosa is ubiquitous in nature. It is widely distributed in the soil, water, sewage, the mammalian gut, and plants, and is frequently isolated from infusion fluids, disinfectants, cosmetics, and foodstuffs (Lory, 1990; Gilardi, 1991). The organism may cause disease in humans, certain animals, insects and plants (Lory, 1990; Gilardi, 1991).

In humans, *P. aeruginosa* rarely causes disease in healthy individuals (very low incidence of community acquired infections) but may establish infection in hospitalized patients whose natural immunity has been compromised. *P. aeruginosa* is an opportunistic pathogen which typically causes infections, frequently fatal, in victims of severe burns, in cancer patients who have been treated with immunosuppressive drugs, and in victims of cystic fibrosis. *P. aeruginosa* is an agent of meningitis, septicemia, endocarditis, severe epidemic diarrhea of infants, ocular infection, burn wound infection, cystic fibrosis-related lung infection, hot tub and whirlpool-associated folliculitis, osteomyelitis, malignant external otitis, pneumonia, and urinary tract infection (Drusano, 1991; Gilbert, 1995).

P. aeruginosa elaborates a variety of potential virulence factors. They include alginate (a polysaccharide composed of D-mannuronic acid and L-guluronic acid units), two hemolysins (phospholipase C and rhamnolipid hemolysin), toxin A (a secreted protein with ADP-ribosyl transferase activity), exoenzyme S (a secreted protein with ADP-ribosyl transferase activity), a cell associated protein cytotoxin (leukocidin), fibrinolysin, lipase, esterase, DNase, elastase, endotoxin, and fimbriae, all of which may contribute to *P. aeruginosa* pathogenicity (Lory, 1990; Gilardi, 1991).

The majority of scientific literature describing aminoglycoside induced adaptive resistance concerns *P. aeruginosa*. *P. aeruginosa* is an important medical pathogen because of its intrinsic resistance to many common antibiotics. This makes it one of the most difficult to treat nosocomial infections. *P. aeruginosa* is a common nosocomial pathogen and has the highest mortality rate of any bacterium (40 to 93% for *P. aeruginosa* bacteremia) (Korvick and Yu, 1991). Aminoglycoside antibiotics are a cornerstone of *P. aeruginosa* therapy (Korvick and Yu, 1991; Gilbert, 1995). Therefore,

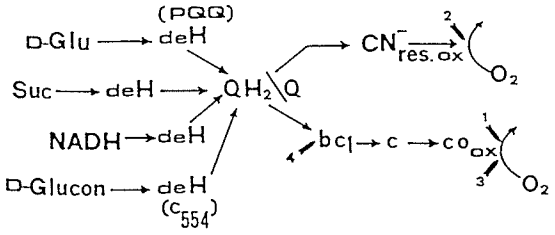
understanding the development of, possible prevention of, and the mechanism of aminoglycoside adaptive resistance is important.

c. Respiratory Pathways of *Pseudomonas aeruginosa*

P. aeruginosa ATP generation arises exclusively from oxidative phosphorylation (Palleroni, 1984). Substrate level phosphorylation does not occur in *P. aeruginosa* (Palleroni, 1984). For this reason, *P. aeruginosa* is commonly, although somewhat misleadingly, classified as a strict aerobe. However, despite its preference for aerobic environments, *P. aeruginosa* is well adapted to conditions of oxygen limitation given the presence of a suitable terminal electron acceptor such as nitrate (NO_3^-) or nitrite (NO_2^-).

Bacterial respiratory pathways generally contain several dehydrogenases (which deliver electrons to the cytoplasmic membrane bound respiratory chains), two or three intermediate reductive enzymes, and one or more types of terminal oxidase (Zannoni, 1989). The components of both the aerobic and anaerobic respiratory pathways of *P. aeruginosa* are presented in Figure 3. Significant overlap exists between the two pathways (Figure 3). The aerobic pathway terminates in cytochrome c oxidase (cyanide sensitive - [KCN 50% inhibitory concentration (IC_{50}) = 5μM KCN]) while the anaerobic pathway terminates in nitrite reductase (cyanide resistant - [KCN (IC_{50}) = 30mM KCN]) (Figure 3). Cytochrome c oxidase reduces O_2 to H_2O, while nitrite reductase can reduce O_2 to H_2O, and NO_2^- (nitrite) to both NO (nitric oxide) and N_2O (nitrous oxide) (Figure 4) (Zannoni, 1989; Cunningham and Williams, 1995). Cytochrome oxidase is completely inhibited by a cyanide concentration of 0.1 mM while nitrite reductase is resistant to cyanide concentrations in excess of 1 mM, as well as to sodium azide (Cunningham and Williams, 1995). The cyanide sensitive cytochrome c oxidase serves as the major terminal electron acceptor under aerobic conditions (Zannoni, 1989). However, the

Figure 3. ***Pseudomonas aeruginosa*** **electron transport pathways**[a]**.** Abbreviations: D-Glu, D-glucose; D-glucon, D-gluconate; deH, dehydrogenase; Suc, succinate; PQQ, pyrroloquinoline quinone; c-554, cytochrome c-554; QH_2/Q, ubiquinol/ubiquinone pool; bc_1, bc_1 oxido-reductase complex; c, soluble cytochrome c; co_{ox}, cytochrome c oxidase of co-type; CN^- res. ox, cyanide resistant oxidase. Short thick arrows indicate sites of inhibition by low- and high-concentrations of KCN (1 and 2, respectively), nitrite (3) and antimycin (4).



[a] Reproduced from Zannoni, 1989.

alternative, cyanide resistant oxidase (nitrite reductase) is also active under normal physiological conditions and contributes to aerobic energy conversion reducing O_2 to H_2O (Matsushita *et al.*, 1983; Silvestrini *et al.*, 1990; Cunningham and Williams, 1995). Nitrite reductase (dimeric form) shows an affinity for O_2 that is approximately 1000 times lower than for NO_2^- (Silvestrini *et al.*, 1990).

P. aeruginosa's resilience to cyanide may initially seem odd. However, *P. aeruginosa* itself produces cyanide in cultures supplemented with glycine, serine or threonine (Collins *et al.*, 1980), as well as in stationary phase and in carbon source limited cultures (Akimenko *et al.*, 1983; Castric, 1983). The cyanide synthase system is membrane bound and cyanide production increases with reductions in oxygen tension (Castric, 1983; Zannoni, 1989). Cyanide concentrations rise to 200 to 300 μM in *P.*

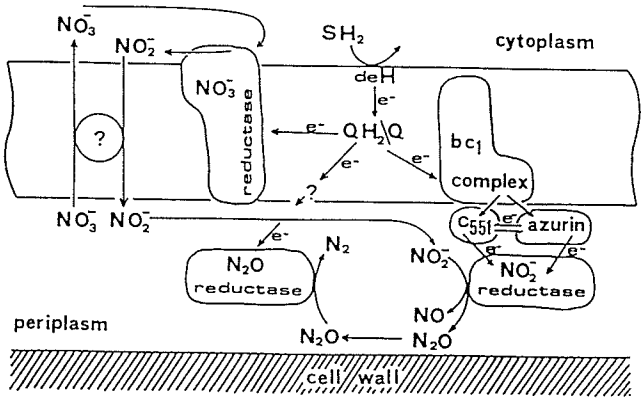
aeruginosa stationary phase cultures (Cunningham and Williams, 1995).

In vitro, *P. aeruginosa* can grow anaerobically in media supplemented with nitrate (NO_3^-) or nitrite (NO_2^-) (Zannoni, 1989). Nitrite serves as the terminal electron acceptor molecule providing a pathway for oxidative phosphorylation (also known as nitrate respiration) (Figure 4). Nitrate respiration depends on nitrate and nitrite reductase activities (Zannoni, 1989). Nitrate reductase of *P. aeruginosa* is an intregral cytoplasmic membrane protein that has been shown to consist of two polypeptides: α (approximately 150 kDa) and ß (approximately 60 kDa) (Carlson *et al.*, 1982). Nitrate reduction to nitrite occurs at a cytoplasmic location on nitrate reductase (Zannoni, 1989). Nitrite reductase is isolated as a homodimer of 120 to 130 kDa with two equivalent subunits of 58 to 65 kDa (Silvestrini *et al.*, 1989). Each monomeric unit contains one c-type haem and one haem designated as d_1 (Silvestrini *et al.*, 1989). Nitrite reduction has been demonstrated to occur in the periplasmic space of *P. aeruginosa* (Brunch and Knowles, 1982) and involves two soluble redox carriers (cytochrome c-551 and azurin, a Cu^{2+} containing protein) (Zannoni, 1989). Reduced cytochrome c-551 serves as an electron donor to nitrite reductase to catalyze the reduction of nitrite. Azurin may act as an electron donor directly to nitrite reductase or to cytochrome c-551. The physiological significance of having two electron substrates for the nitrite reductase of *P. aeruginosa* is unclear (Zannoni, 1989). Nitrous oxide (N_2O) reductase is a water soluble, periplasmic, cytoplasmic membrane associated protein with a mass of approximately 47 kDa (Zannoni, 1989).

The vast majority of nitrite reductase studies have used the dimeric enzyme (2 non-covalently associated identical monomers). A recent study has prepared and tested monomeric (60 kDa) nitrite reductase in an active form (Silvestrini *et al.*, 1995).

Interestingly, the evidence reported in this paper indicated that the dimeric state of *P. aeruginosa* nitrite reductase was not a prerequisite for the O_2 reductase activity of this enzyme. Stopped flow studies showed that the reduced monomer reacts with O_2 with a kinetic pattern similar to that shown by the dimeric enzyme (Silvestrini *et al.*, 1995). However, the reduced monomer demonstrates much higher O_2 affinity (approximately 100 times higher) and a higher reaction rate (approximately twice as fast) than the dimeric enzyme. Investigating the existence of elevated, monomeric (60kDa) nitrite reductase levels may be an area worthy of future study.

Figure 4. **Anaerobic respiratory and denitrification pathways of *Pseudomonas aeruginosa*[a].** Abbreviations: NO_3^-, nitrate; NO_2^-, nitrite; N_2O, nitrous oxide; NO, nitric oxide; N_2, atmospheric nitrogen gas; and ?, putative nitrate-nitrite antiporter. NO_3^- reductase is composed of 2 subunits of 150 and 60 kDa respectively. NO_2^- reductase is non-covalently associated homodimer of identical 60 kDa subunits. N_2O reductase consists of a single 47 kDa subunit.



[a] Reproduced from Zannoni, 1989.

d. Denitrification

Bacterial denitrification returns elemental nitrogen to the atmospheric reservoir by stepwise reductions in N oxides to N_2 gas. Oxygen limitation and the presence of nitrate (NO_3^-) are known to induce denitrification. Denitrification includes the anaerobic respiratory pathway of *P. aeruginosa* (Figure 4) (Carlson and Ingraham, 1983; Davies *et al.*, 1989). Nitrate and two intermediates of the denitrification pathway, nitrite (NO_2^-) and nitrous oxide (N_2O), are used as electron acceptors for anaerobic growth by *P. aeruginosa* (Carlson and Ingraham, 1983; Bazylinski *et el.*, 1986). Nitric oxide (NO), which is an intermediate between nitrite and N_2O in *P. aeruginosa* (Vosswinkel *et al.*, 1991), is reduced to N_2O and then to N_2 by anaerobic cell suspensions of *P. aeruginosa* (Kalkowski and Conrad, 1991). The enzymes participating in the denitrification pathway are dissimilatory nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. It appears that strict anaerobiosis is not a requirement for denitrification in *P. aeruginosa*; in fact, the denitrifying system of this organism can tolerate high oxygen concentrations (Lloyd *et al.*, 1987; Davies *et al.*, 1989).

e. ANR

Recent experiments have shed some light on the molecular mechanisms of transcription initiation during aerobic to anaerobic transition in *P. aeruginosa.* A regulatory gene, designated *anr* (for anaerobic regulation of arginine deiminase and nitrate reduction), that is essential for nitrate respiration and anaerobic arginine degradation in *P. aeruginosa* has recently been identified (Galimand *et al.*, 1991). The identification of *anr* began in 1971 (van Hartingsveldt *et al.*, 1971) with experiments investigating the genetic basis of denitrification in *P. aeruginosa.* A number of nitrosoguanidine-induced mutants defective in dissimilatory nitrate reductase or nitrite reductase were isolated (van

Hartingsveldt *et al.*, 1971). Recent study of one of the nitrite reductase (*nir*) mutants, specifically *nirD*, demonstrated that anaerobic growth of this mutant on nitrite or arginine could be restored by complementation with a 1.3-kb chromosomal fragment inserted into a plasmid vector (Galimand *et al.*, 1991). The insert contained a single gene, which was designated *anr* to indicate its role in global anaerobic regulation and the old genotype symbol *nirD* was abandoned (Galimand *et al.*, 1991).

The molecular mechanisms that operate during the switch from aerobic to anaerobic conditions are best understood in *E. coli*. This bacterium has two oxygen sensing systems. First, during anaerobiosis the FNR protein acts as a transcriptional regulator which positively controls a variety of genes required for anaerobic metabolism. As a consequence, *fnr* mutants are pleiotropically defective in fumarate (another electron acceptor molecule) and nitrate respiration, and other anaerobic functions (Spiro and Guest, 1990; Unden and Trageser, 1991). The second oxygen-sensing system consists of two components, ArcA and ArcB, which turn off the synthesis of many enzymes involved in aerobic metabolism when cells are grown anaerobically. The ArcB protein is a membrane sensor. It transmits a signal (anoxia or the presence of a reduced component of the respiratory chain) to the regulatory protein ArcA. Signal transduction is thought to occur by the transfer of a phosphoryl group from ArcB to ArcA. Phosphorylated ArcA is then thought to repress genes of aerobic respiration directly (Iuchi and Lin, 1988; Iuchi *et al.*, 1990).

anr Maps at about 59 minutes on the genomic map of *P. aeruginosa* PAO1 (Galimand *et al.*, 1991). The molecular weight of the *anr* gene product is 27,129, as deduced from the nucleotide sequence (Zimmermann *et al.*, 1991). However, the ANR protein demonstrates an apparent molecular size of 31 to 32 kDa when electrophoresed

through a sodium dodecyl sulfate-polyacrylamide gel (Zimmermann *et al.*, 1991; Sawers, 1991). The ANR protein of *P. aeruginosa* has 51% amino acid sequence identity with the FNR protein of *E. coli* (Zimmermann *et al.*, 1991). The DNA binding helix-turn-helix motif in the C-terminal portion of FNR has a very similar counterpart in ANR, with 67% identical and another 21% similar residues (Green *et al.*, 1991). FNR dependent promoters of several *E. coli* genes can be anaerobically regulated by ANR, both in *P. aeruginosa* and *E. coli* hosts (Galimand *et al.*, 1991; Sawers, 1991). *anr* Recognition sequences in *P. aeruginosa* promoters have been identified: $TTGATN_4GTCAA$ (*denAB* operon - *denA* codes for nitrite reductase and *denB* codes for cytochrome c-551); $TTGACN_4ATCAG$ (*arcDABC* - anaerobic arginine metabolism operon); and $TTGACN_4ATCAG$ (azurin) (Ye *et al.*, 1995).

In summary, three metabolic functions of ANR have been described to date: induction of anaerobic arginine catabolism, nitrate respiration, and cyanogenesis. Further work on the same *anr* deficient mutant of *P. aeruginosa* described earlier, demonstrated that a basal level of nitrate reductase activity was still present in this mutant, which was non-inducible by oxygen limitation (Galimand *et al.*, 1991). No nitrite reductase or nitrous oxide reductase activities were detectable in the *anr* mutant (Galimand *et al.*, 1991).

5. Thesis Objectives

The objectives of this thesis were to confirm and characterize *P. aeruginosa* adaptive resistance to aminoglycoside antimicrobial agents and to attempt to determine the mechanism(s) by which aminoglycoside adaptive resistance may be arising in *P. aeruginosa.* It is hoped that a more detailed understanding of aminoglycoside adaptive

resistance may provide for improved clinical use of these agents. It is only through the detailed study of antimicrobial agent resistance mechanisms, that the limitations of these agents can be determined, and that suggestions for new and improved agents can be made.

B. MATERIALS AND METHODS

1. Bacterial Strains

Four clinical blood culture isolates (F327, F443, F991, E1481) and two reference (ATCC 27853, PAO1) strains of *P. aeruginosa* were used in many of the experiments characterizing aminoglycoside adaptive resistance. For lipopolysaccharide studies four *P. aeruginosa* strains were used (PAO1, AK1401, dps89, rd7513). *P. aeruginosa* PAO6261 (*anr*⁻ *P. aeruginosa* PAO1), pME3580 (*anr*⁺) in *E. coli* ED8767, and pRK2013 (mobilizing plasmid) in *E. coli* HB101 were used in functional studies. One clinical isolate (G1307) and one reference strain (ATCC 25922) of *Escherichia coli* were used in postantibiotic effect (PAE) and adaptive resistance determinations. Isolates were frozen (-70°C) in skim milk and transferred onto blood agar plates monthly. At weekly intervals, isolates were subcultured onto fresh blood agar plates and streaked for purity.

2. Antimicrobials

The aminoglycoside antibiotics used were amikacin (Bristol-Myers Pharmaceutical Group, Ottawa, Ontario, Canada), gentamicin (Schering Corp. Ltd., Pointe-Claire, Quebec, Canada) and tobramycin (Eli Lilly, Toronto, Ontario, Canada). As well the fluoroquinolones ciprofloxacin (Miles Pharmaceuticals, Etobicoke, Ontario, Canada) and pefloxacin (Rhone Poulenc Rorer, Montreal, Quebec, Canada) were used. The ß-lactams used were ceftazidime (Glaxo, Mississauga, Ontario, Canada), cefotaxime (Hoechst-Roussel Pharmaceuticals, Montreal, Quebec, Canada), and pipercillin (Cyanamid, Baie d'Urfe, Quebec, Canada). As well the carbapenem, imipenem (Merck Sharp and Dohme, Rayway, New Jersey, U.S.A.) was used. Chloramphenicol, polymyxin B and tetracycline (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) were also used. Stock solutions

Table 4. Strains of *Pseudomonas aeruginosa* and *Escherichia coli* and plasmids.

Bacteria or Plasmid	Strain	Phenotype or Genotype	Source or Reference
P. aeruginosa	ATCC 27853	wild-type	American Type Culture Collection
	PAO1	wild-type, 05 (B-Band) LPS, A-Band LPS	Kadurugamuwa *et al.*, 1993
	F327	bacteremia isolate	HSC, Dept. of Clin. Microbiol.
	F443	bacteremia isolate	HSC, Dept. of Clin. Microbiol.
	F991	bacteremia isolate	HSC, Dept. of Clin. Microbiol.
	E1481	bacteremia isolate	HSC, Dept. of Clin. Microbiol.
	AK1401	A-Band LPS only	Kadurugamuwa *et al.*, 1993
	dps89	revertant of rd7513, B-Band LPS	Kadurugamuwa *et al.*, 1993
	rd7513	A-Band deficient mutant of AK1401	Kadurugamuwa *et al.*, 1993
	PAO6261	PAO1 with *anr* internal 0.33 kb deletion	Ye *et al.*, 1995
	PAO6261(*anr*$^+$)	PAO6261 complemented with pME3580	thesis
E. coli	ATCC 25922	wild-type	American Type Culture Collection
	G1307	bacteremia isolate	HSC, Dept. of Clin. Microbiol.
	ED8767	*metB, hsdS, supE, supF*	Galimand *et al.*, 1991
	HB101	*hsdS20, recA13, proA2, leu-6, thi-1, rpsL20, ara-14, galK2, lacY1, xyl-5, mtl-1*	Galimand *et al.*, 1991
Plasmid	pME3580	Amp^R, Cb^R Tet^R Mob IncQ (13.8 kb); pKT240 with 1.3 kb *SacII* fragment carrying *anr*	Galimand *et al.*, 1991
	pRK2013	Tet^R Km^R Tra ColE1 replicon (ca. 50 kb)	Galimand *et al.*, 1991

HSC - Health Sciences Centre
Amp^R - ampicillin resistant
Cb^R - carbenicillin resistant
Tet^R - tetracycline resistant
Km^R - kanamycin resistant

of antimicrobial agents were prepared from standard powders and stored at -70°C. On the day of use, antimicrobial agent concentrates were thawed and then diluted into appropriate media to achieve desired concentrations. Imipenem was an exception to this practice because of its lability. Imipenem stock solutions were prepared fresh daily according to the manufacturer's instructions.

3. Radiolabelled Antimicrobials and Reagents

^{3}H-gentamicin was obtained (Amersham Canada Limited, Oakville, Ontario, Canada) which had a specific activity of 18.9 GBq/mmol and was diluted by the addition nonradioactive (cold) gentamicin to a final specific activity of 0.69 GBq/mmol prior to use. ^{3}H-proline was obtained (Dupont, Markham, Ontario, Canada) which had a specific activity of 1.4TBq/mmol and was diluted by the addition of nonradioactive (cold) proline to a final specific activity of 0.29TBq/mmol and was used at a final proline concentration of 1 µM. ^{3}H-tetraphenylphosphonium (^{3}H-TPP^{+}) was obtained (DuPont, Markham, Ontario, Canada) which had a specific activity of 1.4TBq/mmol and was diluted by the addition of nonradioactive TPP^{+} to a final specific activity 0.29TBq/mmol and was used at a final TPP^{+} concentration of 20 µM. ^{14}C-inulin was obtained (Amersham Canada Limited, Oakville, Ontario, Canada) which had a specific activity of 0.33GBq/mmol and was used at a final concentration of 73.3 KBq/ml. ^{3}H-H_2O was obtained (Amersham Canada Limited, Oakville, Ontario, Canada) which had a specific activity of 0.67 MBq/mmol and was used at a final concentration of 0.37 MBq/ml.

4. Culture Media

Cation (25 mg of $CaCl_2$ per litre, 12.5 mg of $MgSO_4$ per litre) supplemented

Mueller-Hinton Broth (MHB) (pH 7.2 to 7.4) (Difco Laboratories, Detroit, Mich.) was used as culture media for MIC, MBC, PAE, adaptive resistance, and accumulation studies unless otherwise noted. Colony counts were performed by using trypticase soy agar (Scott Laboratories, Fiskeville, R.I.) supplemented with 5% defibrinated sheep blood (blood agar).

5. Antimicrobial Susceptibility Testing

Determination of MICs for all strains tested were made initially by the macrodilution broth method with doubling dilutions as described by the National Committee for Clinical Laboratory Standards (NCCLS, 1990). Following this, arithmetic MICs were determined for selected strains (*P. aeruginosa* F327, F443, F991, E1481, ATCC 27853). Arithmetic antimicrobial dilutions were prepared in 1 ml of MHB and MICs determined to the nearest 0.1 mg/l. The inoculum was adjusted from an exponential phase culture to yield an initial concentration of approximately 5×10^5 cfu/ml. After 18 hours of incubation at 37°C, the MIC was determined as the lowest concentration of antimicrobial that prevented visible growth. Arithmetic MIC determinations were performed on at least 3 separate occasions. The MICs determined in all multiple exposure PAE and MIC reversion time experiments were based on doubling dilutions using multiples of the known arithmetically determined MIC.

MBCs were determined following the preparation of cultures in 1 ml of MHB to yield final concentrations of 0.5, 1, 2, 3, 4, 5 and 8 times the arithmetically determined MIC values. After 18 hours of incubation at 37°C all tubes with no visible growth were subcultured by spreading aliquots of 10ul from each tube onto half plates of blood agar. The MBC was determined as the lowest concentration of antibiotic showing less than

0.1% survival of the inoculum (10 colonies).

6. Minimum Inhibitory Concentration (MIC) Reversion Time Determinations

Given the limitation of MICs as a single measure of antimicrobial activity, it was the intent of the experiments described here to measure MICs repeatedly at fixed intervals, in order to assess susceptibility changes in bacterial cultures over time (i.e. MIC reversion time [MRT]) (Karlowsky *et al.*, 1994b). Experiments were conducted to evaluate the impact of gentamicin and tobramycin concentration on the time required for the MICs of five *P. aeruginosa* strains (ATCC 27853, F327, F443, F991, E1481) to revert to their original values (MRT) following single and multiple two hour aminoglycoside exposures.

An overnight broth culture incubated in a shaking water bath (Labline Instruments Inc., Melrose Park, Ill.) at 37°C was diluted 1:10 into fresh MHB and allowed to regrow until the optical density of the culture at 580 nm approached 0.3 (Spectronic 1201 spectrophotometer; Milton Roy, Rochester, N.Y.). The resultant logarithmic phase culture was then diluted 1:10 into MHB containing either the desired concentration of aminoglycoside (1X MIC, 8 or 24 mg/l) (test culture) or MHB alone (control culture). This procedure resulted in a final bacterial concentration of approximately 10^7 cfu/ml. Following a 2 hour exposure, aminoglycosides were removed by centrifugation. Test cultures and growth controls were centrifuged at 4000 x g for 10 minutes, the supernatant above the pellet decanted, and the pellet resuspended in prewarmed (37°C) MHB to the initial volume. Two additional centrifugations were sufficient to reduce extracellular antimicrobial to inactive levels. Residual antimicrobial controls (RAC), containing 1/1000 of the test antimicrobial concentration, and treated in the same manner as the test solution,

ensured that aminoglycoside concentrations, present in washed cultures, had been reduced to inactive levels (demonstrated by viable colony counting) (Karlowsky *et al.*, 1993). In addition, TDX analysis (sensitivity, 0.1 mg of amikacin, gentamicin, netilmicin, and tobramycin per litre; Abbott Laboratories, Ltd., Mississauga, Ontario, Canada) was also used to confirm that aminoglycoside levels had been reduced to inactive levels. Cultures were allowed to regrow, with aliquots transferred to fresh MHB as necessary to maintain the cultures in logarithmic phase.

Experiments to determine the MRT were performed in triplicate for each strain following both single and multiple aminoglycoside exposures. The MICs determined in all single and multiple exposure MRT experiments were based on doubling dilutions by using multiples of the known arithmetically determined MIC, as described in the antimicrobial susceptibility testing section. Cultures were considered to have reverted to their original susceptibility when the MIC was within one doubling dilution of its original value (NCCLS, 1990). MICs were determined every 4 hours once a sufficient inoculum (approximately 5.5×10^5 cfu/ml) was present (NCCLS, 1990). Sufficient inocula were attainable at approximately 8 hours following exposure to 1X MIC and 8 mg/l, and 12 to 16 hours following exposure to 24 mg/l.

Multiple exposure MRTs were determined by a modification of the single exposure method described above. For 1X MIC and 8 mg/l, when a total of 8 hours had passed from the start of the initial 2 hour aminoglycoside exposure, the culture was diluted, if necessary, to approximately 10^6 to 10^7 cfu/ml and re-exposed to the same aminoglycoside concentration. The entire procedure was repeated again 8 hours later. Multiple exposure MRTs were determined following the third exposure. Similarly, three 2 hour aminoglycoside exposures to 24 mg/l, every 24 hours, were performed and the MRTs

determined.

To test the limits of MRT, and also adaptive resistance induction, *P. aeruginosa* strains were passed against increasing amikacin, gentamicin and tobramycin concentrations until stable growth was no longer observed. The previous culture (exhibited stable growth) was then used as the starting point for MIC reversion time experiments. As well these transient, highly aminoglycoside resistant cells were used to determine MICs against other antimicrobial agents, from different chemical classes, to see if cross resistance existed.

7. Postantibiotic Effect (PAE) Determinations

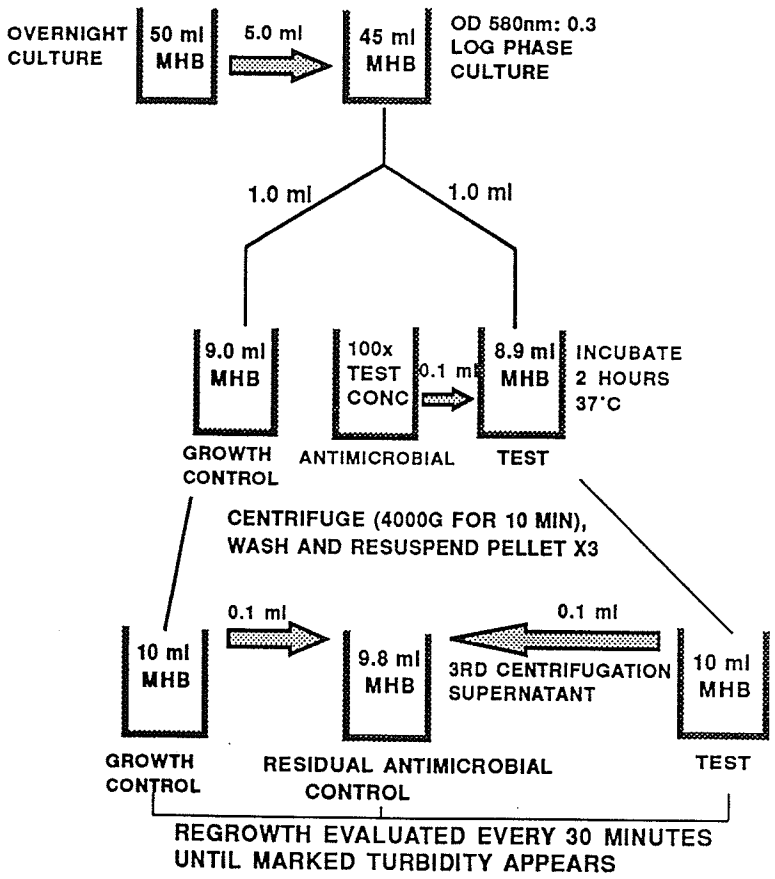
a. Single Exposure PAE Determination

All PAE studies were performed with cultures of the organism in the logarithmic phase of growth at the time of initial antimicrobial agent exposure. Logarithmic phase cells were prepared as described in the MRT determination section. Following a 2 hour antimicrobial agent exposure, the test cultures and growth control were centrifuged, as described in the MRT determination section, to remove extracellular antibiotic. A residual antimicrobial control (RAC) was also maintained. Viable counts of cfu/ml were made for all cultures immediately before antimicrobial agent exposure, following two hour antimicrobial agent exposure, after centrifugation to remove the antimicrobial agent and every 30 minutes thereafter until marked turbidity was noticed. Serial 10-fold dilutions were made using sterile, 4°C, 0.85% NaCl. Aliquots of 0.01, 0.10 and 1.00 ml of the appropriate dilutions were plated using a spread plate technique. Plates were incubated at 37°C and read after 18 to 24 hours.

The PAE was determined using the equation PAE = T - C, where T is the time

(minutes) required for the $\log_{10}$ cfu/ml count in the test culture to increase one $\log_{10}$ above the count immediately following dilution, and where C is the time (minutes) required for the $\log_{10}$ cfu/ml count in the control culture to increase one $\log_{10}$ above the count immediately following dilution (Craig and Gudmundsson, 1991).

Figure 5. PAE Method



b. Multiple Exposure PAE Determinations

Multiple antimicrobial agent exposure PAE determinations were made using the aforementioned single exposure method with the following revisions. When a total of 8 hours had passed from the time of the initial antimicrobial agent exposure the culture

was diluted, if necessary, to 10^6-10^7 cfu/ml and re-exposed to the same antimicrobial agent concentration. This entire process was repeated once more eight hours later. Cultures of both reference and clinical strains were exposed to antimicrobial agents, for two hour periods, at eight hour intervals over 24 hours (i.e. 3 doses). In addition, MICs were determined for all cultures just prior to each of the three antimicrobial agent exposures, at 24 hours following the initial antimicrobial agent exposure, and at daily intervals thereafter until cultures returned to their initial sensitivities. One hundred microlitres of culture was transferred to approximately 100 ml of fresh MHB every 4 to 12 hours, as necessary, to maintain the organism in log phase growth. In a related set of experiments, *P. aeruginosa* cultures were re-exposed to their (previously determined) induced aminoglycoside MICs on second and third exposures. In order to acquire the appropriate number of bacteria for re-exposure (10^6-10^7 cfu/ml) larger initial inocula, 5 x 10^7 cfu/ml, and larger initial culture volumes, were used as necessary. All multiple exposure experiments were performed at least twice, on separate days, with each bacterial strain and individual antimicrobial agent.

8. Adaptive Resistance Determinations

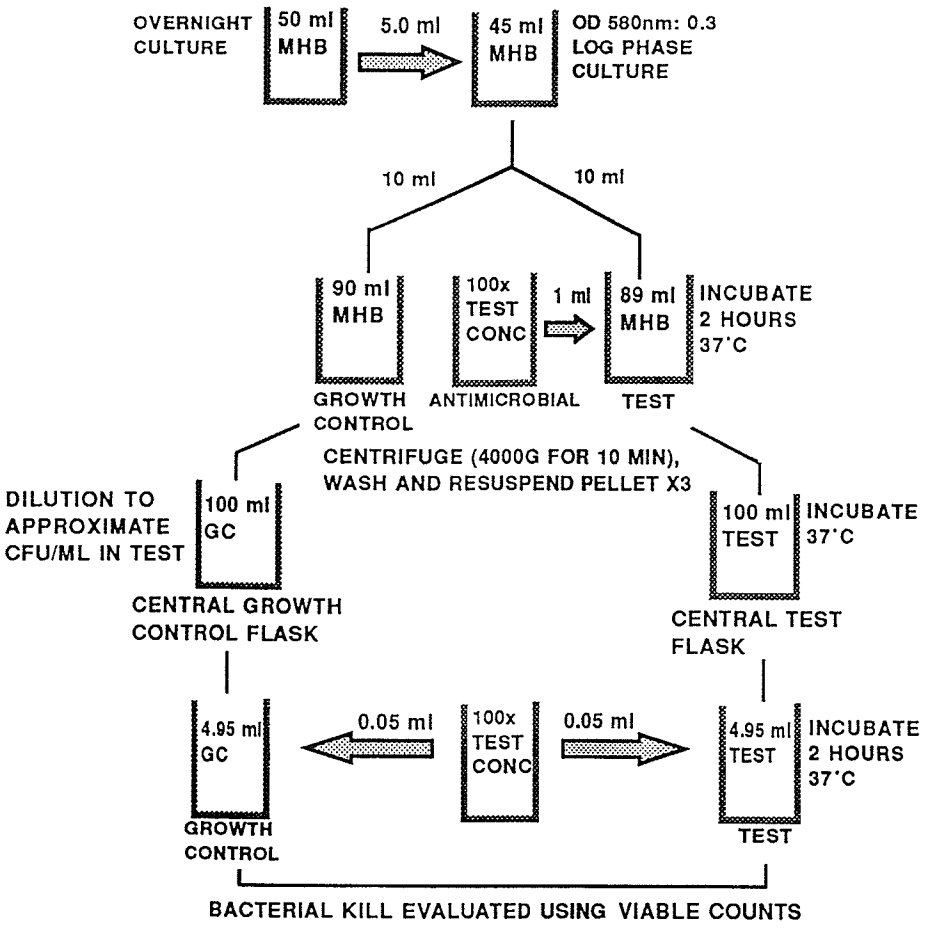
All adaptive resistance experiments were performed, as previously described (Daikos *et al.*, 1990b), with cultures of the organism in the logarithmic phase of growth at the time of the initial antimicrobial exposure. These cultures were prepared identically to those described above for MRT determinations. Test cultures were exposed to 1X MIC for 2 hours at 37°C. Growth control cultures were maintained simultaneously in drug free MHB. After 2 hours, the aminoglycoside was removed by triplicate centrifugation and pellet resuspension as described for MRT determinations (Karlowsky *et al.*, 1994b). The

final resuspension of the test culture yielded a suspension of approximately 10^4 viable cfu/ml which was incubated at 37°C. The growth control was adjusted to approximate this inoculum. Aliquots from both test and control cultures were removed, immediately following aminoglycoside removal, and at 2 hour intervals thereafter, re-exposed to aminoglycoside at 1X MIC for 2 hours, and bacterial killing noted by viable counting. The extent of bacterial killing in cultures with prior aminoglycoside exposure was compared over a period of up to 12 hours to bacterial killing in control cultures, that had not been previously exposed.

9. Aminoglycoside Killing Curves

Time-kill curves provide a dynamic picture of antimicrobial killing, the time to maximal killing, and regrowth kinetics of survivors in the continued presence of the antimicrobial agent. Time-kill curves were performed by a previously described method (Schoenknecht *et al*, 1985). All time-kill curve studies were done in a final volume of 10 ml of MHB in 50 ml glass Erlenmeyer flasks. Both the antimicrobial agent to be tested and the inoculum were added to maintain a final volume of 10 ml. Logarithmic phase cultures were prepared as described previously and used with an initial inoculum of 10^7 cfu/ml. Bacterial strains were exposed to subinhibitory (1/8X, 1/4X and 1/2X MIC) and inhibitory (1X MIC) concentrations of aminoglycosides. All cultures were incubated with shaking as described previously. Samples were obtained for colony counts at 0, 1, 2, 3, 4, 6, 12, and 24 hours of incubation. Time-kill curves were plotted showing time versus cfu/ml of broth. Experiments were performed on at least two occasions for each aminoglycoside concentration/*P. aeruginosa* strain combination.

Figure 6. Adaptive resistance method



10. *P. aeruginosa* Doubling Times

It is often convenient to express growth rate in terms of doubling times (t_D), also called the mean generation time (MGT). Doubling times were calculated as previously described (Koch, 1994). The growth rate constant (k) was first determined for each one or two hour time interval using the following equation, $k = (x-y)2.303/hours$, where x and y are $\log_{10}$ bacterial colony counts at the beginning and end of each one or two hour time interval. The doubling time was then calculated using the equation $t_D = 0.693/k$ as a bacterial culture undergoing balanced growth mimics a first order autocatalytic chemical reaction (i.e. the rate of increase in bacteria at any particular time is proportional to the

number or mass of bacteria present at that time) (Koch, 1994). Doubling times were determined at least ten times for each time point on separate occasions.

11. Susceptibility of Adaptively Aminoglycoside Resistant *Pseudomonas aeruginosa* to Other Antimicrobial Agents

Each of five *P. aeruginosa* strains (F327, F443, F991, E1481, ATCC 27853) was grown in the presence of doubling concentrations of aminoglycoside, beginning with a concentration of 1 mg/l, until MICs could no longer be increased at the expense of cell viability. The aminoglycoside resistant cells were allowed to replicate in antimicrobial free broth for 24 hours and then the MICs to aminoglycosides and other antimicrobials, of different chemical classes, determined as previously described. All high level aminoglycoside resistant variants repeatedly subcultured in fresh antimicrobial free Mueller-Hinton broth returned to their initial susceptibilities.

12. Gentamicin Accumulation in *Pseudomonas aeruginosa*

Accumulation of ^{3}H-gentamicin (Amersham Canada Limited, Oakville, Canada) by *P. aeruginosa* was measured using a previously described method (Fraimow *et al.*, 1991). The radiolabelled gentamicin had a specific activity of 1.1 mCi/mg and was diluted by the addition of nonradioactive (cold) gentamicin to a final specific activity of 40 µCi/mg. Non-specific binding controls were used for each accumulation experiment by treating an aliquot of cells with the protonophore carbonylcyanide *m*-chlorophenyl-hydrazone (CCCP) at a concentration of 50 µM, for 10 minutes. Accumulation assays were performed at 37°C. At defined times, 0.5 ml samples were removed and passed through nylon filters (0.45 µm pore, 25 mm diameter, Micron Separations Inc.) using

vacuum filtration. Filters were pretreated with a 3 ml aliquot of cold gentamicin (200 mg/l). Ionically bound gentamicin was eluted from the bacteria on the filters with 20 ml of a 3% (wt/vol) NaCl solution. Filters were dried at 60°C for 1 hour and placed in 10 ml Cytoscint scintillation cocktail (ICN) and the radioactivity counted in a LKB Rackbeta 1217 scintillation counter. All ^{3}H-gentamicin accumulation experiments were performed at least twice, on separate days, with each *P. aeruginosa* strain tested. Cell associated radioactivity was determined and the results expressed as ng gentamicin accumulation/mg cell protein.

13. Protein Determination

The Lowry method was used to quantify cell protein (Lowry *et al.*, 1951). In this method 0.1 ml of sample or standard was added to an equal volume of 2N NaOH and hydrolyzed at 100°C for 10 minutes in a boiling water bath. The hydrolyzate was then cooled to room temperature, 1 ml of complex-forming reagent added, and the solution let stand at room temperature for 10 minutes. Using a vortex mixer, 0.1 ml of folin reagent (Phenol Reagent [BDH Inc.] at a concentration of 1N) was added and the mixture let stand at room temperature for 30 minutes. The absorbance was then read at 750 nm using a Spectronic 1201 spectrophotometer. The amount of protein was determined from a previously constructed standard curve using bovine serum albumin (BSA) as the protein standard. Complex-forming reagent was prepared immediately before use by mixing the following 3 stock solutions: A (2% w/v Na_2CO_3 in distilled water), B (1% w/v $CuSO_4 \cdot 5H_2O$ in distilled water) and C (2% w/v sodium potassium tartrate in distilled water) in the proportion 100:1:1, respectively.

14. Gentamicin Accumulation in Adaptively Resistant *Pseudomonas aeruginosa*

In order to ensure sufficient numbers of viable cells were available for uptake experiments, the gentamicin accumulation assay, as described above, was modified. In addition, to ensure organisms were adaptively resistant during the accumulation assays, adaptive resistance determinations were simultaneously performed on aliquots taken from the same culture.

Initially a 500 ml culture of logarithmic phase *P. aeruginosa* was exposed to aminoglycoside at 1X MIC for 2 hours. Aminoglycoside, cell fragments and non-viable cells (theoretically less dense than live cells) were removed by triplicate 5 minute centrifugations at 1000 rpm and the final pellet resuspended in 5 ml of fresh 37°C Mueller-Hinton broth. Cell densities were adjusted to between 10^7 to 10^8 cfu/ml by optical density measurement and confirmed by viable colony counts. Following cell preparation, ^{3}H-gentamicin accumulation experiments were performed as described previously. Accumulation was measured, over 30 minutes, at 2 hour intervals, for 12 hours, following the initial 2 hour exposure and removal of aminoglycoside.

15. Spheroplast Preparation

Spheroplasts were prepared by the method of Bryan and van den Elzen (1977). Fifty millilitres of exponential phase (wild-type control), adaptively resistant, or post-adaptively resistant culture was pelleted by centrifugation at 4500 rpm for 15 minutes. The pellet was resuspended in approximately 20 ml of 15% sucrose (w/v) in 4 mM Tris-HCl, pH 8.0. Lysozyme was then added to a final concentration of 20 mg/l (made fresh daily as a 1 mg/ml solution) and the suspension incubated at 28°C for 20 minutes. Four millilitres of 200 mM Tris-HCl, pH 8.0, buffer was then added to the suspension followed

by sodium EDTA to a final concentration of 2 mM. Incubation was continued at 28°C until approximately 99% of cells were spheroplasts as determined by phase contrast microscopy. The suspension was then chilled in an ice bath and centrifuged at 4500 rpm for 12 minutes at 4°C. The pellet was then resuspended in 10 ml of 15% sucrose in 4 mM Tris-HCl, pH 7.6, and incubated with DNase I (20 mg/l) and RNase (40 mg/l) for 30 minutes at 28°C. The suspension was then chilled in an ice bath, centrifuged at 4500 rpm for 12 minutes at 4°C, and resuspended in 10 ml of 15% sucrose (w/v) sucrose in Mueller-Hinton broth. An aliquot of spheroplast culture was then taken for protein determination and viable counting on blood agar.

16. Gentamicin Accumulation in Spheroplasts

Gentamicin accumulation in spheroplasts was performed as described for whole cells, except that 15% sucrose was added to the culture media (Mueller-Hinton broth). Spheroplast cultures were warmed to 37°C, for 30 minutes, prior to gentamicin accumulation assay. Cell densities were adjusted to between 10^7 and 10^8 cfu/ml by optical density measurement and confirmed by viable colony counts. Optical density was monitored throughout each accumulation assay to assess cellular lysis or decrease in cell density.

17. Proline Accumulation in Exponential Phase and Adaptively Resistant *Pseudomonas aeruginosa*

Proline accumulation was assayed using ^{3}H-proline (DuPont, Markham, Canada) (specific activity 1.4 TBq/mmol; final specific activity 0.29 TBq/mmol; final proline concentration 1 µM) as previously described (Korvick and Yu, 1991). One millilitre

aliquots were removed at defined times, filtered through nylon filters, as described previously, and washed with 2 volumes of 0.1 M lithium chloride. Scintillation counting and protein assay were performed as described previously.

18. Determination of $\Delta\Psi$

Proton motive force (pmf, Δp), across a biological membrane (interior negative and alkaline), is the driving force for energy requiring processes such as solute transport and ATP synthesis (Mitchell's chemiosmotic hypothesis). Proton motive force is calculated using the following equation, $\Delta p=\Delta\Psi-Z\Delta pH$ where $\Delta\Psi$ is the electrical potential across the membrane, ΔpH is the chemical difference in proton concentration across the membrane, and Z is a constant (Z=RT/F, Z=61.5 at 37°C). All experiments were performed at pH 7.5, were $\Delta pH\approx 0$ and therefore $\Delta p\approx\Delta\Psi$ (Fraimow *et al.*, 1991). $\Delta\Psi$ was calculated from the Nernst equation, $\Delta\Psi=\log[TPP^+]_{in}/[TPP^+]_{out}$, where TPP^+ is the lipophilic cation ^{3}H-tetraphenylphosphonium, and in and out concentrations were determined from the steady state distribution values of TPP^+ (Fraimow *et al.*, 1991). Concentration gradients were calculated using a value of 3.15 µl of intracellular fluid/mg cell protein as determined by ^{14}C-inulin and ^3H_2O distribution (described in following section) (Daikos *et al.*, 1991). ^{3}H-TPP^+ (DuPont, Markham, Ontario) (specific activity 1.4 TBq/mmol; final specific activity 0.29 TBq/mmol) was used at a final concentration of 20 µM.

19. Determination of Intracellular Volume

Intracellular volume was determined, as the difference between total and extracellular volumes in cell pellets, by a previously described method (Rottenberg, 1979;

McCaffrey *et al.*, 1992). The total volume was measured with the permeant probe ^{3}H-H_2O and the extracellular volume by the large, nonmetabolizable, outer membrane impermeant probe ^{14}C-inulin. Intracellular volume was calculated using the following equation: intracellular volume = $volume_{supernatant}$ x ($^{3}H_{pellet}/^{3}H_{supernatant}$ - $^{14}C_{pellet}/^{14}C_{supernatant}$) (McCaffrey *et al.*, 1992). The intracellular volumes of five strains of *P. aeruginosa* (F327, F443, F991, E1481, ATCC 27853) were determined in triplicate.

20. Determination of Aminoglycoside Threshold $\Delta\Psi$

The aminoglycoside threshold $\Delta\Psi$ was determined by a previously described method (Gilman and Saunders, 1986). Valinomycin alone, at a final concentration of 2 µM, and in combination with variable KCl concentrations (0.5 to 10 mM K^+) was used to adjust $\Delta\Psi$. Valinomycin is a cyclic depsipeptide ionophore that specifically increases membrane permeability to K^+. In the presence of sufficient K^+ (50 to 100 mM K^+), valinomycin causes the complete collapse of $\Delta\Psi$ (Gilman and Saunders, 1986). ^{3}H-Gentamicin accumulation experiments were performed using logarithmic phase *P. aeruginosa* ATCC 27853, in the presence of 2.0 µM valinomycin and KCl concentations of 0.5 mM, 1 mM, 2 mM, 4 mM, 6 mM, 8 mM, and 10 mM. Proton motive force determinations were performed on another aliquot of the cells by the method described earlier. The threshold $\Delta\Psi$ was estimated by comparing these two groups of data.

21. The Influence of Metabolic Inhibitors on Aminoglycoside Accumulation in *Pseudomonas aeruginosa*

a. DNP, CCCP, NaN_3, KCN, NEM

The effect of the metabolic inhibitors 2,4 dinitrophenol (DNP), carbonyl cyanide

m-chlorophenylhydrazine (CCCP), sodium azide (NaN_3), potassium cyanide (KCN) and N-ethylmaleimide (NEM) on the accumulation of ^{3}H-gentamicin in *P. aeruginosa* was examined. Inhibitors were added at final concentrations of 5 mM DNP, 50 µM CCCP, 15 mM NaN_3, 1 mM KCN and 1 mM NEM 10 minutes after the addition of the radiolabelled antimicrobial. The concentrations of inhibitors were determined not to be bactericidal over the course of the assay.

DNP and CCCP are protonophores that dissipate the cytoplasmic membrane proton gradient (i.e. pmf) by uncoupling ATP synthesis from electron transport. KCN and NaN_3 are electron transport chain inhibitors which bind iron molecules (redox centres) found within cytochromes. KCN and NaN_3 inactivate cytochromes and prevent electron movement through respiratory pathways. NEM binds free sulfhydryl groups inhibiting the function of proteins with these surface functional groups.

b. DCCD

N,N'-dicyclohexylcarbodiimide (DCCD) is a H^+-ATPase inhibitor (Azzi *et al.*, 1984). DCCD reacts with acidic amino acid residues in the F_o portion of the H^+-ATPase which decreases the proton conductance of the membrane with resultant hyperpolarization (Azzi *et al.*, 1984). DCCD creates a concentration dependent increase in $\Delta\Psi$ with no effect on ΔpH (Azzi *et al.*, 1984). The effect of DCCD on $\Delta\Psi$ and aminoglycoside accumulation was tested to investigate the relationship between aminoglycoside induced adaptive resistance, pmf and bacterial killing in *P. aeruginosa.*

A concentrated DCCD stock solution (50 mM) was prepared in 95% ethanol and the appropriate volume added to 10 ml of test organism 15 minutes before ^{3}H-gentamicin or ^{3}H-TPP^+. DCCD was tested at concentrations from 0 µM to 300 µM. The quantities of ethanol added were determined not to be bactericidal over the course of the assay.

A DCCD concentration of 200 μM demonstrated maximum increases in pmf and gentamicin accumulation in both growth controls and adaptively resistant cells. The effect of 200 μM DCCD was then tested to determine its effect on adaptive resistance induction and duration using the previously described methodology.

22. Oxidase Test of Adaptively Resistant *Pseudomonas aeruginosa*

P. aeruginosa are oxidase positive bacteria (Lory, 1990). They utilize cytochrome c oxidase as the terminal electron donor in their aerobic respiratory pathway. Difco oxidase slides (Detroit, MI) were used, according to the manufacturer's instructions, to determine the presence of cytochrome c oxidase in adaptively resistant *P. aeruginosa.* The oxidation of di- or tetramethyl-p-phenylenediamine plus α-naphthol to indophenol blue (positive oxidase reaction) generally reflects the presence of a membrane bound high potential cytochrome c linked to an active cytochrome c oxidase.

23. Morphology of Adaptively Resistant *Pseudomonas aeruginosa*

Both phase contrast microscopy and electron microscopy were used to assess the morphology of wild-type control, adaptively resistant and post-adaptively resistant cells. Negative stain electron microscopy was performed according to a previously described method (Beveridge *et al.*, 1994). Bacterial cells were negatively stained with 2.5 mM phosphotungstic acid (PTA), adjusted to pH 7.0 with sodium hydroxide, and viewed on a Phillips model 201 electron microscope.

Cells were prepared for electron microscopy using the following method. An aliquot of bacterial suspension was removed from a culture at a designated time and the cells pelleted by gentle centrifugation (1000 rpm for 5 minutes). The supernatant was

then drawn off, discarded, and 25-50 µl of 0.1 M sodium cacodylate buffer, pH 7.2, added to resuspend the pellet and form an opalescent or even turbid suspension. The cells were mounted by floating the suspension on a 200 mesh Hex Formvar coated grid (the grid held in self-closing forceps) for approximately one minute. The suspension was then drawn off the grid with a Pasteur pipette and excess suspension removed by touching the edge of the grid with the freshly torn edge of a piece of filter paper. The preparation was then stained by applying 10-15 µl of 2.5 mM phosphotungstic acid (PTA), adjusted to pH 7.0 with sodium hydroxide, on the specimen for approximately 30 seconds. Excess PTA stain was then removed by touching the edge of the grid with the freshly torn edge of a piece of filter paper and the specimen viewed.

24. Lipopolysaccharide (LPS) Analysis

Aminoglycoside accumulation in susceptible bacterial cells is dependent upon outer membrane and cytoplasmic membrane characteristics (Davis, 1987; Taber *et al.*, 1987). Therefore outer membrane components (lipopolysaccharide and outer membrane proteins) and cytoplasmic membrane proteins were analyzed from wild-type control, adaptively resistant, and post-adaptively resistant cells of each *P. aeruginosa* clinical isolate (F327, F443, F991, E1481) and the ATCC 27853 reference strain.

The LPS of *P. aeruginosa* is chemically and structurally heterogenous (e.g. 17 O-antigen serotypes) (Kropinski *et al.*, 1985). Recent work indicates that *P. aeruginosa* produces two immunologically and chemically distinct forms of LPS known as A-band and B-band LPS (Lightfoot and Lam, 1991). B-band LPS is the O-antigen containing LPS and determines the O specificity of the bacterium, while A-band LPS contains shorter chains of predominantly neutral polysaccharide (Lightfoot and Lam, 1991).

a. Isolation

Lipopolysaccharide was isolated by the method of Darveau and Hancock (1983). One litre of wild-type control, adaptively resistant, or post-adaptively resistant *P. aeruginosa* were prepared as previously described. The cells were collected by centrifuging at 7000 rpm for 15 minutes and then resuspended in approximately 15 ml of 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$. DNase (100 µg/ml) and RNase (25 µg/ml) were added and the suspension then passed twice through a French press at 15000 psi and sonicated for two bursts at a probe intensity of 75 (Vibracell®, Sonics and Materials Inc., Danbury, CT). DNase and RNase were then added to final concentrations of 200 µg/ml and 50 µg/ml respectively and the suspension incubated at 37°C for 1 hour. Following the 1 hour incubation, 5 ml of 0.5 M EDTA/10 mM Tris, pH 8.0, 2.5 ml of 20% SDS/10 mM Tris, pH 8.0, and 2.5 ml of 10 mM Tris-HCl, pH 8.0, were added to the suspension to give a final volume of 25 ml. The suspension was then vortexed and centrifuged at 26,000 rpm (50,000 g) (70 Ti) for 30 minutes at 20°C to remove peptidoglycan. The supernatant was saved, proteinase K added to give a final concentration of 200 µg/ml, and incubated overnight at 37°C with constant shaking. The next day two volumes of 0.375 M $MgCl_2$/95% ethanol were added and the solution then allowed to cool to 0°C in a -20°C refrigerator. The sample was then centrifuged at 13000 rpm at 0°C for 15 minutes, the pellet resuspended in 25 ml of 0.1 M EDTA/2% SDS/10 mM Tris-HCl, pH 8.0, sonicated for two 30 second bursts at a probe intensity of 75, and incubated for 30 minutes at 85°C. The solution was then cooled to room temperature, the pH adjusted to 9.5 by the addition of 4 N NaOH, proteinase K added to give a final concentration of 25 µg/ml and incubated overnight at 37°C with constant shaking. The following day two volumes of 0.375 M $MgCl_2$/95% ethanol were again added and the solution then allowed

to cool to 0°C as before. The sample was then centrifuged at 13,000 rpm at 0°C for 15 minutes, the pellet resuspended in 15 ml of 10 mM Tris-HCl, pH 8.0, and sonicated for two 30 second bursts at a probe intensity of 75. The suspension was then centrifuged at 1000 rpm for 5 minutes to remove insoluble Mg/EDTA complexes. $MgCl_2$ was then added to a final concentration of 25 mM and the solution centrifuged at 51,000 rpm (200,000 g) (70 Ti) for 2 hours. The pellet was then resuspended in distilled water and stored at -70°C. After isolation LPS samples were resolved on SDS-PAGE.

b. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were electrophoresed in discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gels by the method of Laemmli and Favre (1973). SDS-polyacrylamide gels separate LPS species according to molecular weight. All reagents used were electrophoresis grade (Bio-Rad, Mississauga, Ontario). Separating gel concentrations ranged from 12% to 15% polyacrylamide. Stacking gels were 4% polyacrylamide. The Mini Protean II (Bio-Rad) electrophoresis apparatus with gel dimensions of 8 cm x 10 cm x 0.75 cm was used. The gels were run in 0.025 M Tris/0.192 M glycine/0.1% SDS at 100 volts until the tracking dye reached the bottom of the gel.

c. Silver Staining

LPS gels were silver stained by the method of Morrisey (1981). Following a SDS-PAGE run, a gel was rinsed three times with distilled H_2O, immersed in 100 ml of 50% methanol/10% acetic acid/40% distilled H_2O for 15 minutes with gentle shaking, and then poured off. One hundred millilitres of 5% methanol/ 7% acetic acid /88% distilled H_2O was then added for 15 minutes with shaking, removed, and followed by identical treatment with 100 ml of 10% glutaraldehyde. The gel was then rinsed three times with distilled H_2O, and immersed in distilled H_2O with gentle shaking for 15 minutes. The

H_2O was then poured off and 100 ml of dithiothreitol solution (5 µg/ml in distilled H_2O) was added for 15 minutes with shaking. This solution was poured off and 100 ml of silver nitrate solution (1 mg/ml in distilled H_2O) was added for 15 minutes with shaking. The silver stain then was then decanted and the gel rinsed once with distilled H_2O, and subsequently washed in distilled H_2O twice, for 15 minutes each, to remove unbound silver nitrate. Fifty millilitres of developer (250 ml of 3% sodium carbonate, 6 mM formaldehyde) was then added to rinse the gel. The developer was poured off and this step repeated. The developer was again removed followed by the addition of 100 ml of fresh developer. The gel was then allowed to develop with shaking until the desired level of staining was attained. The developer was then poured off and the gel rinsed three times with distilled H_2O and then washed for 15 minutes in distilled H_2O with shaking.

d. LPS Quantitation

2-keto-3-deoxyoctulosonic acid (KDO) was assayed as previously described (Osborn *et al.*, 1972) using *Escherichia coli* lipopolysaccharide 055:B5 (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) as a standard. Initially a standard curve was constructed using known volumes of an *E. coli* LPS 055:B5 stock solution (200 µg/ml) diluted with distilled water to 50 µl. The 50 µl aliquot was then added to 50 µl of 0.5 N H_2SO_4, vortexed, placed in a 100°C heating block for 15 minutes, and then cooled to room temperature. Fifty microlitres of H_5IO_6 was then added, vortexed, and the sample allowed to stand at room temperature for 10 minutes. Two hundred microlitres of arsenite reagent (2.0 g $NaAsO_2$ in 50 ml 0.5 N HCl) was added to the sample and vortexed. Eight hundred microlitres of thiobarbituric acid reagent (150 mg thiobarbituric acid in 25 ml distilled water, made fresh daily) was added to the sample, vortexed, and placed in a 100°C heating block for 10 minutes. Following cooling to room temperature, 1.5 ml of

butanol reagent (5.0 ml concentrated HCl added to 95 ml n-butanol) was added to the sample, vortexed, and then centrifuged at 2000 rpm for 5 minutes. The upper butanol layer (800 µl) was aspirated and its optical density measured at 549 nm. LPS isolated from *P. aeruginosa* strains was treated identically and the concentration of KDO in the samples determined by reference to the standard curve.

25. LPS A-Band and B-Band Immunoblotting

LPS samples were transferred from SDS-polyacrlyamide gels to nitrocellulose (Bio-Rad, 0.45 micron) and reacted with monoclonal antibody (MAb) MF 15-4 (specific for B band serotype O5) or MAb N1F 10 (specific for A band polysaccharide) as described by Lightfoot and Lam (1991).

Two identical LPS gels were prepared. One was silver stained and the other was used for blotting. A Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories, Mississauga, Ontario, Canada) was used. The blotting buffer contained 25 mM Tris/192 mM glycine/20% v/v methanol and was stored at 4°C prior to use. Nitrocellulose membrane (Bio-Rad Laboratories, Richmond, California, U.S.A) and Whatman filter paper (4 per gel) were cut to the size of the gel and soaked in blotting buffer as were scotch brite pads (2 per gel). Once the gel had finished running it was soaked in blotting buffer for at least 5 minutes to remove the SDS. The blotting apparatus was assembled ensuring that the nitrocellulose was closest to the positive electrode while the gel was closest to the negative electrode. The blot was run overnight at 30 V, at room temperature.

The following day the nitrocellulose membrane was blocked in a solution containing 5% skim milk in 1X phosphate buffered saline (PBS). The blocking solution was then poured off and the membrane washed three times, for 10 minutes each, in 1X

PBS containing 0.1% Tween 20 (wash buffer), at room temperature. The wash buffer was poured off and 50 ml of primary antibody added, (monoclonal antibody [MAb] MF 15-4 [specific for B band serotype O5] or MAb N1F 10 [specific for A band polysaccharide]), as supplied by Dr. Joseph Lam, University of Guelph, Guelph, Ontario, Canada (Lightfoot and Lam, 1991). The primary antibody hybridoma culture supernatants were used directly as supplied. The blot and primary antibody were incubated for 4 hours at 37°C with shaking. The primary antibody was then poured off and the membrane wash three times, for 10 minutes each, with wash buffer. Ten millilitres of secondary antibody (goat antimouse $F(ab)_2$ conjugated to IgG horse radish peroxidase at a 1:5000 dilution, i.e. 2µl in 10 ml PBS) was added and the blot incubated for 2 hours at 37°C with shaking. The secondary antibody was then poured off and the membrane washed three times, for 10 minutes each, with wash buffer. The blot was then stained with a solution containing 25 ml PBS, 10 µl 3% H_2O_2, 500 µl 1% cobalt chloride, and 12.5 mg diaminobenzidine. Once staining was satisfactory the blot was rinsed with distilled water to terminate the reaction.

26. Cell Envelope Protein Analysis

a. Outer Membrane Protein (OMP) Isolation

Outer membrane proteins of *P. aeruginosa* strains were isolated by a previously described method (Hancock and Nikaido, 1978). Two litres of wild-type control, adaptively resistant, or post-adaptively resistant *P. aeruginosa* were prepared as previously described. Cells were pelleted by centrifuging at 5,000 rpm for 20 minutes. The pellet was washed with 40 ml of 30 mM Tris, pH 8.0. The cells were repelleted by centrifugation at 10,000 rpm for 15 minutes. The cells were then suspended in 20 ml of

20% sucrose/50 mM Tris/0.2 mM dithiothreitol (DTT), pH 7.9, and passed through a French press at 15,000 psi. Two millilitres of lysozyme (1 mg/ml) was added and the suspension put on ice in a 4°C refrigerator for 10 minutes. One microlitre of 100 mM phenylmethyl sulfonyl fluoride (PMSF) was added to the suspension, followed by triplicate sonication, for 20 seconds each, at power 7. The suspension was then centrifuged at 15,000 rpm for 30 minutes, the supernatant collected, and the pellet discarded. The supernatant was then centrifuged at 33,000 rpm (Ti 70) for 1 hour, the resulting supernatant discarded, and the membrane containing pellet resuspended in 2 ml of 50 mM Tris/0.2 mM DTT, pH 8.0. Two millilitres of 2% sarcosyl was added and the mixture allowed to sit at room teperature for 30 minutes with occasional mixing with a glass rod. The sample was then centrifuged at 33,000 rpm (70 Ti) for 1 hour, the resulting pellet resuspended in 1 ml 50 mM Tris/0.2 mM DTT, pH 8.0, and stored at -70°C.

b. Cytoplasmic Membrane Protein Isolation

A previously described method was used to isolate cytoplasmic membrane proteins (Fukuoka *et al.*, 1991). One litre of exponentially growing cells, adaptively resistant cells, or post-adaptively resistant cells were harvested by centrifugation as described for OMPs. Cells were washed and resuspended in 20 mM HEPES (N-2-hydroxethylpiperazine-N'-2-ethanesulfonic acid), pH 8.0, to a final volume of 20 ml. Cells were cooled to 4°C and PMSF was added to a final concentration of 1 mM. Cells were broken by passing them twice through a French press at 15000 psi and then sonicating 3 times, for 20 seconds each, at power 7. Unbroken cells were removed by centrifugation at 10,000 rpm (6000 x g) for 20 minutes at 4°C. Membranes were pelleted by centrifugation at 36,000 rpm (Ti 70) (100,000 x g) for 1 hour and the pellet washed once with HEPES, pH 8.0, buffer.

The pellet was then resuspended in 4 ml HEPES, pH 8.0, buffer and PMSF added to a final concentration of 2 mM. The inner membrane was solubilized by adding sarcosyl (N-lauroylsarcosine, Sigma Chemical Company, St. Louis, Missouri, U.S.A) to the suspension to a final concentration of 1%, followed by incubation for 30 minutes at 30°C. This was followed by centrifugation at 36,000 rpm for 30 minutes at room temperature. The supernatant was decanted and recentrifuged at 36,000 rpm for 30 minutes at room temperature. The supernatant was assayed for protein content and then directly mixed with sample buffer and applied to SDS-PAGE.

c. Protein Determination

Protein quantitation was performed by the Lowry method as described earlier.

d. SDS-PAGE

Proteins fractions were suspended in SDS-PAGE sample reducing buffer (2% SDS, 10% glycerol, 0.5 M Tris-HCl (pH 6.8), 0.002% bromophenol blue, and 5% 2-mercapto-ethanol) to a final concentration of 1 µg protein/µl. Samples were heated to 100°C for 4 minutes and electrophoresed in 12% discontinuous SDS-polyacrylamide gels by the method of Laemli and Favre (1973). Running time was approximately 1 hour at 150 volts (time for tracking dye to reach the bottom of the gel). Estimation of molecular weights was done using the molecular weight standards lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase B (92,500) supplied by Bio-Rad. After running, the gels were stained with Coomasie blue to facilitate visualization.

e. Coomasie Blue Staining

Following SDS-PAGE the gel was rinsed three times with distilled water. One hundred millilitres of Coomasie Brilliant Blue stain (0.05% Coomasie Brilliant Blue/25%

2-propanol/10% glacial acetic acid/65% distilled water) was added to the gel and allowed to shake for 30 minutes. The stain was then poured off and 100 ml 7% acetic acid (destaining reagent) was added to the gel and allowed to shake for one hour. The 7% acetic acid was poured off and a fresh 100 ml of 7% acetic acid added to the gel and allowed to shake overnight.

f. Anaerobic Growth Conditions

Anaerobic growth conditions were maintained by using an anaerobic chamber (Coy Laboratories Inc.). The chamber was used according to the manufacturer's instructions and was checked daily, using a methylene blue containing indicator, to ensure continued anaerobiosis. The atmosphere inside the anaerobic chamber consisted of a gas mixture of 80% nitrogen (N_2), 10% hydrogen (H_2), and 10% carbon dioxide (CO_2). Mueller-Hinton broth was allowed to degas for 3 days in the anaerobic chamber before use. Mueller-Hinton broth treated in this manner did not support the growth of *P. aeruginosa* ATCC 27853 (negative control) but did support the growth of *E. coli* ATCC 25922 (positive control). Anaerobic Mueller-Hinton broth supplemented with 50 mM KNO_3 supported anaerobic growth of *P. aeruginosa* ATCC 27853 and all other *P. aeruginosa* isolates tested with the exception of an *anr* deficient strain, *P. aeruginosa* PAO6261 (discussed in detail in a later section).

27. Northern Analysis of *denA* and *anr*

a. Isolation of RNA

All water and solutions used in this procedure were first treated with diethylpyrocarbonate (DEPC) to inhibit RNase activity (an exception was any solutions containing Tris). Similarly, all glassware was baked at 300°C for 4 hours to inhibit

RNase activity. A method from Current Protocols in Molecular Biology was used to isolate total RNA from *P. aeruginosa* (Ausubel *et al.*, 1994). Briefly, 10 ml of *P. aeruginosa* culture was centrifuged (10 minutes, 4500 rpm, 4°C) to pellet the cells. The cells were resuspended in 10 ml protoplasting buffer (15 mM Tris-HCl pH 8.0, 8 mM EDTA, 0.45 M sucrose), 80 µl of 50 mg/ml lysozyme added, then incubated 15 minutes on ice. Protoplasts were centrifuged (5 minutes, 4500 rpm, 4°C) and the pellet resuspended in 0.5 ml Gram-negative lysing buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 1 mM sodium citrate, 1.5% SDS) with 15µl DEPC. This suspension was mixed well, transferred to a microcentrifuge tube, incubated for 5 minutes at 37°C, and chilled on ice. Two hundred and fifty microlitres of saturated NaCl (16 g NaCl in 40 ml DEPC treated water) was added, mixed, and incubated for 10 minutes on ice. The sample was then microcentrifuged (10 minutes, 13,000 rpm, 4°C) and the supernatant removed into two clean microcentrifuge tubes. One millilitre of ice cold 100% ethanol was added to each tube and stored overnight at -20°C. The following day tubes were microcentrifuged (15 minutes, 13,000 rpm, 4°C), the pellet rinsed in ice cold 70% ethanol, air dried, and resuspended in 95 µl DNase digestion buffer (20 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$), 4 µl of a 2.5 mg/ml solution of RNase-free DNase I added, and the solution incubated for 60 minutes at 37°C. RNA was then re-extracted with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol. The aqueous layer was removed and 100 µl DEPC treated water was added to the remaining organic layer. Following mixing and microcentrifugation the two aqueous layers were pooled and a 24:1 chloroform:isoamyl alcohol extraction performed. Ten microlitres of saturated NaCl solution and 600 µl of 100% ethanol were added to each tube and mixed. Tubes were stored overnight at -20°C. The following day tubes were microcentrifuged (15 minutes, 13,000 rpm, 4°C), the pellet

rinsed in ice cold 70% ethanol, air dried, and resuspended in 100 µl DEPC treated water.

b. RNA Quantitation

The purity and quantity of RNA was determined spectrophotometrically. A 1:50 dilution of RNA was made in $T_{10}E_1$ and the optical density measured at 260 nm and 280 nm. Purity was determined by the 260 nm/280 nm ratio. The quantity of RNA was determined with the following formula:

$$[RNA]_{\mu g/ml} = \Sigma_{260} \times OD_{260} \times \text{dilution factor}$$

where the dilution factor was 1:50 and Σ_{260} = 40 µg/ml.

c. Agarose/Formaldehyde Gel Electrophoresis

A method from Current Protocols in Molecular Biology was used to electrophorese isolated total RNA from *P. aeruginosa* (Ausubel *et al.*, 1994). Twenty micrograms of total RNA (measured spectrophotometrically) was loaded per lane, onto a 1% agarose gel containing 0.22 M formaldehyde and electrophoresed in 1X MOPS buffer. Duplicate samples were loaded on the other side of the gel and visualized with ethidium bromide staining following electrophoresis.

d. Transfer of RNA to Membrane

The unstained portion of the gel was rinsed several times in sterile DEPC treated water to remove the formaldehyde. The gel was soaked in 10 volumes of 0.05 M NaOH/1.5 M NaCl for 30 minutes and subsequently replaced with 10 volumes of 0.5 M Tris-HCl, pH 7.4/1.5 M NaCl. After soaking for 20 minutes the gel was placed into 10 volumes of 10X SSC (made as a 20X SSC stock: 3 M NaCl, 0.3 M sodium citrate, pH adjusted to 7.0 with 1 M HCl) and allowed to soak for 45 minutes.

The transfer was set up in 10X SSC using the following technique. A glass dish was filled with 10X SSC and a glass plate placed across it. Two strips of 3MM paper

(wicks) were placed across the plate and into the 10X SSC solution on each side. The strips of paper were then soaked with 10X SSC. Three pieces of 3MM paper were cut to the size of the gel, soaked in 10X SSC and placed onto the 3MM paper on the top of the glass plate. Air bubbles were removed and the gel then placed evenly onto the top of the three 3MM paper pieces making note of the gel's orientation for later reference. Following air bubble removal, a piece of Boehringer Mannheim nylon membrane, cut to fit the gel and presoaked in 10X SSC, was placed onto the gel and air bubbles once again removed. The membrane was surrounded with even edged pieces of parafilm, draping over the soaked 3MM paper. Three more trimmed pieces of 3MM paper were placed on top of the membrane, soaked with 10X SSC, air bubbles removed, and a large stack of paper towels placed on top. The paper towels were compressed with a weight for 18 to 24 hours.

With the membrane still moist from transfer, UV crosslinking was performed for 3 minutes, the membrane soaked in 2X SSC to reduce salt content, and the membrane baked for 2 hours, at 80°C, in a vacuum oven.

e. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was used to prepare digoxigenin labelled DNA probes corresponding to internal portions of the *P. aeruginosa* nitrite reductase gene, *denA*, and anaerobic regulatory gene, *anr*. PCR was also employed to amplify the promoter regions of both *denA* (which is present in the *denAB* operon) and *anr*, to facilitate DNA sequencing.

α. Preparation of *P. aeruginosa* Lysates for PCR

One or two colonies from a fresh blood agar plate were suspended in 100 µl of distilled sterile water. Alternatively, *P. aeruginosa* grown in suspension was also used

for lysate preparation. Following centrifugation of a suitable aliquot, cells were resuspended in 100 µl of distilled sterile water. A 20 µl aliquot of prepared suspension was transferred to a 0.5 ml tube and heated for 5 minutes at 95°C. Ten microlitres of this bacterial lysate was used as DNA template in PCR reactions. Fresh lysates were prepared daily as needed.

ß. PCR Reaction Protocols

The following basic reaction mixture was used.

distilled sterile water	25.5 µl
10x reaction buffer (200 mM NaCl, 100 mM Tris-HCl pH 8.9, 25 mM $MgCl_2$, 0.05% gelatin, 0.5% Triton X-100)	5 µl
1.25 mM dNTPs (dATP, dCTP, dGTP, dTTP)	8 µl
100 µM 5' oligonucleotide	0.5 µl
100 µM 3' oligonucleotide	0.5 µl
template DNA (approximately 0.3 µg total DNA)	10 µl
Taq polymerase (1 unit/µl)	3.5 µl
	50 µl

The 50 µl volume was placed in a Perkin-Elmer GeneAmp PCR 9600 series thermocycler (Perkin-Elmer Corporation, Norwalk, CT), heated at 94°C for 3 minutes, and then cycled through the following protocol.

Denaturation step	94°C for 60 seconds
Annealing step	60°C - 65°C for 60 seconds*,**
Extension step	72°C for 120 seconds

* the annealing temperature depended on the Tm of the oligonucleotide

** Tm, melting temperature, of the oligonucleotide was determined using the following formula: $Tm = 69.3 + 0.41 (G + C\%) - 650/L$

where L = length of oligonucleotide

After completion of 35 cycles, a final 4 minute extension step was performed at 72°C. Five microlitres (10%) of the completed PCR reactions were removed and loaded onto a 1% agarose gel containing ethidium bromide to assess the products. The remaining 45 µl was extracted with 25:24:1 phenol:chloroform:isoamyl alcohol followed by a 24:1 chloroform:isoamyl alcohol extraction. The PCR products were then precipitated with a 1/10 volume of 3 M sodium acetate and 2 volumes of cold absolute ethanol. The pellet was dried and then resuspended in 50 µl $T_{10}E_1$.

γ.Gel Purification of PCR Products

Electrophoresis in 1% agarose gels was used to remove primers from PCR-amplified DNA. Gel portions containing the PCR products were cut from the gels, and the DNA fragments harvested using the Prep-A-Gene matrix system, protocol 2.1 (Bio-Rad Laboratories, Inc.).

f. Probe Selection for Northern Analysis

The complete DNA sequence of the *denAB* operon and its flanking regions has been published (Silvestrini *et al.*, 1989; Arai *et al*, 1990; Arai *et al.*, 1991). A 699 bp internal *denA* (nitrite reductase) region was PCR amplified (digoxigenin labeled), using primers selected by PC-Gene computer software. Primer 1 was 5' ATG CGC GAA TCG TGG AAG GTG 3' and primer 2 was 5' GAC GGT CCT TGG AGT CGA TCA 3'. The complete DNA sequence of *anr* and its flanking regions has also been published (Zimmermann *et al.*, 1991). A 615 bp internal *anr* region was PCR amplified (digoxigenin labeled), using primers selected by PC-Gene computer software. Primer 1 was 5' CCC AAG CAC ACT GCA AGG ATT GC 3' and primer 2 was 5' TTC TGC TGG AAG CGG GTG AAG AGC C 3'. All oligonucletide primers described in this thesis were synthesized on an Oligo 1000 DNA Synthesizer (Beckman) in the Department

of Medical Microbiology, Faculty of Medicine, University of Manitoba.

g. Preparation of Digoxigenin Labelled Probes

The Boehringer Mannheim PCR digoxigenin (DIG) probe synthesis kit, which incorporates DIG-11-dUTP label into the PCR product, was used. PCR amplification was identical to that described previously, with one exception. PCR DIG probe synthesis mix, containing dATP, dCTP, dGTP, dTTP and alkali-labile DIG-11-dUTP, replaced the regular dNTP mix. The alkali labile DIG-11-dUTP formulation enables simple removal of the DIG-label after chemiluminescent detection and subsequent rehybridization of blots with multiple DIG-labeled probes. Following PCR, probes were washed and gel purified as described previously. The yield of DIG-labeled probe was then quantitated according to the manufacturer's protocol using DIG-11-dUTP labeled control DNA as a standard.

h. Northern Hybridization Protocol

The blot prepared above (section 27, part d) was placed into a hybridization bag containing 20 ml prehybridization solution (5X SSC, 1.0% salmon sperm DNA, 0.1% N-lauroylsarcosine, 0.2%SDS), the bag sealed, and incubated at the anticipated hybridization temperature (*denA* probe: 65°C, *anr* probe: 65°C) for 2 hours. The double stranded DNA probes used were boiled for 10 minutes to denature the DNA and immediately placed on ice. DIG-11-dUTP labelled probes were diluted in 20 ml of hybridization solution (identical to prehybridization solution) to a final probe concentration of 20 ng/ml. The prehybridization solution was poured from the bag, the hybridization solution containing DIG-11-dUTP labeled probe added, and the blot incubated overnight at the hybridization temperature. The next morning, following stringency washes, the blots were submitted to immunological detection using anti-digoxigenin antibody conjugated to alkaline phosphatase and CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tri-

cyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate) (Boehringer Mannheim). CSPD is a chemiluminescent substrate for alkaline phosphatase that enables extremely sensitive and fast detection of biomolecules by producing visible light which can be recorded with film or instrumentation. Enzymatic dephosphorylation of CSPD by alkaline phosphatase leads to the metastable phenolate anion which decomposes and emits light at a maximum wavelength of 477 nm.

28. PCR Primer Selection for DNA Sequencing

a. Promoter Region Sequencing of *denAB*

As mentioned above, the complete DNA sequence of the *denAB* operon and its flanking regions has been published (Silvestrini *et al.*, 1989; Arai *et al*, 1990; Arai *et al.*, 1991). The entire promoter region of the *denAB* operon was sequenced (215 bp); this included the putative binding sequence of the ANR regulatory protein (Zimmermann *et al*, 1991). Primers were selected by PC-Gene computer software. Primer 1 was 5' CTT GAG CAA TAC CGG CAG GC 3' and primer 2 was 5' GCT TTC ATG TCG TCC TTG GCG TGA 3'. PCR was performed as described previously. Primers 1 and 2 doubled as sequencing primers. The author acknowledges that a nested approach is recommended, however, this method was successful and more cost effective.

b. Promoter Region Sequencing of *anr*

As mentioned above, the complete DNA sequence of *anr* and its flanking regions has been published (Zimmermann *et al.*, 1991). Two hundred and twenty-nine nucleotides were sequenced 5' to the *anr* start codon (promoter region). Primers were selected by PC-Gene computer software. Primer 1 was 5' AGG GTC AAC ATT CCA GTC ACT CCG 3' and primer 2 was 5' GCA ATC CTT GCA GTG TGC TTG G 3'.

PCR was performed as described previously. The aforementioned primers doubled as the sequencing primers. Again, the author acknowledges that a nested approach is recommended, however, this method was successful and more cost effective.

29. DNA Sequencing Protocols

a. Exo⁻ Pfu Cycle Sequencing (Stratagene)

Gel purified DNA template was dissolved in double distilled water and sequenced using the Cyclist Exo⁻ Pfu DNA Sequencing Kit (Stratagene). Briefly, 7 µl of prereaction mix (prereaction mix contained 200 fmol DNA template, 1 pmol sequencing primer, 4 µl 10X sequencing buffer, 10 µCi radioactive label [^{35}S], 1 µl Exo⁻ Pfu DNA polymerase, distilled sterile water [to a prereaction mix volume of 26 µl], and 4 µl DMSO [final prereaction mix volume 30 µl]) was added to each of four tubes containing 3 µl of one ddNTP (A,C,G,T), the tubes incubated for 5 minutes at 95°C, followed by 30 cycles of 95°C for 30 seconds (denaturation), 62°C to 65°C for 30 seconds (annealing), and 72°C for 60 seconds (extension). The exact annealing temperature used depended on the Tm of the oligonucleotide used. Following the run, 5 µl stop solution was added to each tube. Samples were denatured for 2 to 5 minutes at 95°C prior to loading the sequencing gel. Two to four microlitres of samples were double loaded onto a 4% polyacrylamide (acrylamide:bis-acrylamide 19:1) sequencing gel and an 8% (acrylamide:bis-acrylamide 19:1) polyacrylamide wedge gel. Gels were fixed in a solution containing 7.5% glacial acetic acid and 7.5% methanol for 30 minutes. The gels were rinsed with distilled water, dried on Whatman filter paper using a Bio-Rad gel drier for 1 hour, exposed to x-ray film for a period of up to seven days and the film developed.

b. PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing (Applied Biosystems)

The PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.) was used to prepare PCR amplified, gel purified, DNA for sequence analysis on the Applied Biosystems model 373A automated DNA sequencing system. The performance of this kit relies on G, A, T, and C dye-labeled ddNTP terminators. The manufacturer's protocol was followed exactly. Centri-Sep (Princeton Separations, Inc.) spin columns were used to remove excess DyeDeoxy terminators from completed sequencing reactions. Reaction products were run on a 6% (acrylamide:bis-acrylamide 19:1) polyacrylamide gel and automatically analyzed in an Applied Biosystems model 373A DNA sequencer.

31. *Pseudomonas aeruginosa* PAO6261: An *anr* Deletion Mutant of *Pseudomonas aeruginosa* PAO1

An *anr* deletion mutant of *P. aeruginosa* PAO1, PAO6261, was obtained from Dr. Dieter Haas, Universite de Lausanne, Lausanne, Switzerland (Ye *et al.*, 1995). PAO6261 was constructed by introducing a defined *anr* deletion from plasmid pME3585 into the chromosomal *anr* locus of *P. aeruginosa* PAO1 by gene replacement (Ye *et al.*, 1995). PAO6261 was constructed based on the mobilizable, ColE1-based suicide plasmid pME3087. A 2.9 kb SalI-BamHI fragment carrying *anr* with an internal 0.33 kb deletion was inserted, with flanking sequences, into pME3087. In the resulting construct, pME3585, the sequences upstream and downstream of the mutated *anr* gene provided sufficient homology for marker exchange. Plasmid pME3585 was mobilized from an exponential culture of transfer proficient *E. coli* S17-1 cells into *P. aeruginosa* PAO1

grown to stationary phase at 43°C. The mating conditions were described previously (Rella *et al.*, 1985). Selection for tetracycline resistance (50 µg/ml tetracycline) at 37°C produced PAO1 transconjugates carrying a chromosomally integrated copy of pME3585 at a frequency of approximately 10^{-7} per donor. Tetracycline sensitive derivatives were then obtained by enrichment with cycloserine (Ye *et al.*, 1995). Excision of pME3585 gave the *anr* mutant PAO6261. The 0.33 kb deletion in the *anr* gene was confirmed by Southern hybridization (Ye *et al.*, 1995). The *anr* mutant, PAO6261, was shown to be stable and not to grow anaerobically on arginine, nitrate, or nitrite, while PAO1 did (Ye *et al.*, 1995). Growth characteristics were confirmed in our laboratory.

32. Complementation of *Pseudomonas aeruginosa* PAO6261

A plasmid, pME3580, replicated in *E. coli* ED8767, was obtained from Dr. Marc Galimand, Mikrobiologisches Institut, Eidgenossische Technische Hochschule, Zurich, Switzerland (Galimand *et al.*, 1991). To complement the *anr* mutation in *P. aeruginosa* PAO6261, pME3580, in *E. coli* ED8767, was mobilized by pRK2013, in *E. coli* HB101, to *P. aeruginosa* PAO6261 by triparental mating (Jeenes *et al.*, 1986; Ye *et al.*, 1995). Modifications were made to the original method. The *E. coli* HB101 (pRK2013) donor was mated for 4 hours with *E. coli* ED8767 (pME3580) on 0.45 µm Nalgene membrane filters placed on Mueller-Hinton agar. Cells were washed from the filters and grown to exponential phase in Mueller-Hinton broth supplemented with 100 µg/ml ampicillin and 50 µg/ml tetracycline hydrochloride. The exponential phase culture was washed with 0.9% NaCl solution and resuspended to its original volume in 0.9% NaCl solution. One hundred microlitre aliquots of this donor suspension were then added to 100 µl aliquots of *P. aeruginosa* PAO6261, previously grown at 43°C without aeration (growth at 43°C

without aeration reduces restriction activity), and concentrated ten times in 0.9% NaCl solution. The mixtures were filtered using 0.45 µm Nalgene filters, the filters removed, transferred to Mueller-Hinton agar filter side up, and incubated overnight at 30°C. The next day bacteria were resuspended in 2 ml 0.9% NaCl solution. A 1:100 dilution of this suspension was made in 0.9% NaCl solution and 100 µl of the undiluted and diluted samples spread on selection plates (Pseudomonas isolation agar supplemented with 500 µg/ml carbenicillin). Plates were incubated for 1 to 2 days at 37°C and colonies restreaked twice, on selection plates, to ensure purity and presence of antibiotic selection marker. Colonies were then tested for anaerobic growth in the presence of 50 mM nitrate and 50 mM nitrite. Two *anr* complemented PAO6261 strains were then used in adaptive resistance experiments.

Previous work performed using the original method (Jeenes *et al.*, 1986) suggested that recombination between chromosomal mutations and fragments carried on various recombinant plasmids is unlikely, and the return of function most probably reflects complementation (Galimand *et al.*, 1991).

32. Statistical Data Analysis

All data are expressed with means ± standard deviations when possible. Paired t-tests were used to compare two data groups. Comparisons involving three or more data groups were analyzed initially using standard analysis of variance calculations, or multi-way analysis of variance calculations where necessary, to check for significant differences between groups within given data sets. If a significant difference was detected between groups, Tukey's multiple comparison test was then used to identify the specific location of the difference(s) between groups. Statistical significance was determined when $P<0.05$

(two-tailed tests).

C. RESULTS

Part 1. Aminoglycoside Pharmacodynamics: Focus on Adaptive Resistance

1. Antimicrobial Susceptibility Testing

Arithmetic aminoglycoside MICs were determined for four clinical isolates (F327, F443, F991, E1481) and one reference strain (ATCC 27853) of *P. aeruginosa.* They ranged from 4.7 to 6.3 mg/l for amikacin, 2.5 to 3.6 mg/l for gentamicin, and 0.8 to 1.1 mg/l for tobramycin (Table 4). MBCs were also identified for these strains. All MBCs were the same as, or within one multiple dilution of the MIC with a single exception, F991, which was within two multiple dilutions of the MIC.

Doubling dilution MICs were determined for all other strains tested (Table 5). The gentamicin and tobramycin MICs of the four *P. aeruginosa* strains used in lipopolysaccharide (LPS) studies are presented in Table 5. Aminoglycoside MICs for strain rd7513 was modestly but significantly lower than the PAO1 control, while AK1401 and dps89 exhibited no significant differences. Doubling dilution MICs are considered significantly different if their values differ by at least four fold (NCCLS, 1990). The aminoglycoside MICs for the three *P. aeruginosa* strains used in *anr* functional analysis were not significantly different (Table 5). Table 6 provides MICs for other antimicrobial agents tested against the four clinical isolates (F327, F443, F991, E1481) and the reference strain ATCC 27853 of *P. aeruginosa.* The MICs of cefotaxime, ciprofloxacin and gentamicin for the *E. coli* reference strain ATCC 25922 and the clinical isolate tested (G1307) are given in Table 7.

Table 5. Aminoglycoside MICs and MBCs against *Pseudomonas aeruginosa.*

Strain	Amikacin		Gentamicin		Tobramycin	
	MIC[a,b] (mg/l)	MBC[a,b] (mg/l)	MIC (mg/l)	MBC (mg/l)	MIC (mg/l)	MBC (mg/l)
ATCC 27853	6.9	6.9	3.6	7.2	0.9	1.8
F327	4.9	4.9	3.2	3.2	1.0	2.0
F443	4.7	9.4	2.5	5.0	0.8	1.6
F991	6.5	13.0	3.5	14.0	0.9	0.9
E1481	6.3	12.6	3.2	3.2	1.1	2.2
mean	**5.9**	**9.4**	**3.2**	**6.5**	**0.9**	**1.7**
PAO1	4.0	ND	2.0	ND	1.0	ND
AK1401	ND	ND	4.0	ND	2.0	ND
dps89	ND	ND	4.0	ND	2.0	ND
rd7513	ND	ND	0.5	ND	0.25	ND
PAO6261 (*anr*$^-$)	2.0	ND	1.0	ND	0.5	ND
PAO6261 (*anr*$^+$)	4.0	ND	2.0	ND	1.0	ND

[a] MICs and MBCs determined arithmetically or by doubling dilution on a minimum of 3 different occasions.

[b] ND - not determined

LPS phenotypes - PAO1 (A-band LPS present, B-band LPS present); AK1401 (A-band LPS present, B-band LPS absent); dps89 (A-band LPS absent, B-band LPS present); rd7513 (A-band LPS absent, B-band LPS absent).

Table 6. Other antimicrobial agent MICs against *Pseudomonas aeruginosa.*

Antimicrobial Agent	MIC[a,b] (mg/l)
Ciprofloxacin	0.30 (0.25-0.50)
Pefloxacin	3.6 (2-4)
Ceftazidime	3.6 (2-4)
Cefotaxime	64
Imipenem	3.6 (2-4)
Pipercillin	4
Chloramphenicol	256
Polymyxin B	1.4 (1-2)
Tetracycline	22.4 (16-32)

[a] MICs determined by doubling dilution on a minimum of 3 different occasions with each of the *P. aeruginosa* clinical isolates (F327, F443, F991, E1481) and ATCC 27853.
[b] Range of MIC values given in brackets.

Table 7. Antimicrobial agent MICs against *Escherichia coli.*

Strain	Cefotaxime	Ciprofloxacin	Gentamicin
ATCC 25922	0.125	0.016	1.0
G1307	0.063	0.008	1.0

[a] MICs were determined by doubling dilution on a minimum of 3 different occasions.

2. Minimum Inhibitory Concentration (MIC) Reversion Time Determinations

Individual MIC reversion times (MRTs) were taken as the midpoints of the 4 hour intervals in which the MICs of the culture returned to within one doubling dilution of their original value. A minimum of 15 midpoint values was used to calculate each mean MRT and the standard deviation. MRT values are presented in Tables 8 and 9 for both gentamicin and tobramycin for all aminoglycosides concentrations and intervals tested. Following a single 2 hour treatment with 1X MIC, it took between 8 and 20 hours for MICs to return to their original values (gentamicin, 11.7 ± 1.7 hours; tobramycin, 12.7 ± 2.7 hours) (Table 8). Following a single 2 hour treatment with 8 mg/l, MICs at 8, 12 and 24 hours were four- to eightfold, four- to eightfold, and two- to fourfold greater, respectively, than the original MIC and did not return to their original values for between 16 and 32 hours (gentamicin, 21.5 ± 4.0 hours; tobramycin, 22.3 ± 2.8 hours). Following a single 2 hour treatment with 24 mg/l, MICs at 12 and 24 hours posttreatment were four- to eightfold and two- to fourfold greater, respectively, than the original MIC and did not return to their original values for 20 to 40 hours (gentamicin, 28.9 ± 3.8 hours; tobramycin, 26.8 ± 3.1 hours). Multiple aminoglycoside exposures significantly increased the MRTs for cultures exposed to 1X MIC and 8 mg/l. Following triplicate 2 hour treatments with 1X MIC every 8 hours, MICs at 8, 12, and 24 hours preceding the last treatment were two- to fourfold greater than the original MIC of both gentamicin and tobramycin and did not return to their original values for between 32 and 56 hours (gentamicin, 44.3 ± 4.4 hours; tobramycin, 39.2 ± 5.1 hours). Following triplicate 2 hour treatments with 8 mg/l every 8 hours, MICs at 8, 12, and 24 hours preceding the last treatment were 8- to 16-fold greater than the original MIC of both gentamicin and tobramycin and did not return to their original values for between 56 and 96 hours

(gentamicin, 68.1 ± 5.2 hours; tobramycin, 77.8 ± 7.8 hours). Following the triplicate exposure of cultures to 24 mg/l for 2 hours, every 24 hours, MICs at 12 and 24 hours posttreatment were four- to eightfold and two- to fourfold greater, respectively than the original values, with 28 to 40 hours (gentamicin, 36.1 ± 3.0 hours; tobramycin, 34.5 ± 3.0 hours) required for MICs to revert to their original values.

Table 8. MIC reversion times (MRT) and times for MICs to fall below or to the aminoglycoside dosing concentration following single 2 hour aminoglycoside exposures in *Pseudomonas aeruginosa*[a].

Aminoglycoside	Aminoglycoside dosing concentration	MRT (h)[b]	Time for MIC to fall below or to aminoglycoside dosing concentration (h)[b]
Gentamicin	1X MIC	11.7 ± 1.7 (8-16)	11.7 ± 1.7 (8-16)
	8 mg/l	21.5 ± 4.0 (16-32)	12.4 ± 2.0 (8-16)
	24 mg/l	28.9 ± 3.8 (24-40)	<12-16[c]
Tobramycin	1X MIC	12.7 ± 2.7 (8-20)	12.7 ± 2.7 (8-20)
	8 mg/l	22.3 ± 2.8 (16-28)	<8-8[c]
	24 mg/l	26.8 ± 3.1 (20-32)	<12[c]

[a] Values are means ± standard deviations for all five strains tested. Determinations were repeated at least three times for each strain.
[b] Values in parentheses are ranges for all strains tested.
[c] Mean ± standard deviation was not calculable because some or all individual values did not exceed the aminoglycoside dosing concentration.

Table 9. MIC reversion times (MRT) and times for MICs to fall below or to the aminoglycoside dosing concentration following multiple 2 hour aminoglycoside exposures in *Pseudomonas aeruginosa*[a].

Amino-glycoside	Aminoglycoside dosing concentration	MRT (h)[b]	Time for MIC to fall below or to aminoglycoside dosing concentration (h)[b]
Gentamicin	1X MIC, 2h, q8h, 3 times	44.3 ± 4.4 (36-56)	44.3 ± 4.4 (36-56)
	8 mg/l, 2h, q8h, 3 times	68.1 ± 5.2 (56-80)	60.1 ± 7.1 (44-72)
	24 mg/l, 2h, q24h, 3 times	36.1 ± 3.0 (28-40)	15.1 ± 3.2 (12-24)
Tobramycin	1X MIC, 2h, q8h, 3 times	39.2 ± 5.1 (32-52)	39.2 ± 5.1 (32-52)
	8 mg/l, 2h, q8h, 3 times	77.8 ± 7.8 (64-96)	32.4 ± 3.3 (24-40)
	24 mg/l, 2h, q24h, 3 times	34.5 ± 3.0 (28-40)	<8[c]

[a] Values are means ± standard deviations for all five strains tested. Determinations were repeated at least three times for each strain.
[b] Values in parentheses are ranges for all strains tested.
[c] Mean ± standard deviation was not calculable because some or all individual values did not exceed the aminoglycoside dosing concentration.

The experiments performed using 8 and 24 mg/l were intended to compare a simulated traditional gentamicin and tobramycin dosing regimen (2 mg/kg every 8 hours [q8h], resulting in an approximate maximum plasma concentration [Cp_{max}] of 8 mg/l), with a once daily dosing regimen (6 mg/kg every 24 hours [q24h], resulting in an approximate Cp_{max} of 24 mg/l), on the basis of the time required for MICs to revert to

their original values (MRT) following single and multiple 2 hour aminoglycoside exposures. A single exposure to 8 mg/l produced significantly shorter MRTs than exposure to 24 mg/l for gentamicin ($P<0.01$) and tobramycin ($P<0.05$). No significant differences were observed between gentamicin and tobramycin after single two hour exposures to either 8 or 24 mg/l. MRTs following multiple exposures to 24 mg/l were significantly shorter than those following exposures to 8 mg/l for both gentamicin ($P<0.005$) and tobramycin ($P < 0.005$). Significant differences were seen between gentamicin and tobramycin in only one instance. MRTs following multiple exposures to tobramycin at 8 mg/l were significantly longer ($P<0.05$) than those for gentamicin.

The mean time for the MIC of a culture to fall below or to the aminoglycoside dosing concentration was defined as the midpoint of the 4 hour interval in which the MIC fell below or to the aminoglycoside concentration used. Tables 8 and 9 show the mean times for the MICs for the cultures to fall below or to the aminoglycoside dosing concentration at which the MICs were calculable. Often for one or more strains, the aminoglycoside dosing concentration was not exceeded at the earliest MIC determination, and therefore means and standard deviations cannot be accurately provided. In these cases, only ranges are provided. Comparable data were obtained for multiple exposures to gentamicin at 8 and 24 mg/l. The time required for the gentamicin concentration to fall below or to the aminoglycoside dosing concentration was significantly shorter ($P<0.005$) for cultures exposed in triplicate to 24 mg/l (q24h) than for cultures exposed in triplicate to 8 mg/l (q8h). In addition, MICs for cultures exposed to gentamicin at 8 mg/l in triplicate required significantly more time ($P<0.005$) to fall below the aminoglycoside dosing concentration than the MICs for identical cultures exposed to tobramycin.

The design of the study also allowed for the in vitro assessment of aminoglycoside concentration/MIC ratios. The calculation and consideration of in vitro aminoglycoside concentration/MIC ratios are important because Cp_{max}/MIC ratios have been correlated with clinical cure (Moore *et al.*, 1987; Drusano *et al.*, 1991). The simulated once-daily dosing regimen (24 mg/l, q24h) consistently produced cultures for which the aminoglycoside concentration/MIC ratios were significantly higher ($P<0.005$) compared with those for cultures reexposed to 8 mg/l every 8 hours (Table 10). All tobramycin concentration/MIC ratios determined were significantly ($P<0.001$) larger than the gentamicin concentration/MIC ratios measured over the same dosage interval at both 8

Table 10. Comparison of aminoglycoside concentration/MIC ratios on multiple gentamicin and tobramycin exposures at 8 and 24 mg/l[a].

Aminoglycoside	Dose (mg/l) and interval	Aminoglycoside Concentration/MIC Ratio		
		First Exposure	Second Exposure	Third Exposure
Gentamicin	8 mg/l, 2h, q8h	2.5 ± 0.4	0.4 ± 0.3	0.2 ± 0.3
	24 mg/l, 2h, q24h	7.5 ± 1.1	2.1 ±0.3	1.9 ± 0.4
Tobramycin	8 mg/l, 2h, q8h	8.9 ± 1.0	2.7 ± 0.2	1.0 ± 0.3
	24 mg/l, 2h, q24h	26.7 ± 2.8	8.0 ± 1.0	6.2 ± 0.5

[a] Values are means ± standard deviations for all five strains tested. Determinations were repeated at least three times for each strain.

mg/l (q8h) and 24 mg/l (q24h). All first exposure aminoglycoside concentration/MIC ratios were significantly ($P<0.005$) greater than second exposure aminoglycoside concentration/MIC ratios, regardless of the aminoglycoside or the regimen tested. In a comparison of second and third aminoglycoside exposures, only a single statistically significant difference was determined. Tobramycin exposed to 8 mg/l, as one exposure every 8 hours, had a significantly ($P<0.01$) higher concentration/MIC ratio just preceding the second exposure compared with that measured just prior to the third exposure. With respect to these experiments, the reader should appreciate the limitations of conducting in vitro experiments with 2 hour fixed aminoglycoside concentrations. Principal among these limitations is the knowledge that in vivo concentrations fluctuate constantly and that the half-lives of these agents are on the order of 2 to 3 hours in patients with normal renal function (Gilbert, 1995).

A further study was conducted to assess the limits of transient aminoglycoside resistance induction and MRTs. In this study the clinical isolates (F327, F443, F991, E1481) and the reference strain ATCC 27853 of *P. aeruginosa* were systematically passed against doubling aminoglycoside concentrations until stable growth was no longer attained (Table 11). Stable growth was no longer attained with amikacin concentrations of 1024 to 4096 mg/l, gentamicin concentrations of 512 to 2048 mg/l, and tobramycin concentrations of 128 to 256 mg/l (Table 11). These concentrations ranged from 142 to 694 times the original MIC. All wild-type control cultures of the identical strains were sterilized by an aminoglycoside concentration of 30 to 40 times the MIC. The transiently resistant cultures appeared to demonstrate step-wise increases in aminoglycoside resistance and were able to grow in concentrations of aminoglycoside that would have eradicated them, given no previous aminoglycoside exposure. All cultures reverted to their original

MIC with 144 to 192 hours of growth in aminoglycoside free Mueller-Hinton broth (Table 11).

Table 11. MIC reversion times (MRT) for transient, highly aminoglycoside resistant *Pseudomonas aeruginosa* variants[a,b].

Aminoglycoside	Adaptive Resistant Variant Starting MIC (mg/l)	Time to Revert to Original MIC (hrs)
Amikacin	1024-4096	144-192
Gentamicin	512-2048	144-192
Tobramycin	128-256	144-192

[a] MICs determined every 24 hours.
[b] Organisms maintained in log phase by periodic transfer to fresh MHB.

3. Postantibiotic Effect Determinations

The PAE refers to the period of depressed bacterial growth following an antimicrobial exposure. In the past, it was assumed that because post-PAE cultures resumed logarithmic phase growth, they had returned to their wild-type susceptibility (Craig and Gudmundsson, 1991). However, the experiments performed in this thesis demonstrate that repeated exposure of *P. aeruginosa* to aminoglycoside at times well after the conventional PAE interval was known to have passed, resulted in significant reductions in bacterial killing and PAE. These results suggested that post-PAE-phase cultures do not return to their initial aminoglycoside susceptibilities coincident with resumption of logarthmic phase growth.

a. Single Exposure PAE Determinations

α. Aminoglycosides and *Pseudomonas aeruginosa*

Concentration-dependent PAE and bacterial killing were demonstrated by *P. aeruginosa* following single 2 hour exposures to amikacin, gentamicin, or tobramycin at 1/8, 1/4, 1/2, 1, 3 and 6 times the MIC. The results are presented in Table 12.

ß. Other Antimicrobial Agents and *Pseudomonas aeruginosa*

PAE and bacterial kill determinations were made using four clinical isolates (F327, F443, F991, E1481) and the reference strain ATCC 27853 of *P. aeruginosa* following single 2 hour exposures to the ceftazidime and ciprofloxacin at 1, 3, and 6 times the MIC. The results are presented in Table 13. The ß-lactam ceftazidime demonstrated limited bacterial kill (1.1 ± 0.4 $\log_{10}$ cfu/ml at 6X MIC) and no PAE against the *P. aeruginosa* strains tested. On the other hand, the fluoroquinolone ciprofloxacin demonstrated concentration dependent bacterial killing and PAE. These results are in agreement with previously published data (Craig and Gudmunsson, 1991).

γ. *Escherichia coli*

Bacterial killing and PAE determinations resulting from a 2 hour cefotaxime, ciprofloxacin or gentamicin exposure against *E. coli* are presented in Table 14. The ß-lactam cefotaxime produced limited bacterial killing and an insignificant PAE against *E. coli* at 2X MIC. Ciprofloxacin and gentamicin were both bactericidal (99.9% or more bacterial kill) and produced PAEs of longer than 1 hour at 2X MIC. These results are in agreement with previously published data (Craig and Gudmunsson, 1991).

Table 12. Aminoglycosides PAEs against *Pseudomonas aeruginosa.*

Aminoglycoside	Concentration	PAE (min)[a]	Bacterial Kill[a,b,c] (log_{10} cfu/ml)
Amikacin	1/8x MIC	4±5	+0.7±0.3
	1/4 x MIC	7±5	0.1±0.3
	1/2x MIC	24±10	1.0±0.2
	1x MIC	70±8	2.2±0.2
	3x MIC	96±10	3.4±0.2
	6x MIC	169±11	5.0±0.3
Gentamicin	1/8x MIC	8±8	+0.8±0.2
	1/4x MIC	10±6	0.2±0.4
	1/2x MIC	40±8	1.0±0.3
	1x MIC	68±9	2.1±0.2
	3x MIC	108±12	3.3±0.2
	6x MIC	176±10	5.2±0.3
Tobramycin	1/8x MIC	2±4	+0.6±0.3
	1/4x MIC	15±6	0.1±0.3
	1/2x MIC	40±10	0.5±0.3
	1x MIC	86±15	1.7±0.2
	3x MIC	117±7	3.1±0.3
	6x MIC	187±7	4.9±0.4

[a] Means of four clinical isolates (F327, F443, F991, E1481) and one reference strain (ATCC 27853) of *P. aeruginosa* ± standard deviation. Determinations were repeated in triplicate with each strain.

[b] Bacterial kill is the difference between the colony count of the original inoculum and the colony count following a 2 hour aminoglycoside exposure measured in log_{10} units.

[c] Positive values, at 1/8X MIC, signify bacterial culture growth.

Table 13. Other antimicrobial agent PAEs against *Pseudomonas aeruginosa.*

Antimicrobial Agent	Concentration	PAE (min)[a]	Bacterial Kill[a,b,c] (log_{10} cfu/ml)
Ceftazidime	1X	<0	0.6±0.3
	3X	<0	0.6±0.4
	6X	<0	1.1±0.4
Ciprofloxacin	1X	57±9	2.3±0.4
	3X	85±10	3.0±0.3
	6X	162±10	4.1±0.3

[a] Means of four clinical isolates (F327, F443, F991, E1481) and one reference strain ATCC 27853 of *P. aeruginosa* ± standard deviation. Determinations were repeated in triplicate with each strain.

[b] Bacterial kill is the difference between the colony count of the original inoculum and the colony count following a 2 hour aminoglycoside exposure measured in log_{10} units.

[c] Positive values signify bacterial culture growth.

Table 14. Cefotaxime, ciprofloxacin, and gentamicin PAEs against *Escherichia coli.*

Antimicrobial Agent	Concentration	PAE (min)[a]	Bacterial Kill[a,b] (log_{10} cfu/ml)
Cefotaxime	2X MIC	8±4	1.0±0.1
Ciprofloxacin	2X MIC	82±7	3.0±0.3
Gentamicin	2X MIC	91±5	3.4±0.4

[a] Means for the two *E. coli* strains (ATCC 25922, G1307) ± standard deviation. Determinations were repeated in triplicate with each strain.

[b] Bacterial kill is the difference between the colony count of the original inoculum and the colony count following a 2 hour aminoglycoside exposure measured in log_{10} units.

b. Multiple Exposure PAE Determinations

α. Aminoglycosides and *Pseudomonas aeruginosa*

Cultures re-exposed to their original MIC demonstrated PAE ($P<0.05$) and percent bacterial kill ($P<0.01$) values which were significantly reduced with each subsequent exposure (Table 15). MIC values were determined prior to each exposure. Eight hours following the start of the first two hour aminoglycoside exposure (i.e. just prior to the second exposure) MICs were two to four times the original MIC for all aminoglycosides. At 16 hours, just prior to the third exposure, amikacin MICs were four to eight times their original values, gentamicin MICs were all four times their original values and tobramycin MICs were two to four times their original values. MICs at 24 hours were between four and eight times their original value for amikacin and gentamicin and two to four times their original value for tobramycin. All cultures returned to their original MIC between 96 and 144 hours following their first aminoglycoside exposure (data shown in MRT section). Once cultures were known to have returned to their original susceptibilities they were once again exposed to aminoglycoside at their MIC. PAE and bacterial killing were not significantly different from that demonstrated on initial exposures for all strains and aminoglycosides (data not shown).

To gain insight into the reduction in PAE and bacterial killing noted for re-exposures at the original MIC, cultures were re-exposed to their induced MICs, which ranged from two to four times the original MIC following first exposure, to four to 16 times the original MIC following the second exposure (Table 16). A significant increase in PAE ($P<0.05$) was demonstrated between first and second exposures, but not between second and third exposures, for all aminoglycosides tested. Similarly, statistically significant ($P<0.05$) decreases in bacterial killing were only seen between first and second

Aminoglycoside	First Exposure			Second Exposure			Third Exposure			
	MIC (mg/l)[b] 0h	PAE (min)[b]	Bacterial Kill[b,c]	MIC (mg/l) 24h	PAE (min)	Bacterial Kill	MIC (mg/l) 48h	PAE (min)	Bacterial Kill	MIC (mg/l) 72h
Amikacin	5.9±0.8	70±8	2.2±0.2	20.7±2.6	50±10	0.4±0.2	36.8±3.9	22±8	+0.7±0.5	36.8±3.9
Gentamicin	3.2±0.4	68±9	2.1±0.2	10.4±1.1	38±7	0.6±0.3	12.8±1.4	25±5	+0.1±0.3	14.2±1.5
Tobramycin	0.9±0.1	86±15	1.7±0.2	3.0±0.2	56±11	0.4±0.3	3.3±0.3	30±6	+0.4±0.3	3.3±0.3

[a] 10^7 cfu/ml were exposed to aminoglycoside for 2 h, every 8 h, over a 24 h period.
[b] Values given are means of four clinical isolates (F327, F443, F991, E1481) and one reference strain ATCC 27853 of *P.aeruginosa* ± standard deviation. Experiments were repeated twice with each strain of *P. aeruginosa.*
[c] Positive values signify bacterial culture growth.

Table 15. **PAEs following multiple aminoglycoside exposures at 1X original MIC against *Pseudomonas aeruginosa*[a].**

Aminoglycoside	First Exposure			Second Exposure			Third Exposure			
	MIC (mg/l)[b] 0h	PAE (min)[b]	Bacterial Kill[b]	MIC (mg/l) 24h	PAE (min)	Bacterial Kill	MIC (mg/l) 48h	PAE (min)	Bacterial Kill	MIC (mg/l) 72h
Amikacin	5.9±0.8	70±8	2.2±0.2	20.7±2.6	103±15	1.5±0.5	61.1±5.5	116±16	1.3±0.5	61.1±5.5
Gentamicin	3.2±0.4	68±9	2.1±0.2	10.4±1.1	109±17	1.4±0.3	25.6±3.1	124±14	1.2±0.3	25.6±3.1
Tobramycin	0.9±0.1	86±15	1.7±0.2	3.0±0.2	135±14	1.1±0.4	6.1±0.7	140±18	1.1±0.4	7.2±0.8

[a] 10^7 cfu/ml were exposed to aminoglycoside for 2 h, every 8 h, over a 24 h period.
[b] Values given are means of four clinical isolates (F327, F443, F991, E1481) and one reference strain ATCC 27853 of *P.aeruginosa* ± standard deviation. Experiments were repeated twice with each strain of *P. aeruginosa*.

Table 16. **PAEs following multiple aminoglycoside exposures at 1X induced MIC against *Pseudomonas aeruginosa*[a].**

exposures, but not between second and third exposures, for all aminoglycosides tested. All cultures required between 120 and 192 hours to return to their initial susceptibilities following the first aminoglycoside exposure (data shown in MRT section).

Multiple exposure experiments were performed simulating traditional (thrice daily) and once daily dosing regimens with gentamicin and tobramycin. Triplicate gentamicin and tobramycin re-exposures at 8 mg/l (traditional peak) for 2 hours every 8 hours, and 24 mg/l (once daily dosing peak) for 2 hours every 24 hours, were used. Cultures re-exposed thrice at 8 and 24 mg/l of gentamicin and tobramycin demonstrated a pattern similar (Table 17 and 18) to that shown in Table 15. Significant decreases ($P<0.05$) were demonstrated in PAE and bacterial kill on subsequent exposures for gentamicin and tobramycin at both concentrations with a single exception. At 8 mg/l there was no significant difference between the first and second exposure PAEs ($P>0.05$). Using 8 mg/l, gentamicin MICs just prior to the second exposure were four to eight times their original values while tobramycin gave MIC values between eight and sixteen times their original values. Gentamicin and tobramycin MICs just prior to the third exposure were eight to sixteen times their original values. At 24 mg/l, MICs just prior to the second exposure were four to eight times their original values for gentamicin, and two to four times their original values for tobramycin. Gentamicin and tobramycin MICs just prior to the third exposure were eight times their original values for gentamicin and four to eight times their original values for tobramycin. All cultures exposed in triplicate at 8 and 24 mg/l required approximately 240 to 288 hours to return to their initial susceptibilities following their first aminoglycoside exposure (data shown in MRT section). Cultures known to have returned to their original susceptibilities demonstrated PAEs and bacterial killing which was not significantly different to that seen following

Aminoglycoside	First Exposure			Second Exposure			Third Exposure			
	MIC (mg/l)[b] 0h	PAE (min)[b]	Bacterial Kill[b,c]	MIC (mg/l) 24h	PAE (min)	Bacterial Kill	MIC (mg/l) 48h	PAE (min)	Bacterial Kill	MIC (mg/l) 72h
Gentamicin	3.2±0.4	100±12	3.7±0.4	19.0±2.6	88±12	0.8±0.4	43.2±2.9	37±11	+0.5±0.5	43.2±2.9
Tobramycin	0.9±0.1	230±16	4.0±0.4	7.8±1.1	110±15	0.6±0.5	11.4±1.5	64±15	0.1±0.4	11.4±1.5

[a] 10^5-10^7 cfu/ml were exposed to aminoglycoside for 2 h, every 8 h, over a 24 h period.
[b] Values given are means of four clinical isolates (F327, F443, F991, E1481) and one reference strain (ATCC 27853) of *P.aeruginosa* ± standard deviation. Experiments were repeated twice with each strain of *P. aeruginosa.*
[c] Positive values signify bacterial culture growth.

Table 17. **PAEs following multiple aminoglycoside exposures at 8 mg/l against *Pseudomonas aeruginosa*[a].**

Aminoglycoside	First Exposure			Second Exposure			Third Exposure			
	MIC (mg/l)[b] 0h	PAE (min)[b]	Bacterial Kill[b]	MIC (mg/l) 24h	PAE (min)	Bacterial Kill	MIC (mg/l) 48h	PAE (min)	Bacterial Kill	MIC (mg/l) 72h
Gentamicin	3.2±0.4	208±17	4.7±0.4	15.6±1.2	106±13	3.2±0.3	25.6±2.6	64±14	0.7±0.5	25.6±2.6
Tobramycin	0.9±0.1	343±16	5.2±0.5	3.0±0.2	200±14	4.0±0.5	5.7±0.7	120±15	1.0±0.4	6.0±0.8

[a] 10^7 cfu/ml were exposed to aminoglycoside for 2 h, every 24 h, over a 72 h period.
[b] Values given are means of four clinical isolates (F327, F443, F991, E1481) and one reference strain (ATCC 27853) of *P.aeruginosa* ± standard deviation. Experiments were repeated twice with each strain of *P. aeruginosa*.

Table 18. PAEs following multiple aminoglycoside exposures at 24 mg/l against *Pseudomonas aeruginosa*[a].

initial aminoglycoside exposures (data not shown). Both the traditional and once daily aminoglycoside regimens demonstrated the identical trend of reductions in PAE and bacterial killing. However, the once daily regimen may be seen as more advantagous as it produced much larger initial PAEs and, relative to the traditional regimen, has significantly greater PAE and bacterial killing on subsequent exposures.

ß. Other Antimicrobial Agents and *Pseudomonas aeruginosa*

The PAEs following multiple ceftazidime and ciprofloxacin exposures at 1X MIC against *P. aeruginosa* are presented in Table 19. No significant changes were observed in ceftazidime MIC ($P>0.05$), PAE ($P>0.05$) or bacterial kill ($P>0.05$) following analysis of variance determinations. Ciprofloxacin demonstrated significant differences in MICs ($P<0.05$) and PAEs ($P<0.05$) between first and third exposures, and in bacterial kill ($P<0.05$) between first and second, and first and third exposures. Other differences were not statistically significant ($P>0.05$).

γ. Escherichia coli

PAE and bacterial kill determinations following multiple cefotaxime, ciprofloxacin and gentamicin exposures against *E. coli* are presented in Table 20. Cefotaxime demonstrated a PAE of less than 10 minutes with all three exposures in the *E. coli* strains tested. The extent of cefotaxime killing was not significantly different ($P>0.05$) (approximately 1 $\log_{10}$ cfu/ml) with all three exposures. Ciprofloxacin and gentamicin demonstrated significant increases in MIC ($P<0.05$) and decreases in PAE ($P<0.05$), between first and second, and first and third exposures, but not between second and third exposures. Ciprofloxacin and gentamicin demonstrated significant decreases ($P<0.01$) in bacterial killing between first and second, and second and third exposures (Table 20).

Antimicrobial Agent	First Exposure			Second Exposure			Third Exposure			
	MIC (mg/l)[b] 0h	PAE (min)[b]	Bacterial Kill[b]	MIC (mg/l) 24h	PAE (min)	Bacterial Kill	MIC (mg/l) 48h	PAE (min)	Bacterial Kill	MIC (mg/l) 72h
Ceftazidime	3.6±0.2	<0	0.6±0.3	3.6±0.2	<0	0.5±0.4	3.6±0.2	<0	0.6±0.4	3.6±0.2
Ciprofloxacin	0.3±0.1	57±9	2.3±0.4	0.6±0.4	46±7	1.7±0.3	0.8±0.5	38±5	1.2±0.4	0.9±0.5

[a] 10^7 cfu/ml were exposed to antimicrobial agent for 2 h, every 8 h, over a 24 h period.

[b] Values given are means of four clinical isolates (F327, F443, F991, E1481) and one reference strain (ATCC 27853) of *P.aeruginosa* ± standard deviation. Experiments were repeated twice with each strain of *P. aeruginosa.*

Table 19. **PAEs following multiple ceftazidime and ciprofloxacin exposures at 1X original MIC against *Pseudomonas aeruginosa*[a].**

Antimicrobial Agent	First Exposure			Second Exposure			Third Exposure			
	MIC (mg/l)[b] 0h	PAE (min)[b]	Bacterial Kill[b]	MIC (mg/l) 24h	PAE (min)	Bacterial Kill	MIC (mg/l) 48h	PAE (min)	Bacterial Kill	MIC (mg/l) 72h
Cefotaxime	0.094	8±4	1.0±0.1	0.094	8±3	0.9±0.2	0.094	9±4	1.0±0.3	0.094
Ciprofloxacin	0.012	82±7	3.0±0.3	0.048	60±10	2.2±0.3	0.096	51±9	1.3±0.4	0.096
Gentamicin	1.0	91±5	3.3±0.4	4.0	70±9	2.5±0.5	4.0	60±8	1.1±0.4	4.0

[a] 10^7 cfu/ml were exposed to antimicrobial agent for 2 h, every 8 h, over a 24 h period.
[b] Values given are means for 2 strains (ATCC 25922, G1307). Experiments were repeated in triplicate with each strain of *E. coli*.

Table 20. **PAEs and bacterial kill following multiple exposures of cefotaxime, ciprofloxacin, and gentamicin against *Escherichia coli*.**

4. Adaptive Resistance Determinations

a. Inhibitory Aminoglycoside Concentrations and *Pseudomonas aeruginosa*

Figure 7 depicts aminoglycoside adaptive resistance following a single, 2 hour gentamicin exposure at 1X MIC against *P. aeruginosa* ATCC 27853. Several observations can be made from Figure 7. In the control culture, the addition of 1X MIC (3.6 mg/l - final gentamicin concentration) consistently ($P>0.05$) produced bacterial killing of approximately 2 $\log_{10}$ cfu/ml in 2 hours, during 7 consecutive 2 hour intervals (Figure 7, lower left panel). The test culture (2 hour gentamicin pre-exposure at 1X MIC) showed the appearance and disappearance of unstable adaptive resistance (reduced bacterial killing) to the re-addition of gentamicin, at 1X MIC, during growth for 12 hours in antimicrobial free medium (Figure 7, lower right panel). From 0 to 4 hours after aminoglycoside removal (growth in drug free medium), the readdition of 1X MIC of gentamicin had decreasing bactericidal action. From 4 to 10 hours the culture gradually regained its aminoglycoside susceptibility. Ten hours after the culture was removed from the first aminoglycoside exposure, when the bacterial population had increased approximately 3 $\log_{10}$ cfu/ml (99.9% of the culture are new progeny in aminoglycoside free media) in aminoglycoside free medium, the culture again was susceptible to the bactericidal action of gentamicin with bacterial killing of approximately 2 $\log_{10}$ cfu/ml in 2 hours. Clinical isolates F327, F443, F991, and E1481 each demonstrated very similar results to those depicted in Figure 7. Each clinical isolate and the reference strain ATCC 27853 was tested on 3 separate occasions. Analysis of variance, followed by Tukey's multiple comparison test, indicated that bacterial killing was significantly different from wild-type controls from 0 to 8 hours following aminoglycoside removal ($P<0.05$). Adaptive resistance was greatest (least bacterial killing) ($P<0.05$) when logarithmic growth

of the pre-exposed culture had resumed (4 hours following aminoglycoside removal), generally considered the end of the postantibiotic effect (PAE) period.

b. Subinhibitory Aminoglycoside Concentrations and *Pseudomonas aeruginosa*

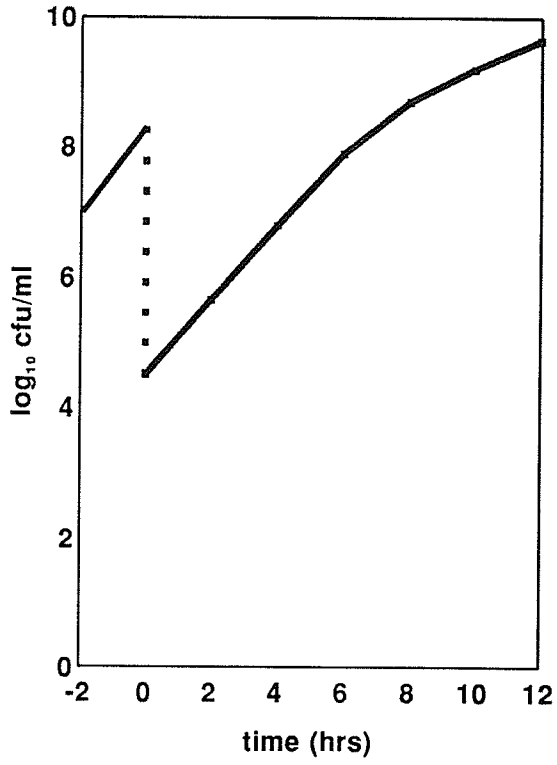
Adaptive resistance intervals were also determined at 1/2X, 1/4X and 1/8X the gentamicin MIC. The results are presented for *P. aeruginosa* 27853 at 1/2X MIC (Figure 8) and 1/8X MIC (Figure 9). The clinical isolates tested (F327, F443, F991, E1481) demonstrated very similar results to those depicted in Figures 8 and 9. An identifiable adaptive resistance interval, with significant ($P<0.05$) reductions in bacterial killing, of 6 hours was seen with 1/2X MIC (Figure 8, lower right panel) . No significant ($P>0.05$) adaptive resistance interval was identifiable with 1/4X and 1/8X MIC (Figure 9, lower right panel). Bacterial killing was similar in both the pre-exposed and the control cultures.

c. Others Antimicrobials and *Pseudomonas aeruginosa*

Adaptive resistance determinations were conducted with ceftazidime and ciprofloxacin (Figures 10 and 11 respectively). Ceftazidime did not demonstrate an adaptive resistance interval following an initial 2 hour exposure at 1X MIC (Figure 10). Instead, bacterial killing with ceftazidime appeared inoculum dependent and independent of prior ceftazidime exposure at 1X MIC (Figure 10). Ciprofloxacin demonstrated adaptive resistance with 1X MIC that, although present, was shorter, far less consistently demonstrated in repetition with the same strain, and appeared to lack the induction phase demonstrated with *P. aeruginosa.* Generally, following a 2 hour exposure at 1X MIC, ciprofloxacin produced very limited to no adaptive resistance beyond the end of the PAE interval with the *P. aeruginosa* strains tested (ATCC 27853, F327, F443, F991, E1481).

d. *Escherichia coli*

Adaptive resistance determinations were made using 2 strains of *E. coli* (ATCC 25922 and G1307) exposed to ciprofloxacin or gentamicin at 1X MIC. Neither antimicrobial agent demonstrated a significant adaptive resistance interval in *E. coli* (data not shown). This result suggests that there may be a difference in the manner in which *E. coli* and *P. aeruginosa* respond to an aminoglycoside insult. The results presented here are in agreement with previously published data (Gould *et al.*, 1991).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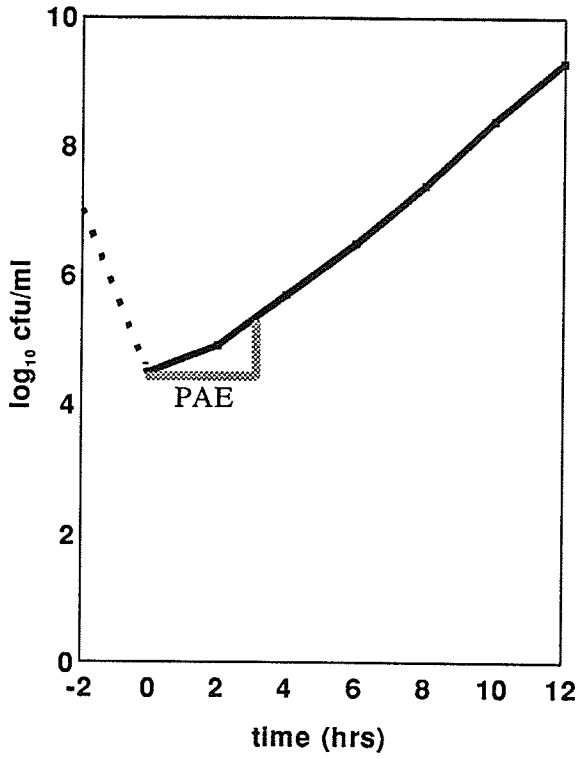
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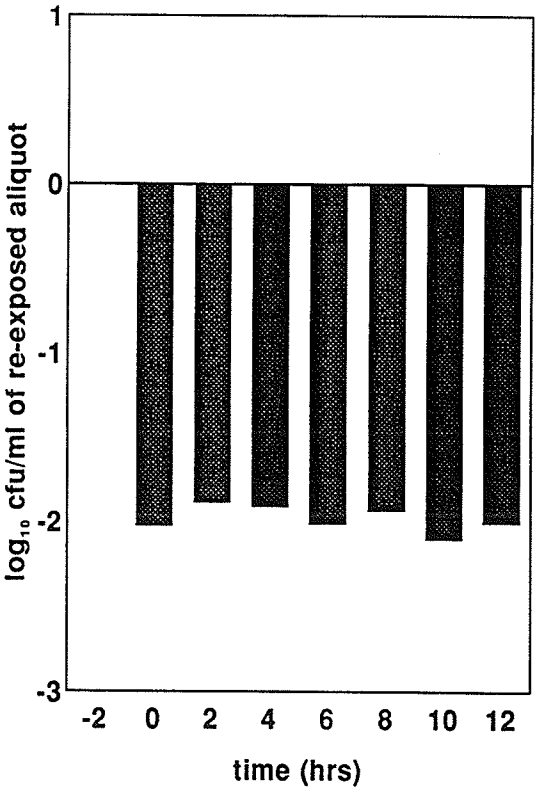
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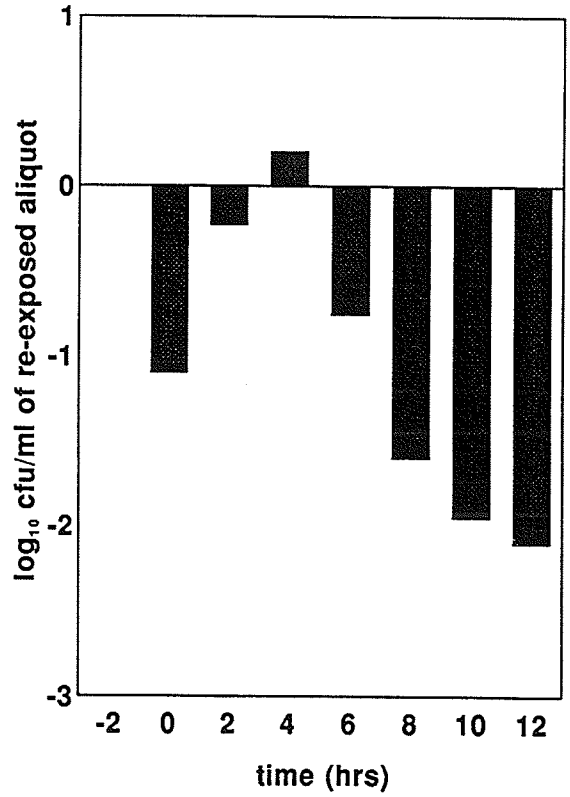
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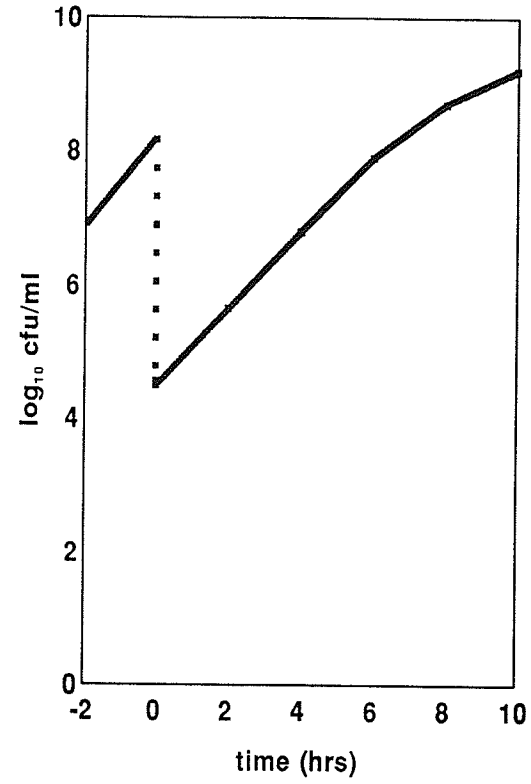
time (hrs)

Figure 7. **Adaptive resistance following a single, 2 hour gentamicin exposure at 1X MIC against *Pseudomonas aeruginosa* ATCC 27853.** Upper left panel shows bacterial growth of control culture with no prior gentamicin exposure. Upper right panel shows bacterial regrowth following a 2 hour gentamicin pre-exposure, at 1X MIC. Extracellular gentamicin was removed by centrifugation following 2 hour pre-exposure. Control (upper and lower left panels) and test (upper and lower right panels) cultures were diluted to approximately 5 x 10^4 cfu/ml at time zero. Lower left panel shows bacterial killing, at successive 2 hour intervals, in control cultures (no previous gentamicin exposure) 2 hours after the addition of gentamicin at 1X MIC. Lower right panel shows bacterial killing, at successive 2 hour intervals, in test cultures (2 hour gentamicin pre-exposure at 1X MIC) 2 hours after the addition of gentamicin at 1X MIC. Values presented are means of 3 experiments performed on separate occasions.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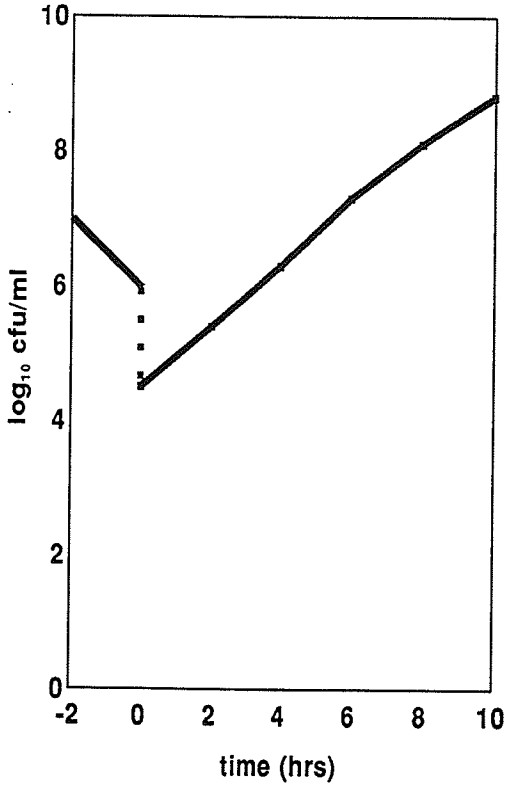
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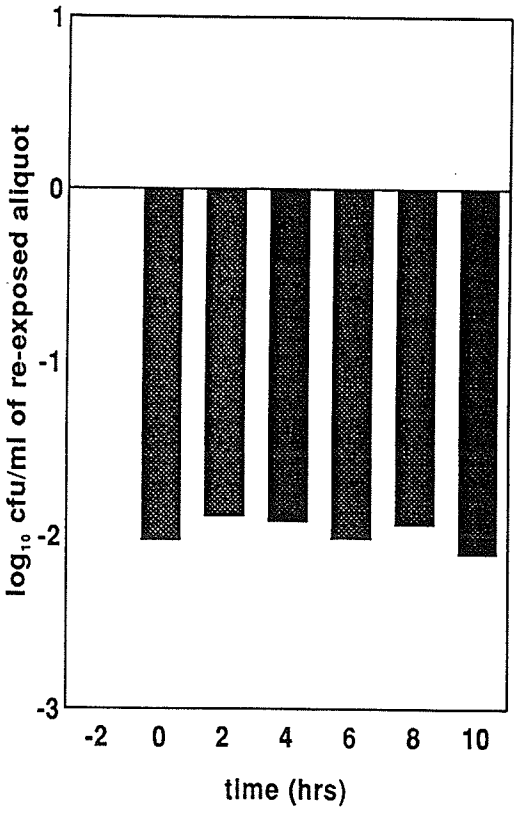
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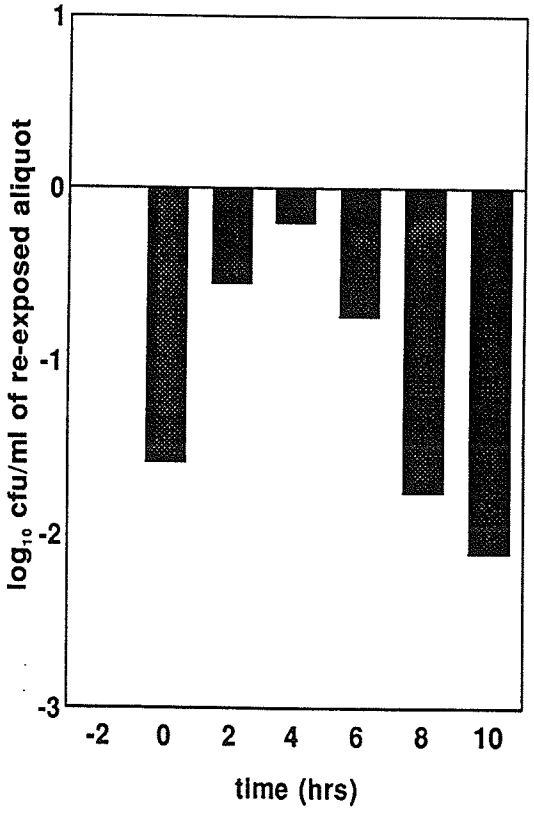
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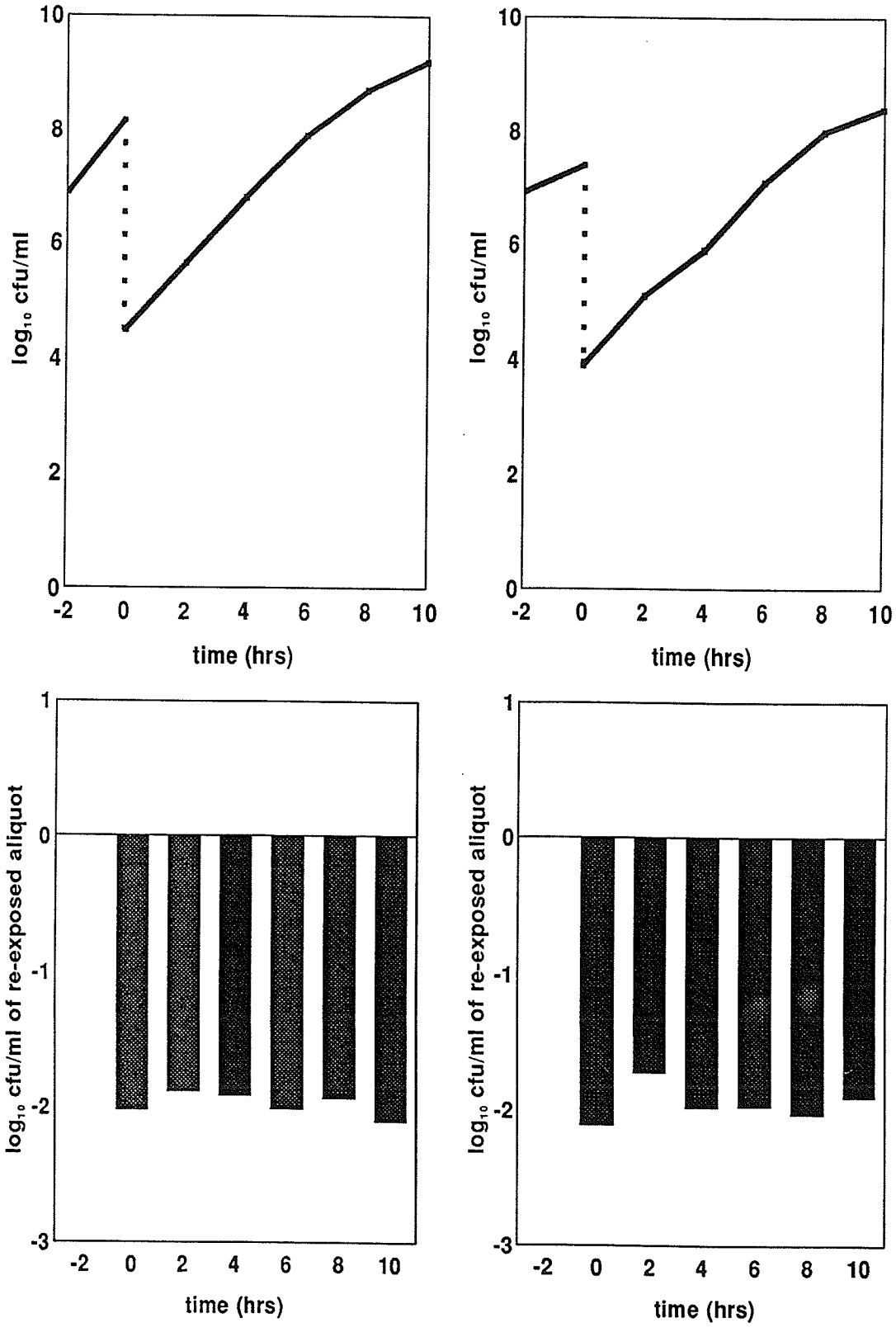
Figure 8. **Adaptive resistance following a single, 2 hour gentamicin exposure at 1/2X MIC against *Pseudomonas aeruginosa* ATCC 27853.** Upper left panel shows bacterial growth of control culture with no prior gentamicin exposure. Upper right panel shows bacterial regrowth following a 2 hour gentamicin pre-exposure, at 1/2X MIC. Extracellular gentamicin was removed by centrifugation following 2 hour pre-exposure. Control (upper and lower left panels) and test (upper and lower right panels) cultures were diluted to approximately 5 x 10^4 cfu/ml at time zero. Lower left panel shows bacterial killing, at successive 2 hour intervals, in control cultures (no previous gentamicin exposure) 2 hours after the addition of gentamicin at 1X MIC. Lower right panel shows bacterial killing, at successive 2 hour intervals, in test cultures (2 hour gentamicin pre-exposure at 1/2X MIC) 2 hours after the addition of gentamicin at 1X MIC. Values presented are means of 3 experiments performed on separate occasions.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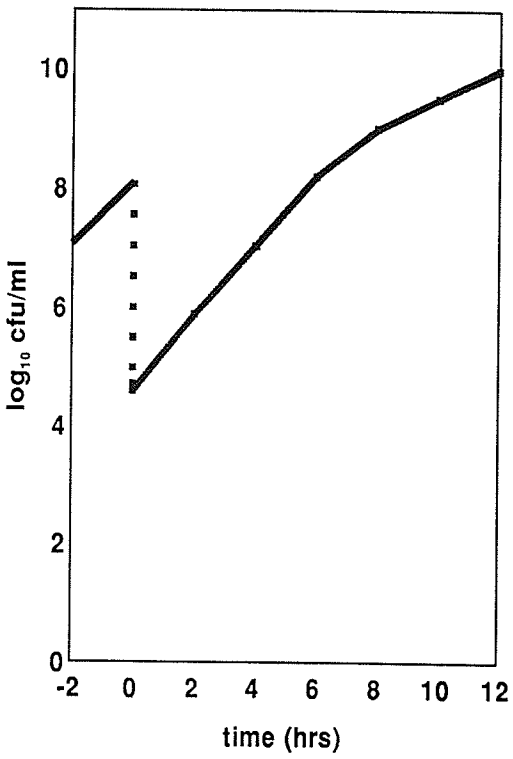
log10 cfu/ml of re-exposed aliquot

Figure 9. **Adaptive resistance following a single, 2 hour gentamicin exposure at 1/8X MIC against *Pseudomonas aeruginosa* ATCC 27853.** Upper left panel shows bacterial growth of control culture with no prior gentamicin exposure. Upper right panel shows bacterial regrowth following a 2 hour gentamicin pre-exposure, at 1/8X MIC. Extracellular gentamicin was removed by centrifugation following 2 hour pre-exposure. Control (upper and lower left panels) and test (upper and lower right panels) cultures were diluted to approximately 5 x 10^4 cfu/ml at time zero. Lower left panel shows bacterial killing, at successive 2 hour intervals, in control cultures (no previous gentamicin exposure) 2 hours after the addition of gentamicin at 1X MIC. Lower right panel shows bacterial killing, at successive 2 hour intervals, in test cultures (2 hour gentamicin pre-exposure at 1/8X MIC) 2 hours after the addition of gentamicin at 1X MIC. Values presented are means of 3 experiments performed on separate occasions.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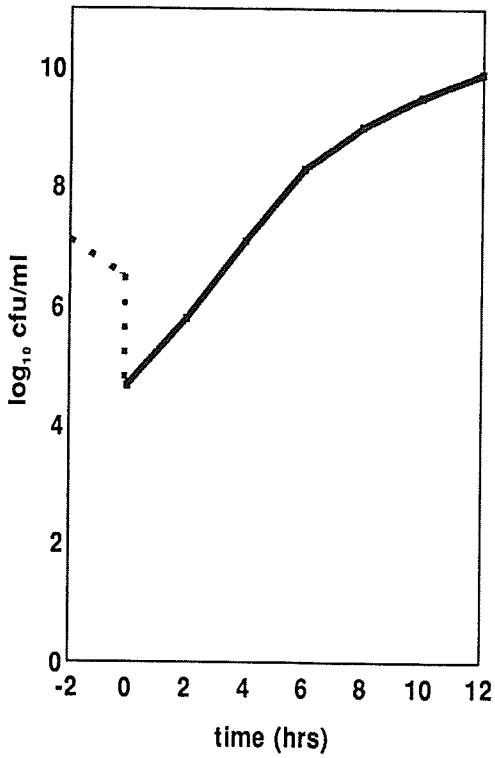
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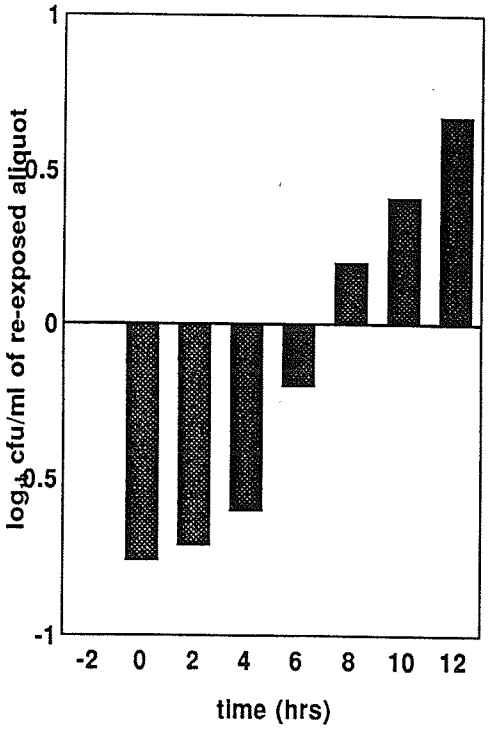
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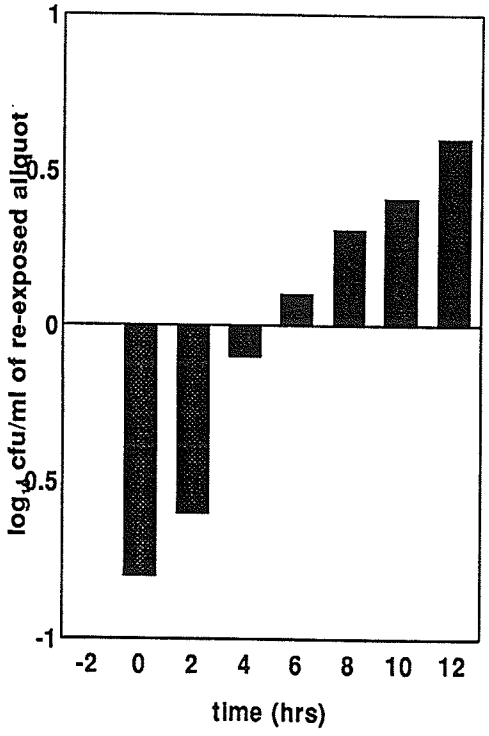
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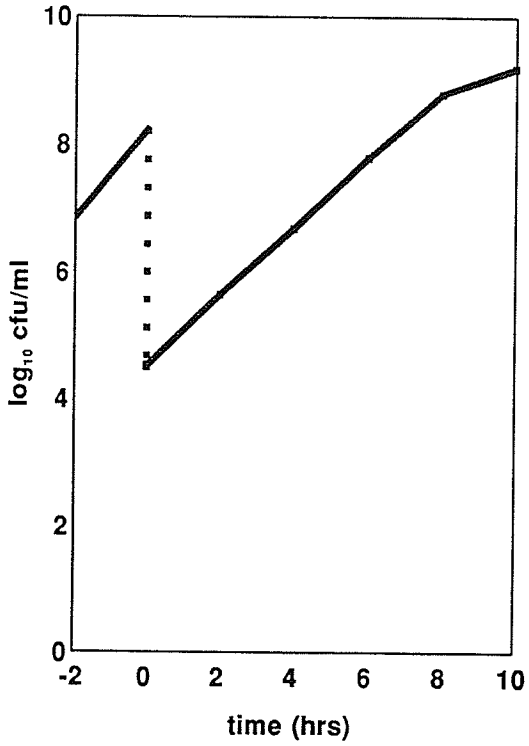
Figure 10. **Adaptive resistance following a single, 2 hour ceftazidime exposure at 1X MIC against *Pseudomonas aeruginosa* ATCC 27853.** Upper left panel shows bacterial growth of control culture with no prior ceftazidime exposure. Upper right panel shows bacterial growth following a 2 hour ceftazidime pre-exposure, at 1X MIC. Extracellular gentamicin was removed by centrifugation following 2 hour pre-exposure. Control (upper and lower left panels) and test (upper and lower right panels) cultures were diluted to approximately 5 x 10^4 cfu/ml at time zero. Lower left panel shows bacterial killing, at successive 2 hour intervals, in control cultures (no previous ceftazidime exposure) 2 hours after the addition of ceftazidime at 1X MIC. Lower right panel shows bacterial killing, at successive 2 hour intervals, in test cultures (2 hour ceftazidime pre-exposure at 1X MIC) 2 hours after the addition of ceftazidime at 1X MIC. Values presented are means of 3 experiments performed on separate occasions.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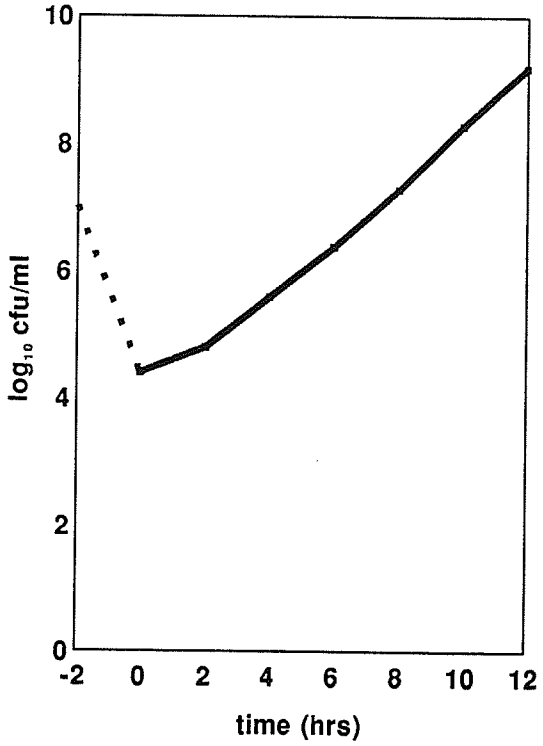
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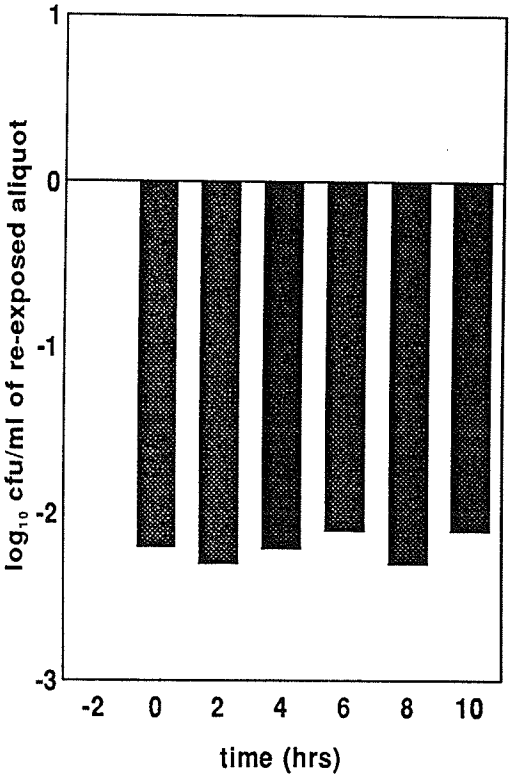
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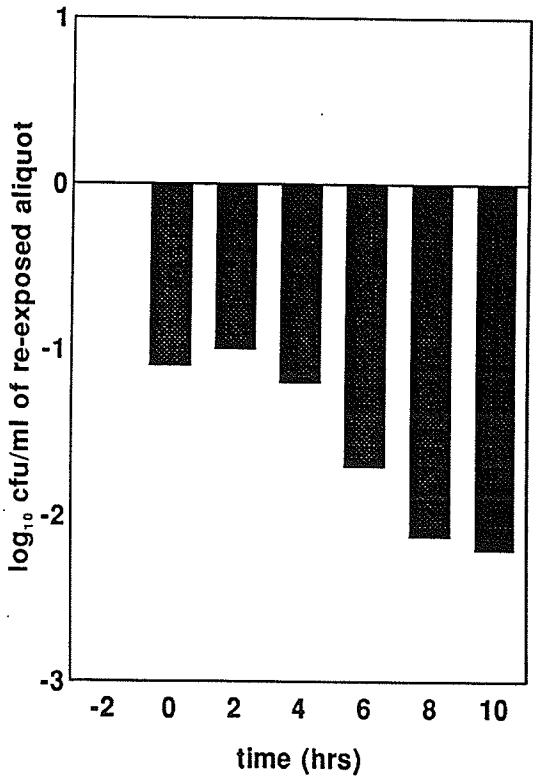
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time (hrs)

Figure 11. **Adaptive resistance following a single, 2 hour ciprofloxacin exposure at 1X MIC against *Pseudomonas aeruginosa* ATCC 27853.** Upper left panel shows bacterial growth of control culture with no prior ciprofloxacin exposure. Upper right panel shows bacterial regrowth following a 2 hour ciprofloxacin pre-exposure, at 1X MIC. Extracellular gentamicin was removed by centrifugation following 2 hour pre-exposure. Control (upper and lower left panels) and test (upper and lower right panels) cultures were diluted to approximately 5 x 10^4 cfu/ml at time zero. Lower left panel shows bacterial killing, at successive 2 hour intervals, in control cultures (no previous ciprofloxacin exposure) 2 hours after the addition of ciprofloxacin at 1X MIC. Lower right panel shows bacterial killing, at successive 2 hour intervals, in test cultures (2 hour ciprofloxacin pre-exposure at 1X MIC) 2 hours after the addition of ciprofloxacin at 1X MIC. Values presented are means of 3 experiments performed on separate occasions.

5. Susceptibility of Adaptively Aminoglycoside Resistant *Pseudomonas aeruginosa* to Other Antimicrobial Agents

To determine if transient resistance to aminoglycosides provides high level cross-resistance to antimicrobial agents of other chemical classes, *P. aeruginosa* strains (ATCC 27853, F327, F443, F991, E1481) were serially passaged against doubling concentrations of gentamicin to a final gentamicin concentration of 1024 mg/l. Cells were then subcultured into fresh antimicrobial free media (Mueller-Hinton broth), and grown with further subculture as necessary, for 24 hours. MIC determinations were then performed using a variety of antimicrobial agents, including amikacin, gentamicin, tobramycin, ciprofloxacin, pefloxacin, ceftazidime, cefotaxime, pipercillin, imipenem, chloramphenicol, polymyxin B and tetracycline. The results are presented in Figure 12. Aminoglycoside MICs increased between 330 and 370 fold for these highly aminoglycoside resistant (highly adaptive resistant) strains. In addition, significant increases in MIC (significance being a 4 fold or greater increase in MIC) were seen for ceftazidime (7-fold increase) and pipercillin (4-fold increase). The highly, aminoglycoside, adaptively resistant strains of *P. aeruginosa* returned to their initial aminoglycoside susceptibility with further subculture in aminoglycoside free Mueller-Hinton broth, as described previously.

6. Aminoglycoside Killing Curves

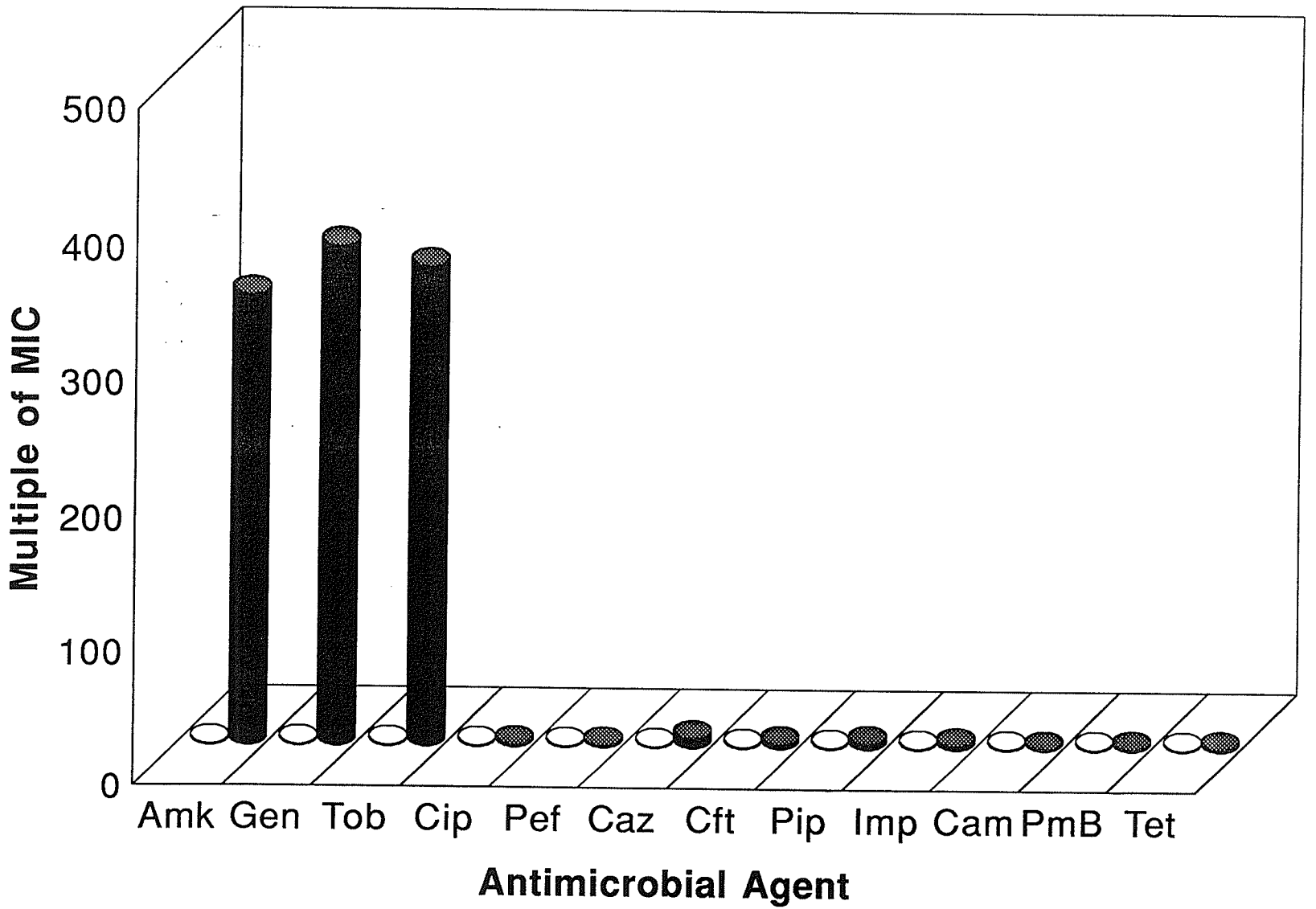
Killing curves demonstrate antimicrobial agent killing of bacteria over time. The killing curve studies performed demonstrated that subinhibitory concentrations of aminoglycosides either decreased bacterial growth for several hours (1/4X and 1/2X MIC) or produce stasis of growth for several hours (1/8X MIC). Inhibitory aminoglycoside concentrations (1X MIC) proved to be bactericidal (3 $\log_{10}$ killing) with approximately

3 hours of exposure. Figures 13, 14, and 15 display the mean inhibitory effect of amikacin, gentamicin and tobramycin at subinhibitory (1/8X, 1/4X and 1/2X MIC) and inhibitory (1X MIC) concentration against five *P. aeruginosa* strains. At a concentration of 1/8X MIC, no agent demonstrated bactericidal activity. All three aminoglycosides delayed bacterial growth at 1 hour. By 2 to 3 hours, all three antimicrobial containing solutions were in logarithmic phase growth, with similar cfu/ml counts when compared to the growth control.

At a concentration of 1/4X MIC, none of these agents demonstrated bactericidal activity. At 1 hour, all three aminoglycosides decreased growth by approximately 0.5 $\log_{10}$ cfu/ml. Bacterial growth in the amikacin and tobramycin solutions returned to baseline (0 hours) at 2 hours, while the gentamicin solution returned to baseline by 3 hours. Gentamicin delayed regrowth more than amikacin and tobramycin up to the 12 hour sample time. Very minor differences in bacterial inhibition were noted between the five isolates at both 1/8X and 1/4X MIC.

With aminoglycosides at 1/2X MIC no agent demonstrated bactericidal activity. Following 3 to 4 hours of exposure, all three aminoglycosides displayed similar killing, with approximately 90% or greater of the initial inoculum being killed. Regrowth was noted by 6 hours for all aminoglycosides, and at 12 hours, the final inoculum was greater than the initial inoculum.

Amikacin, gentamicin and tobramycin proved to be bactericidal at 1X MIC, providing approximately a 3 $\log_{10}$ cfu/ml decrease in bacterial growth at approximately 3 hours. At 1 hour, aminoglycoside killed approximately 1 $\log_{10}$ cfu/ml (90% of the initial inoculum); at 2 hours all three aminoglycosides killed approximately 2 $\log_{10}$ cfu/ml (99% of the initial inoculum). By 3 hours, 3 or more $\log_{10}$ cfu/ml kill was seen. Marked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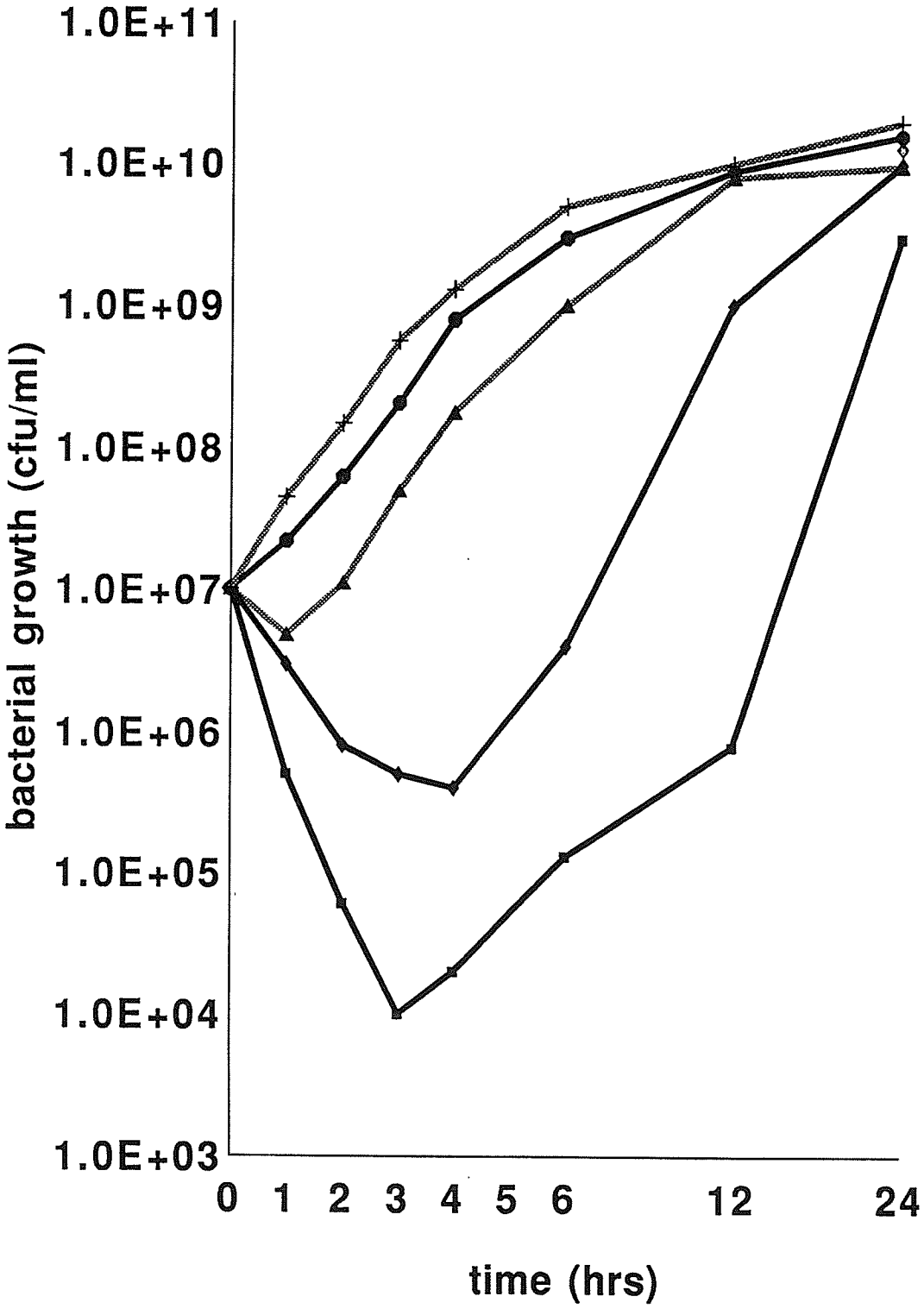
Multiple of MIC
Amk Gen Tob Cip Pef Caz Cft Pip Imp Cam PmB Tet
Antimicrobial Agent

Figure 12. **Susceptibility of Adaptively Aminoglycoside Resistant *Pseudomonas aeruginosa* to other antimicrobial agents.** *P. aeruginosa* strains (ATCC 27853, F327, F443, F991, E1481) were serially passaged against increasing (doubling) concentrations of gentamicin to a final gentamicin concentration of 1024 mg/l. Cells were then subcultured into fresh antimicrobial free media (Mueller-Hinton broth) and grown, with further subculture as necessary, for 24 hours. MIC determinations were then performed using a variety of antimicrobials, including amikacin (Amk), gentamicin (Gen), tobramycin (Tob), ciprofloxacin (Cip), pefloxacin (Pef), ceftazidime (Caz), cefotaxime (Cft), pipercillin (Pip), imipenem (Imp), chloramphenicol (Cam), polymyxin B (PmB) and tetracycline (Tet). The white cylinders represent the MICs of wild-type *P. aeruginosa* strains. The dark cylinders represent the MICs of high level adaptively aminoglycoside resistant *P. aeruginosa* strains. The results are means for the 5 strains of *P. aeruginosa* used. Each MIC was determined in duplicate for each strain.


1.0E+11
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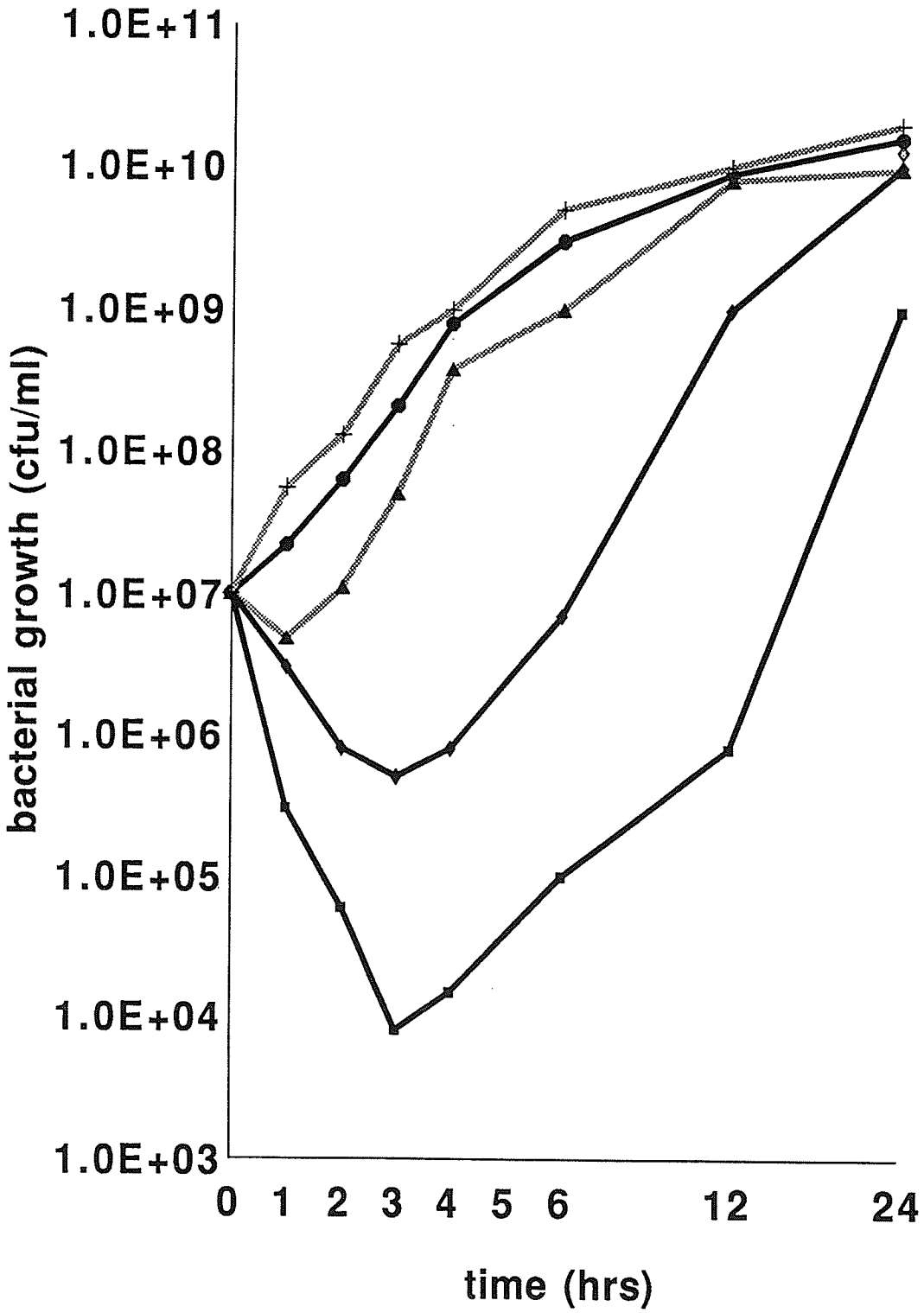
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
bacterial growth (cfu/ml)
0 1 2 3 4 5 6 12 24
time (hrs)

Figure 13. ***Pseudomonas aeruginosa*/Amikacin kill curve.** The curves depicted represent means of viable colony counts taken from 4 clinical isolates (F327, F443, F991, E1481) and the reference strain ATCC 27853 of *P. aeruginosa* incubated with amikacin at 1/8X MIC (•), 1/4X MIC (▲), 1/2X MIC (♦), and 1X MIX (■). The growth control is (+). Viable counts were performed at 0, 1, 2, 3, 4, 6, 12 and 24 hours. Experiments with each strain were performed at least twice, on separate occasions.


1.0E+11
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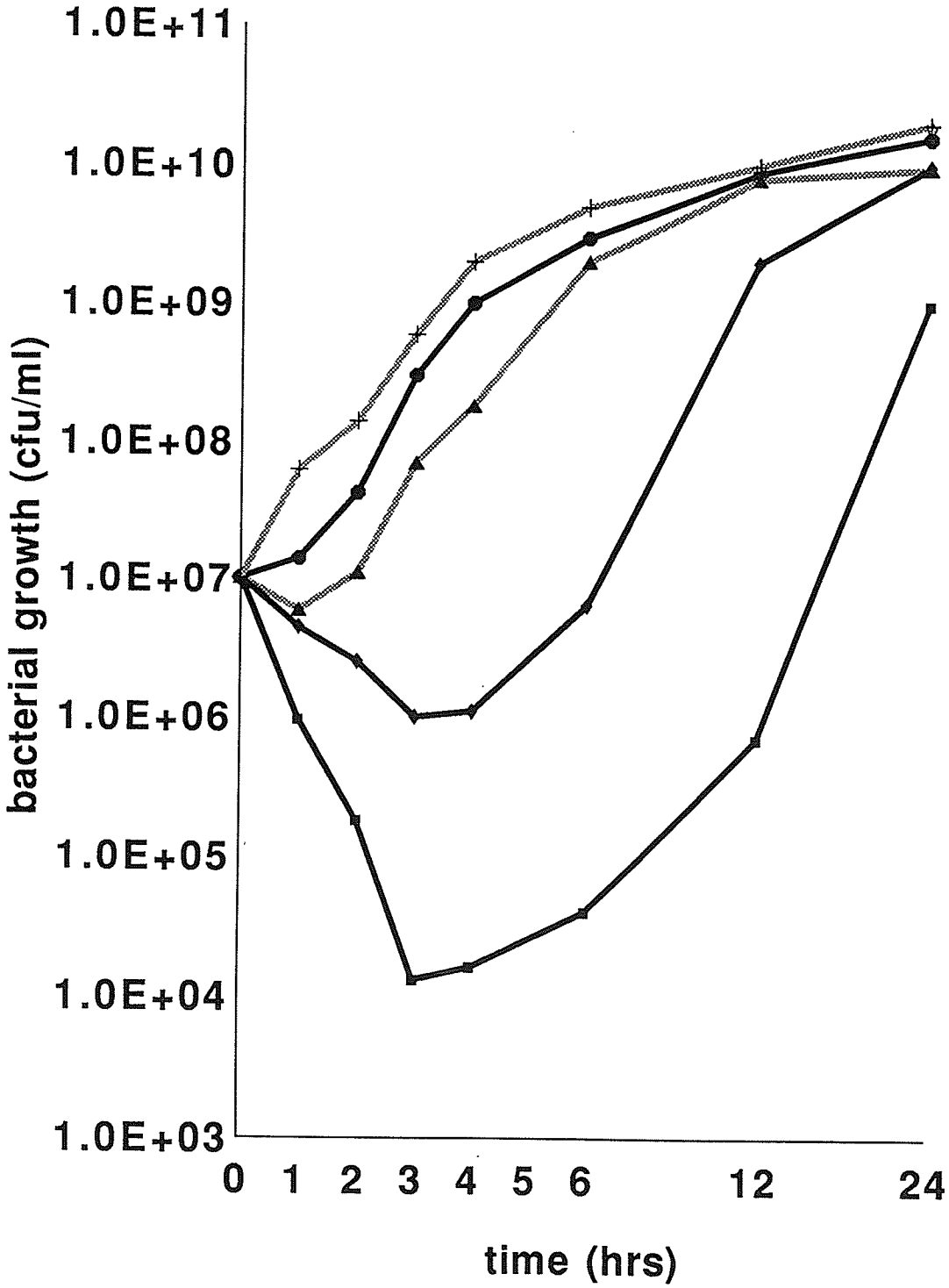
1.0E+04
1.0E+03
bacterial growth (cfu/ml)
0 1 2 3 4 5 6 12 24
time (hrs)

Figure 14. ***Pseudomonas aeruginosa*/Gentamicin kill curve.** The curves depicted represent means of viable colony counts taken from 4 clinical isolates (F327, F443, F991, E1481) and the reference strain ATCC 27853 of *P. aeruginosa* incubated with gentamicin at 1/8X MIC (•), 1/4X MIC (▲), 1/2X MIC (♦), and 1X MIX (■). The growth control is (+). Viable counts were performed at 0, 1, 2, 3, 4, 6, 12 and 24 hours. Experiments with each strain were performed at least twice, on separate occasions.


1.0E+11
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1.0E+07
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1.0E+05
1.0E+04
1.0E+03
bacterial growth (cfu/ml)
0 1 2 3 4 5 6 12 24
time (hrs)

Figure 15. ***Pseudomonas aeruginosa*/Tobramycin kill curve.** The curves depicted represent means of viable colony counts taken from 4 clinical isolates (F327, F443, F991, E1481) and the reference strain ATCC 27853 of *P. aeruginosa* incubated with tobramycin at 1/8X MIC (•), 1/4X MIC (▲), 1/2X MIC (♦), and 1X MIC (■). The growth control is (+). Viable counts were performed at 0, 1, 2, 3, 4, 6, 12 and 24 hours. Experiments with each strain were performed at least twice, on separate occasions.

regrowth was noted by 12 and 24 hours and the final inoculum was greater than the initial inoculum for all three aminoglycosides. As with 1/8X and 1/4X MIC, only minor differences in bacterial killing were noted between the five isolates at 1/2X and 1X MIC.

An important observation that can be made from these graphs is that aminoglycoside killing is not complete at 1 or 2 hours with 1X MIC. It takes approximately 3 hours to reach maximum killing which means that a culture used in an adaptive resistance determination after a 2 hour aminoglycoside exposure still contains susceptible cells.

7. *Pseudomonas aeruginosa* Doubling Times

The mean generation time (doubling time) of the four clinical isolates (F327, F443, F991, E1481) and the reference strain (ATCC 27853) of *P. aeruginosa* tested was 29.9 ± 2.3 minutes (n=30). From PAE and adaptive resistance experiments, mean generation times were determined at one or two hour intervals following an initial two hour exposure at 1X MIC of amikacin, gentamicin or tobramycin (Table 21). Mean generation times were significantly longer ($P<0.05$), compared to growth control, for cells 0 to 3 hours following a two hour aminoglycoside (amikacin, gentamicin, tobramycin) exposure at 1X MIC. At times greater than three hours, mean generation times were similar to growth control. The return to a control mean generation time coincided with the end of the PAE interval, and the maximum reduction in aminoglycoside killing with subsequent exposure (peak adaptive resistance) (Figure 7). In contrast, *P. aeruginosa* grown in the continued presence of aminoglycoside at 1X MIC demonstrated a mean generation time of 52.4 ± 5.0 minutes, which was significantly longer than for control organisms ($P<0.05$) (Zhanel *et al.*, 1991).

Table 21. ***Pseudomonas aeruginosa*** **doubling times.**

Doubling Time Interval[a]	Doubling Time[b] (minutes)
0-1 hour	73.9 ± 12.1[c]
1-2 hours	44.4 ± 7.0[c]
2-3 hours	37.6 ± 8.4[c]
3-4 hours	32.1 ± 5.8
4-5 hours	29.7 ± 7.1
5-6 hours	30.5 ± 5.0
6-8 hours	30.4 ± 5.3
Growth Control	29.9 ± 2.3

[a] Doubling time intervals begin following a two hour aminoglycoside exposure and subsequent removal.
[b] Doubling times are means of gentamicin and tobramycin experiments combined.
[c] Doubling time significantly longer than growth control ($p<0.05$).

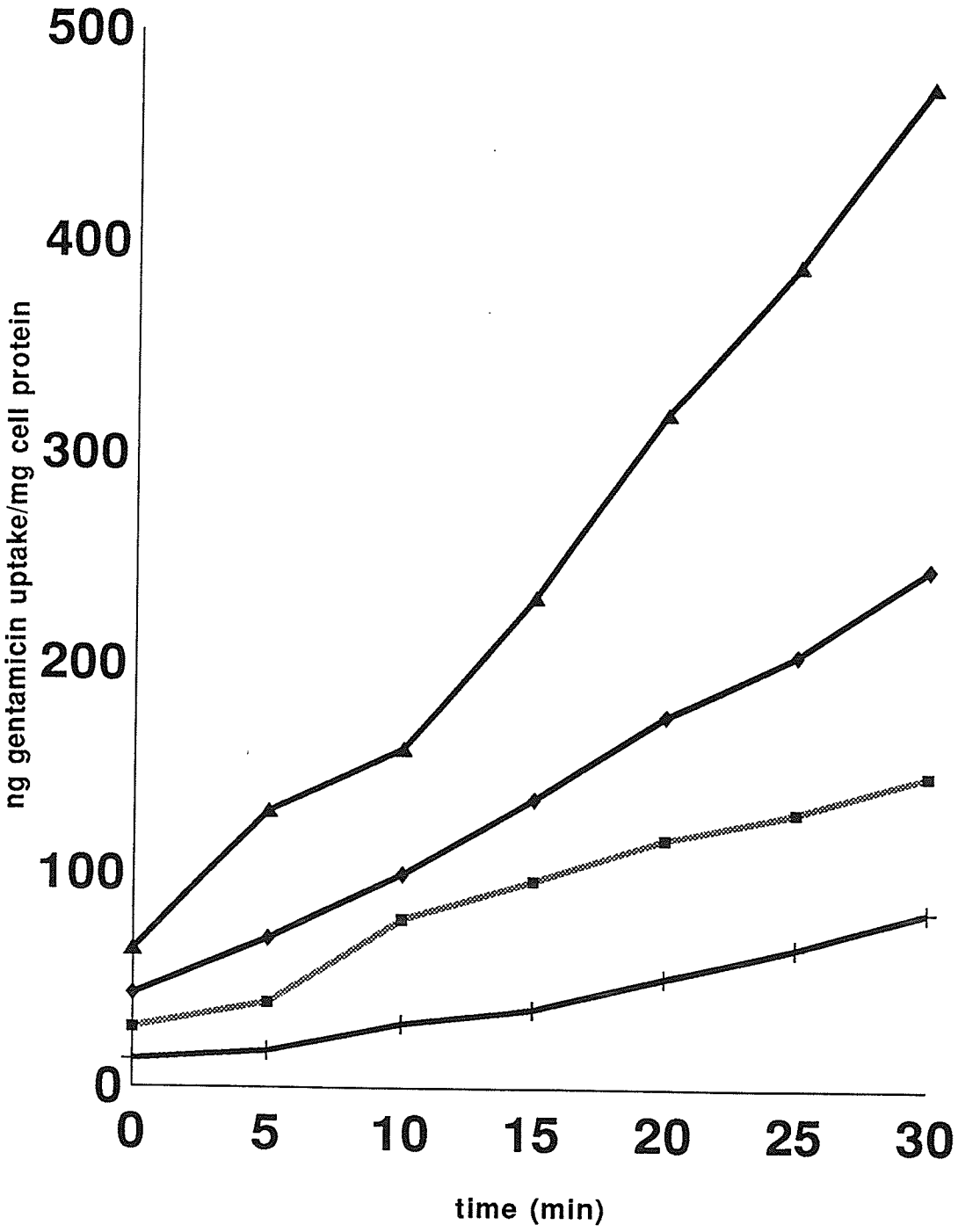
Part 2. Aminoglycoside Accumulation in Adaptively Resistant *Pseudomonas aeruginosa*

1. Gentamicin Accumulation in Exponentially Growing *Pseudomonas aeruginosa*

Gentamicin accumulation in exponentially growing *P. aeruginosa* was measured in the reference strain ATCC 27853 and 4 clinical isolates (F327, F443, F991, E1481). Intracellular accumulation was determined with initial extracellular aminoglycoside concentrations of 4, 8, 16, and 32 mg/l. Gentamicin accumulation in *P. aeruginosa* 27853 is presented in Figure 16. The clinical isolates tested showed similar patterns of gentamicin accumulation. Gentamicin accumulation was concentration dependent and not saturable at the concentrations tested (Figure 16). Peak serum concentrations, even with once daily aminoglycoside dosing, do not approach 32 mg/l. This suggests that in vivo, aminoglycoside accumulation in *P. aeruginosa* would follow a similar concentration dependent, non-saturable pattern.

2. Gentamicin Accumulation in Adaptively Resistant *Pseudomonas aeruginosa*

Gentamicin accumulation in adaptively resistant *P. aeruginosa* was measured in the reference strain ATCC 27853 and 4 clinical isolates (F327, F443, F991, E1481). Figure 17 represents accumulation of gentamicin at 1X MIC by untreated *P. aeruginosa* ATCC 27853, compared to accumulation observed immediately following a 2 hour gentamicin exposure and removal, during peak adaptive resistance, and at 8 and 12 hours following aminoglycoside removal. The clinical strains tested showed similar patterns of gentamicin accumulation. Each clinical isolate and reference strain ATCC 27853 was tested on 3 separate occasions. Statistical analysis was performed on gentamicin accumulation values


500
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ng gentamicin uptake/mg cell protein
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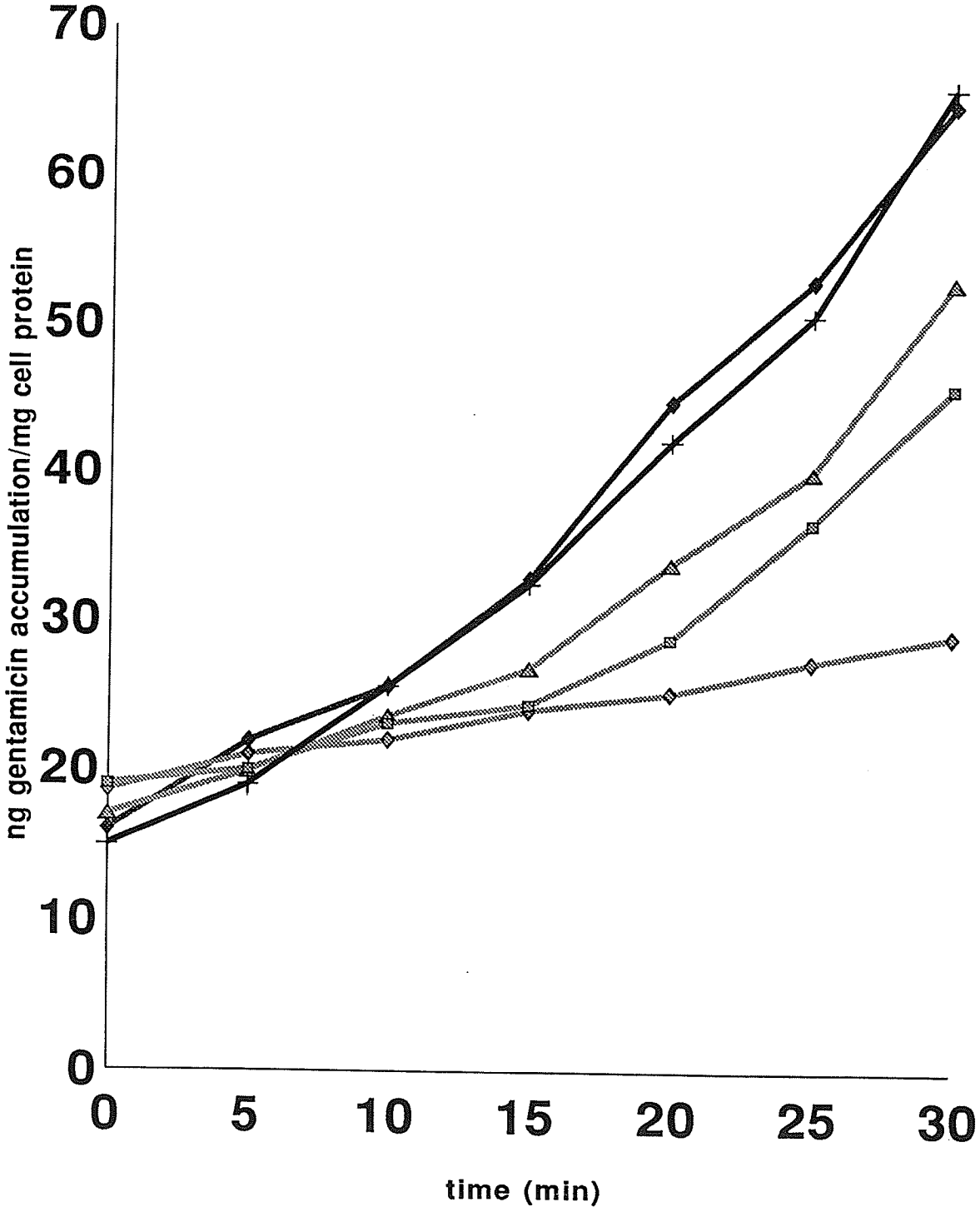
time (min)

Figure 16. **^{3}H-Gentamicin accumulation in exponentially growing *Pseudomonas aeruginosa* ATCC 27853.** Gentamicin accumulation in *P. aeruginosa* ATCC 27853 was determined using extracellular gentamicin concentrations of 4 (+), 8 (■), 16 (♦) and 32 (▲) mg/l over 30 minutes.


70
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ng gentamicin accumulation/mg cell protein
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25
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time (min)

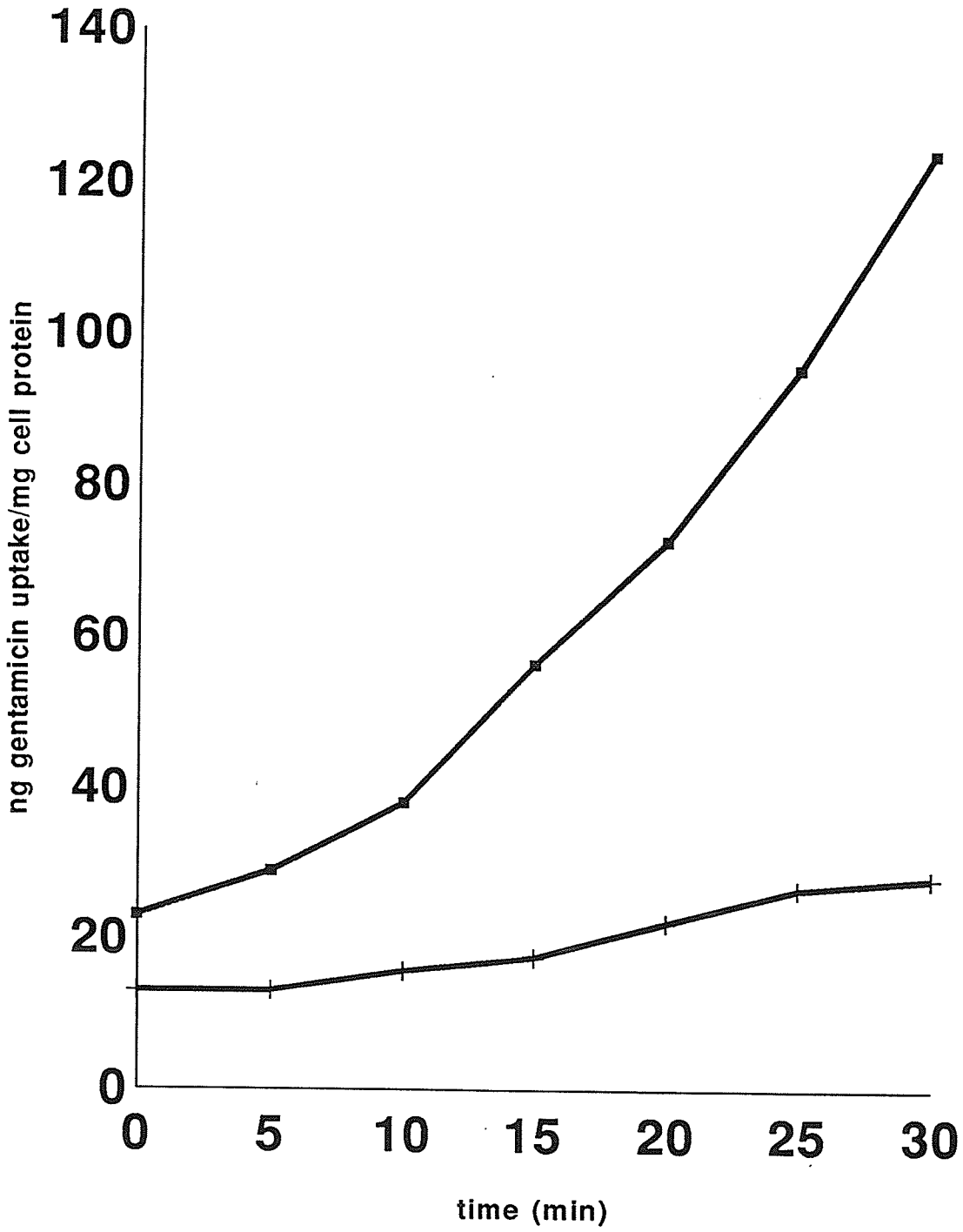
Figure 17. **^{3}H-Gentamicin accumulation in *Pseudomonas aeruginosa* ATCC 27853 following a 2 hour gentamicin pre-exposure at 1X MIC.** Gentamicin accumulation was determined in unexposed controls (+) and compared with accumulation in cultures following a 2 hour exposure to gentamicin at 1X MIC. Gentamicin accumulation was measured in gentamicin pre-exposed cultures immediately following gentamicin removal (0 hour, ■), and then following 4 (♦), 8 (▲) and 12 (•) hours of growth in aminoglycoside free MHB at 37°C.

measured following 30 minutes of incubation. Analysis of variance, followed by Tukey's multiple comparison test, indicated that aminoglycoside accumulation was significantly ($P<0.05$) reduced immediately following the initial 2 hour gentamicin exposure and removal, and at 4 (peak adaptive resistance) and 8 hours thereafter (Figure 17). Peak adaptively resistant cultures (4 hours following 2 hour gentamicin exposure [1X MIC] and removal) demonstrated significantly ($P<0.05$) lower levels of gentamicin accumulation when compared with the other times measured. Aminoglycoside accumulation normalized 12 hours following aminoglycoside removal. Changes in gentamicin accumulation correlated well with fluctuations in aminoglycoside killing (Figure 7).

The effect of increased aminoglycoside concentration on aminoglycoside accumulation in cells that are adaptively resistant to a lower aminoglycoside concentration was also determined (Figure 18). The accumulation of gentamicin at a concentration of 21.6 mg/l (6X MIC) was measured in *P. aeruginosa* ATCC 27853 pre-exposed to 3.6 mg/l (1X MIC) of gentamicin for 2 hours, and incubated in aminoglycoside free MHB at 37°C for 4 hours. Adaptively resistant cultures accumulated significantly ($P<0.001$) more gentamicin at 21.6 mg/l than at 3.6 mg/l (Figure 18). Similarly, the other adaptively resistant strains of *P. aeruginosa* tested (F327, F991, F991, E1481), also demonstrated significantly ($P<0.001$) increased gentamicin accumulation at 6X MIC.

3. Proline Accumulation in *Pseudomonas aeruginosa*

Proline accumulation in adaptively resistant *P. aeruginosa* was also determined, to demonstrate the specificity of adaptive resistance impermeability to aminoglycosides. Proline accumulation in *P. aeruginosa* occurs via active transport directly proportional to the magnitude of the proton motive force (pmf) across the cytoplasmic membrane


140
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ng gentamicin uptake/mg cell protein
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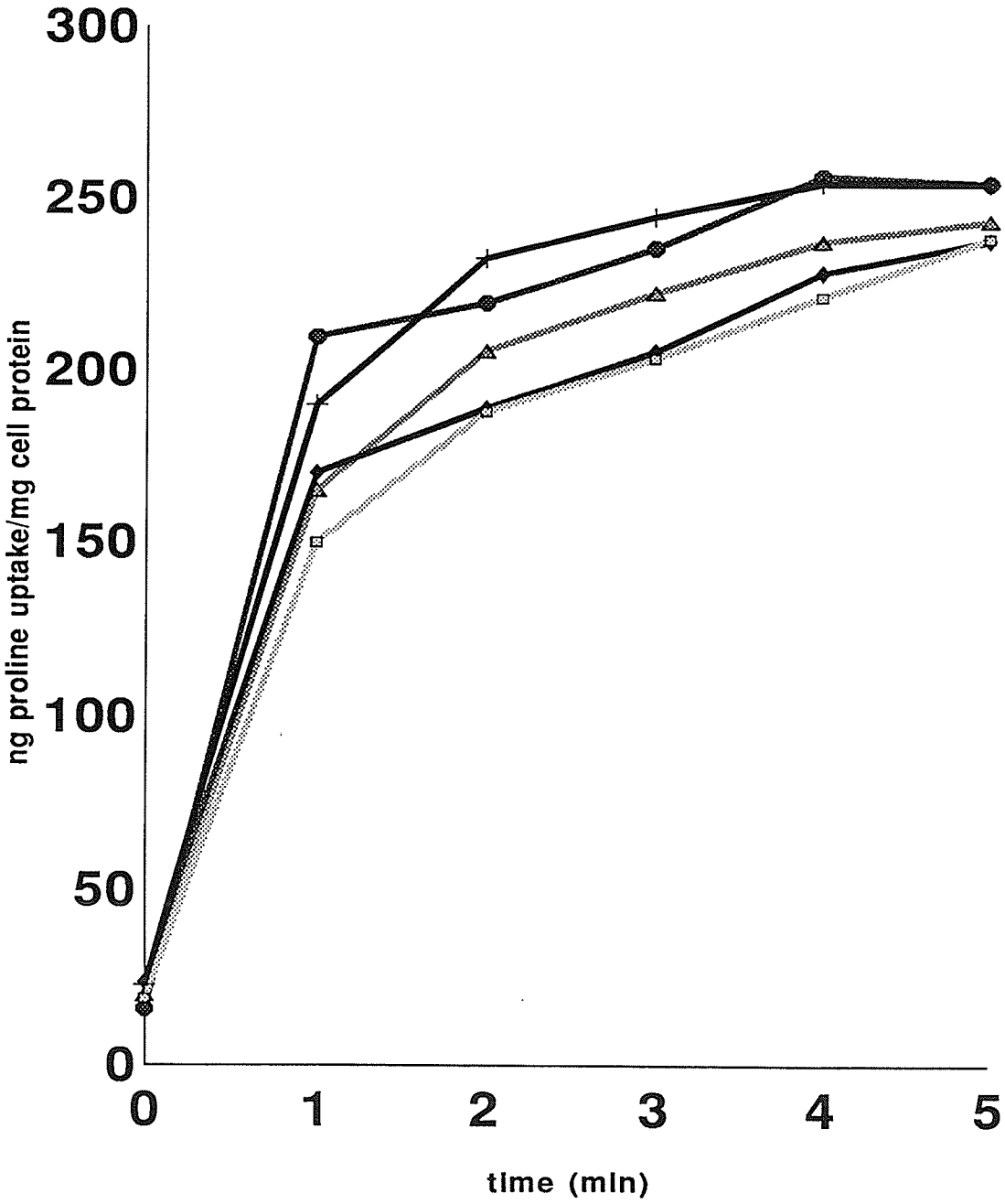
time (min)

Figure 18. **^{3}H-Gentamicin accumulation at 6X MIC (21.6 mg/l) in *Pseudomonas aeruginosa* ATCC 27853 adaptively resistant to 1X MIC (3.6 mg/l) induced by a 2 hour gentamicin pre-exposure at this concentration.** Gentamicin accumulation at 3.6 mg/l (+) and 21.6 (■) was measured following gentamicin removal, and 4 hours of incubation in aminoglycoside free Mueller-Hinton broth at 37°C.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ng proline uptake/mg cell protein
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time (min)

Figure 19. **^{3}H-Proline accumulation in *Pseudomonas aeruginosa* ATCC 27853 adaptively resistant to gentamicin.** Adaptive resistance was induced by a 2 hour gentamicin exposure at 1X MIC against *P. aeruginosa* ATCC 27853. Proline accumulation was determined in unexposed controls (+) and compared with accumulation in cultures following a 2 hour exposure to gentamicin at 1X MIC. Gentamicin accumulation was measured in gentamicin pre-exposed cultures immediately following initial gentamicin removal (0 hour, ■), and then following 4 (♦), 8 (▲) and 12 (•) hours of growth in aminoglycoside free MHB at 37°C.

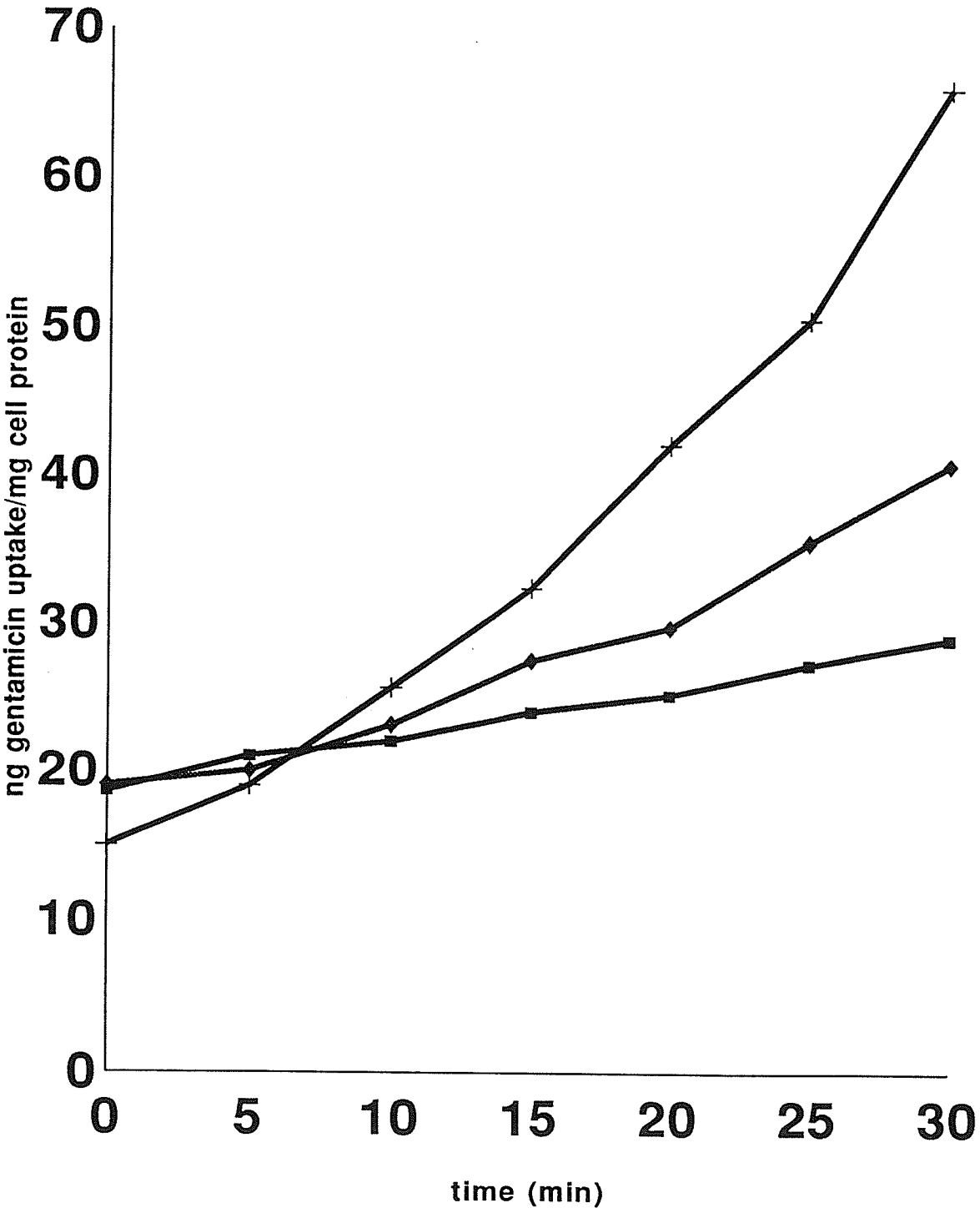
(Fraimow *et al.*, 1991). Aminoglycoside accumulation, as previously discussed, is also dependent upon the magnitude of the pmf. Figure 19 depicts *P. aeruginosa* ATCC 27853 proline accumulation in a wild-type control culture, immediately following a 2 hour gentamicin exposure (1X MIC) and removal, during peak adaptive resistance (4 hours following aminoglycoside removal), and at 8 and 12 hours following aminoglycoside removal. Proline accumulation following a 5 minute incubation was unchanged ($P>0.05$) during the adaptive resistance interval, compared with wild-type controls (Figure 19). This result suggests that aminoglycoside exclusion during the adaptive resistance interval may be pmf independent, at least at the aminoglycoside concentration tested (1X MIC). The clinical isolates tested (F991, E1481) demonstrated similar patterns of proline accumulation.

4. Gentamicin Accumulation in *Pseudomonas aeruginosa* Grown Anaerobically

P. aeruginosa can grow under anaerobic conditions if an alternative, terminal electron acceptor molecule, such as nitrate or nitrite, is available. Growth rates of *P. aeruginosa* grown anaerobically, in the presence of nitrate or nitrite, are similar to growth rates of aerobically maintained cells (Muir *et al.*, 1985). Gentamicin accumulation in logarithmically growing *P. aeruginosa* ATCC 27853 was significantly ($P<0.01$) lower for anaerobically maintained cells compared with aerobically maintained cells (Figure 20). Other strains of *P. aeruginosa* (F991, E1481) tested demonstrated similar results. These results are in agreement with work performed by other researchers (Bryan *et al.*, 1980).

5. Gentamicin Accumulation in *Pseudomonas aeruginosa* Spheroplasts

Spheroplasts were prepared from wild-type control cells and adaptively resistant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ng gentamicin uptake/mg cell protein
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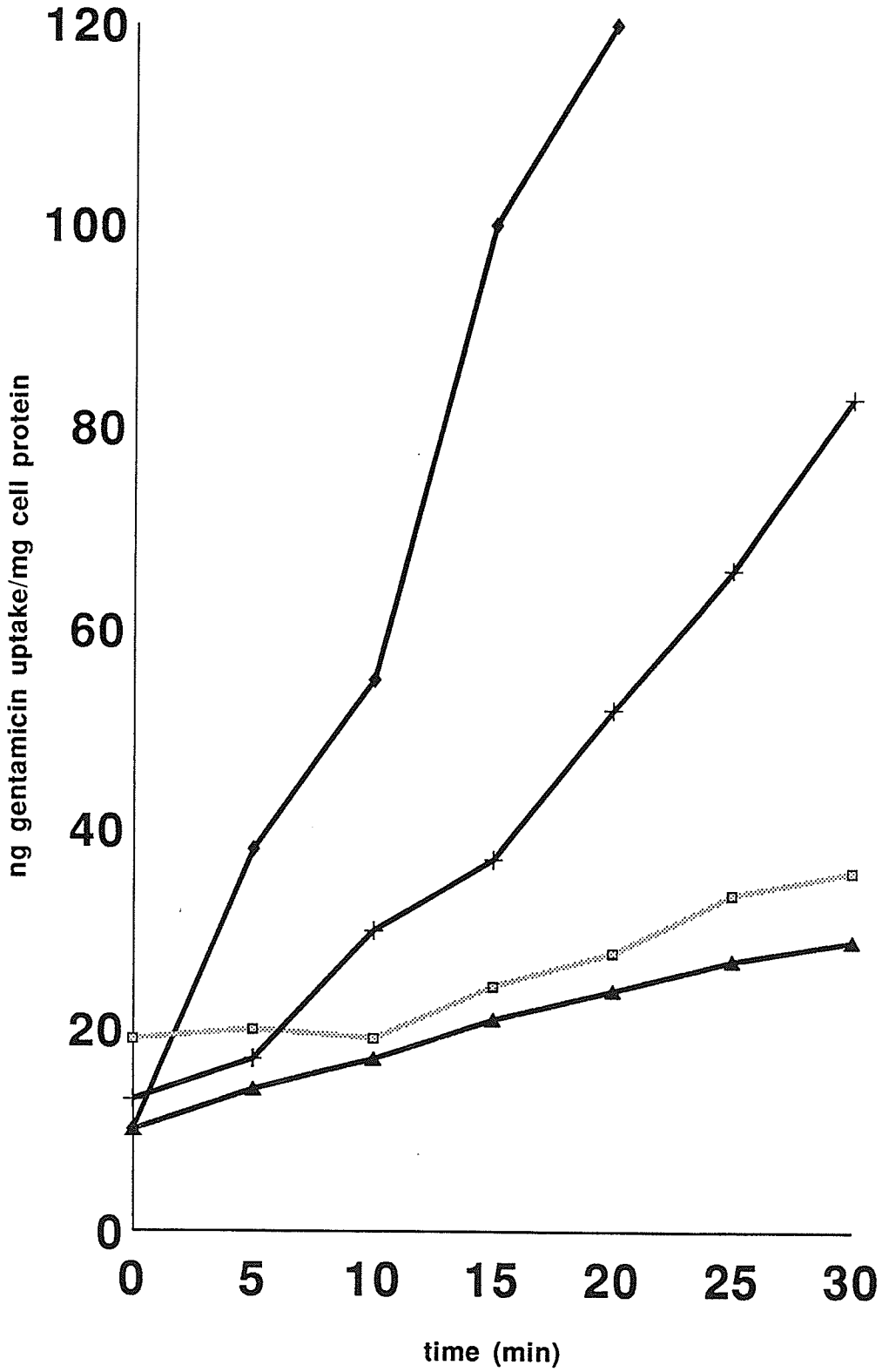
time (min)

Figure 20. **Gentamicin accumulation in *Pseudomonas aeruginosa* ATCC 27853 grown anaerobically in Mueller-Hinton broth supplemented with 50 mM KNO_3.** Gentamicin accumulation was measured in aerobically grown, wild-type control cultures (+), in gentamicin pre-exposed (1X MIC for 2 hours) aerobic cultures following 4 hours of growth in aminoglycoside free MHB (i.e. peak adaptively resistant cells) (■), and in logarithmic phase, anaerobically grown cells (♦).


120
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ng gentamicin uptake/mg cell protein
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time (min)

Figure 21. **^{3}H-Gentamicin accumulation in spheroplasts made from wild-type and adaptively resistant *Pseudomonas aeruginosa* ATCC 27853.** Gentamicin accumulation was determined in spheroplast (♦) and whole cell (+) unexposed, wild-type control cultures and in adaptively resistant cultures of spheroplasts (■) and whole cells (▲).

cells. Gentamicin accumulation was determined in both whole cells and spheroplasts. Figure 21 depicts the differences in gentamicin accumulation when spheroplasts are compared with whole cells. Control spheroplasts accumulated significantly ($P<0.001$) more gentamicin than whole cells suggesting that the outer membrane provides a considerable barrier to aminoglycoside accumulation. However, it appeared that aminoglycoside accumulation in adaptively resistant spheroplasts was similar ($P>0.05$) to adaptively resistant whole cells suggesting that the impediment to aminoglycoside accumulation may be found within, or associated with, the cytoplasmic membrane. Other strains of *P. aeruginosa* tested (F991, E1481) demonstrated similar results. Duplicate or triplicate experiments, with each strain, were performed on separate occasions.

6. Gentamicin Accumulation in *Pseudomonas aeruginosa* and the Electrical Component ($\Delta\Psi$) of the Proton Motive Force

Proton motive force (pmf), measured as $\Delta\Psi$, was determined in the reference strain ATCC 27853 and four clinical isolates (F327, F443, F991, E1481) of *P. aeruginosa*. Concentration gradients were calculated using a value of 3.15 ± 0.42 µL of intracellular fluid/mg cell protein. Table 22 presents mean pmf measurements in wild-type control cells, and in washed cells either immediately following the original 2 hour gentamicin exposure at 1X MIC (0 hour, during PAE), or at 4 (peak adaptive resistance), 8 and 12 hours following gentamicin exposure. Analysis of variance identified significant differences in pmfs measured at different times ($P<0.05$) (Table 22). Tukey's multiple comparison test identified the mean pmf at 4 hours (peak adaptive resistance) to be significantly lower than the others ($P<0.05$).

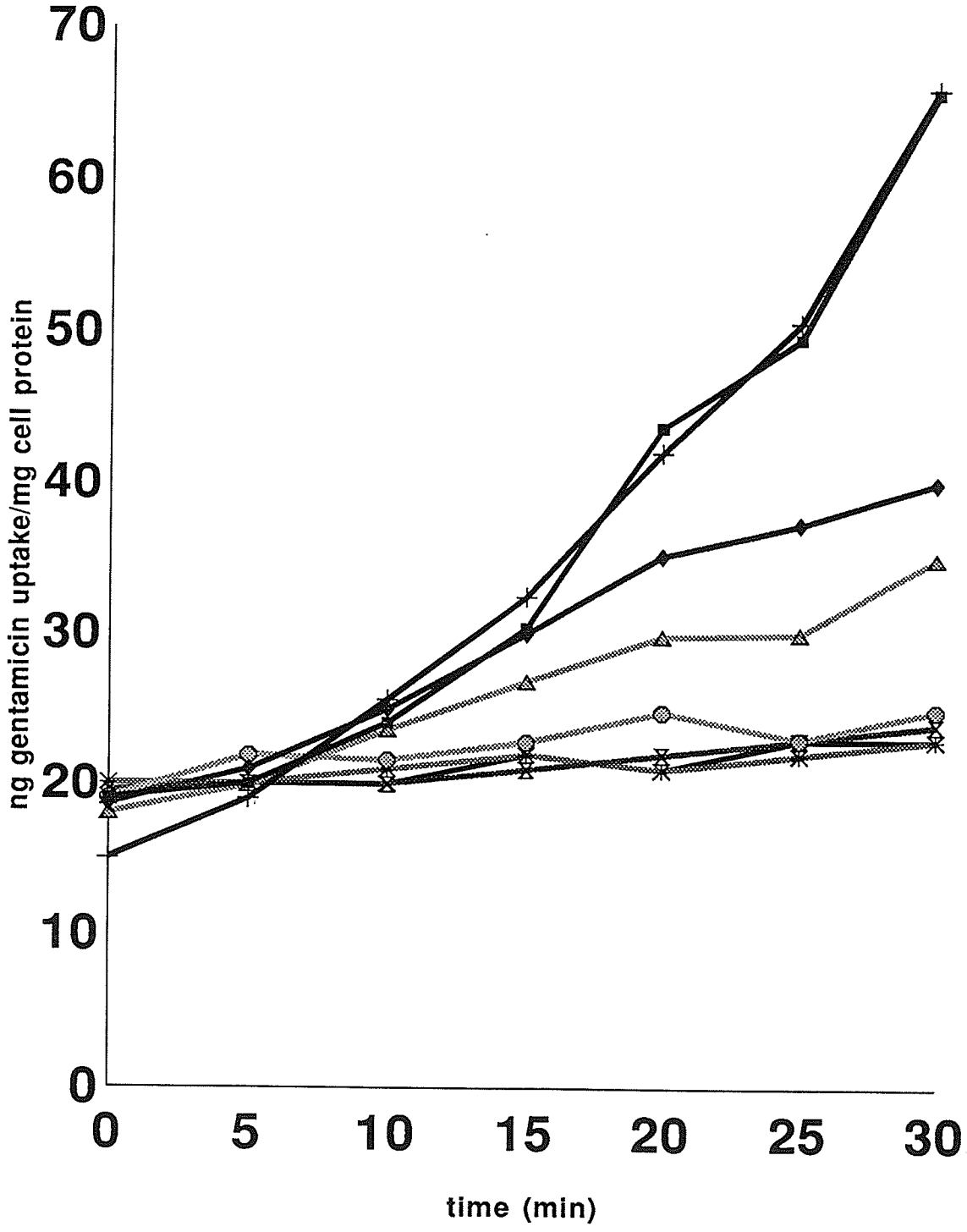
7. Gentamicin Threshold $\Delta\Psi$

Aminoglycoside accumulation within sensitive cells is dependent upon the pmf across the cytoplasmic membrane (Taber *et al.*, 1987). Gentamicin accumulation in *P. aeruginosa* ATCC 27853 appeared to approach zero when incubated with 2 µM valinomycin plus 1 mM KCl (Figure 22). The pmf generated by this combination was -131 ± 5 mV (Table 23). Other strains of *P. aeruginosa* tested (F991, E1481) demonstrated similar results. It appears that the threshold pmf required for gentamicin accumulation in *P. aeruginosa* is approximately -130 mV (interior negative). Threshold $\Delta\Psi$ determinations were performed in triplicate on separate occasions.

Table 22. Proton motive force measurements.

Time	pmf (-mV)[a]
control	156±3
0h	152±3
4h	148±4
8h	153±3
12h	157±3
anaerobic	155±4

[a] Mean of 4 clinical isolates (F327, F443, F991, E1481) and one reference strain (ATCC 27853) of *P. aeruginosa.*


70
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10
0
ng gentamicin uptake/mg cell protein
0
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25
30
time (min)

Figure 22. **Accumulation of ^{3}H-gentamicin (3.6 µg/ml) in *Pseudomonas aeruginosa* ATCC 27853 in the presence of valinomycin (2µM) with varying concentrations of KCl (0.5 to 10 mM KCl).** Gentamicin accumulation was determined in unexposed controls (+), and compared with accumulation in cultures treated with 10 mM KCl alone (■), 2 µM valinomycin alone (♦), 2 µM valinomycin + 0.5 mM KCl (▲), 2 µM valinomycin + 1.0 mM KCl (•), 2 µM valinomycin + 2.0 mM KCl (⧗), 2 µM valinomycin + 5.0 KCl (*), and 2 µM valinomycin + 10.0 mM KCl (X). The clinical isolates tested (F991, E1481) demonstrated similar patterns of gentamicin accumulation. ^{3}H-Gentamicin accumulation was measured at 37°C after 30 minutes of incubation with valinomycin (2 µM) and varying concentrations of KCl.

Table 23. The effect of valinomycin (2 µM) and varying concentrations of KCl on proton motive force (pmf) in *Pseudomonas aeruginosa*.

Condition	pmf (-mV)[a]
control	156 ± 3
10 mM KCl	154 ± 4
2.0 µM valinomycin	145 ± 4
2.0 µM valinomycin + 0.5 mM KCl	134 ± 4
2.0 µM valinomycin + 1.0 mM KCl	131 ± 5
2.0 µM valinomycin + 2.0 mM KCl	127 ± 5
2.0 µM valinomycin + 5.0 mM KCl	120 ± 4
2.0 µM valinomycin + 10.0 mM KCl	113 ± 5

[a] Mean of 2 clinical isolates (F991, E1481) and one reference strain (ATCC 27853) of *P. aeruginosa*. Experiments were performed in triplicate, on separate occasions, with each strain.

8. The Influence of Metabolic Inhibitors on Gentamicin Accumulation in *Pseudomonas aeruginosa*

a. DNP, CCCP, NaN_3, KCN, NEM

The metabolic uncouplers (DNP, CCCP), the electron transport chain inhibitors (NaN_3, KCN), and NEM all abolished ^{3}H-gentamicin accumulation at the concentrations used (Figure 23). The results with DNP, CCCP, NaN_3 and KCN confirm that pmf and

a functioning electron transport are prerequisites for intracellular aminoglycoside accumulation in *P. aeruginosa* (Bryan *et al.*, 1976a; Bryan *et al.*, 1976b; Bryan and van den Elzen, 1977). NEM also prevented aminoglycoside uptake, suggesting that the aminoglycoside transporter is likely a cytoplasmic membrane protein with accessible sulfhydryl groups. NEM has previously been shown to block the uptake of lactose, melibose, and proline by inactivating carrier proteins but has no effect on pmf or growth rate (Leviton *et al.*, 1995).

b. DCCD

DCCD was tested at concentrations from 0 μM to 300 μM. A DCCD concentration of 200 μM demonstrated maximum increases in pmf (maximum pmf in unexposed control cultures was -171 ± 6 mV; maximum pmf in adaptively resistant cultures was -168 ± 4 mV), gentamicin accumulation, and bacterial killing in both growth controls and adaptively resistant cells (Figures 24 and 25). At identical concentrations, adaptively resistant cells responded to DCCD in a manner very similar to wild-type cells suggesting that adaptive resistance is probably not the result of changes in H^+-ATPase activity.

Figure 26 depicts aminoglycoside adaptive resistance following a single, 2 hour gentamicin exposure at 1X MIC against *P. aeruginosa* ATCC 27853. All control culture and test aliquots received 200 μM DCCD pretreatment before their second gentamicin exposure at 1X MIC (Figure 26, lower left and lower right panels). In the control culture the addition of 1X MIC in the presence of 200 μM DCCD consistently ($P>0.05$) produced bacterial killing of approximately 2.5 $\log_{10}$ cfu/ml in 2 hours during 7 consecutive 2 hour intervals (Figure 26, lower left panel). The test culture, in the presence of 200 μM DCCD (Figure 26, lower right panel), demonstrated a pattern of bacterial killing similar to that

seen in test cultures receiving no DCCD pretreatment (Figure 7). Each clinical isolate (F991, E1481) and the reference strain ATCC 27853 were tested thrice, on separate occasions. Analysis of variance, followed by Tukey's multiple comparison test, indicated that bacterial killing was significantly different for wild-type control cells from 0 to 8 hours following aminoglycoside removal ($P<0.05$) despite similar ($P>0.05$) pmf values (unexposed control cultures -171 ± 6 mV and adaptively resistant cultures -168 ± 4 mV). The DCCD results suggest that adaptive resistance may, at least in part, be due to a pmf independent mechanism.

9. Oxidase test of Adaptively Resistant *Pseudomonas aeruginosa*

Wild-type control, adaptively resistant, and post-adaptively resistant cells harvested from cultures of the reference strain ATCC 27853 and four clinical isolates (F327, F443, F991, E1481) of *P. aeruginosa* were all oxidase positive.

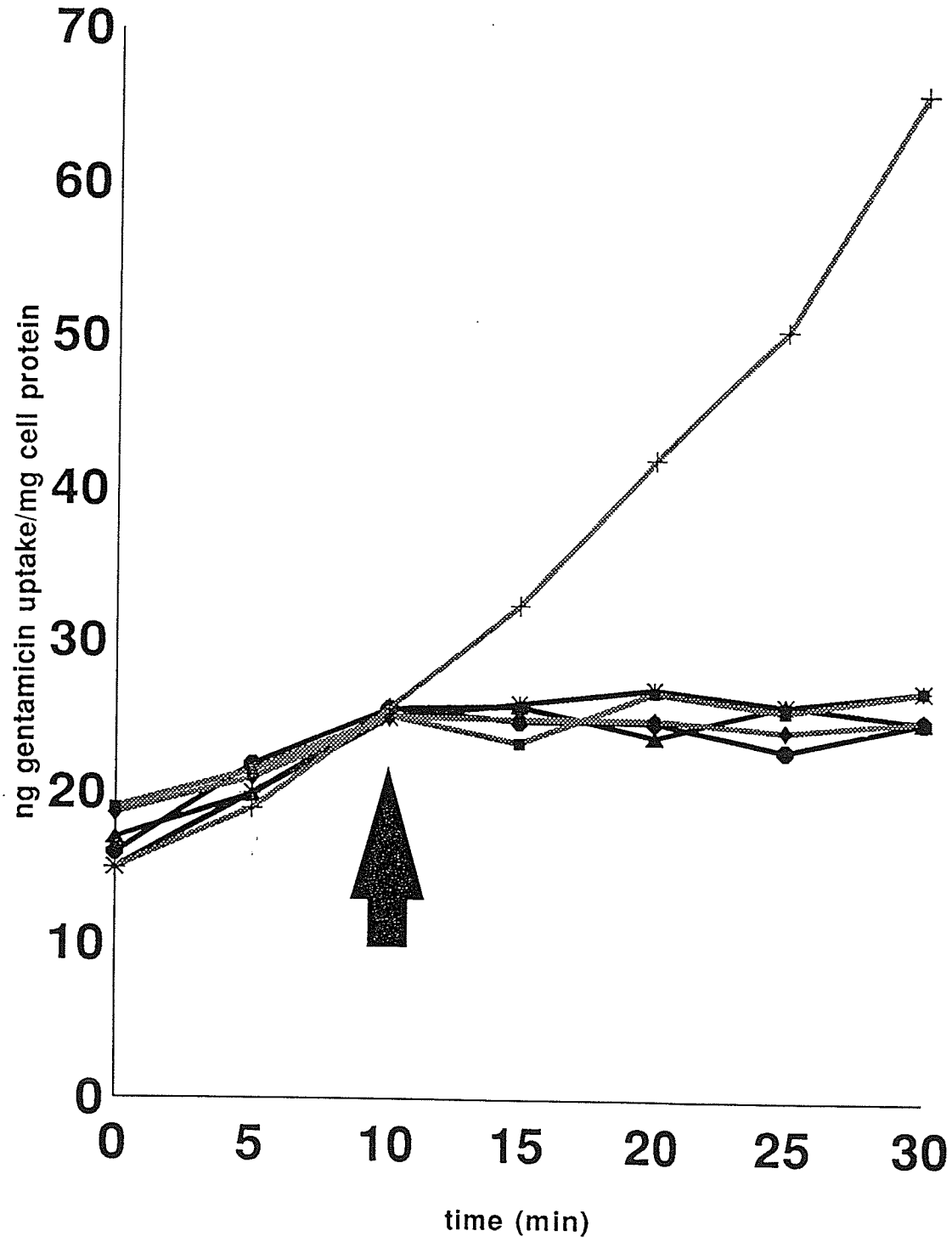

70
60
50
40
30
20
10
0
ng gentamicin uptake/mg cell protein
0
5
10
15
20
25
30
time (min)

Figure 23. Influence of metabolic inhibitors on gentamicin accumulation in *Pseudomonas aeruginosa* ATCC 27853. Gentamicin accumulation was determined in unexposed controls (+) and compared with accumulation in cultures treated with 5 mM DNP (■), 50 µM CCCP (♦), 1 mM KCN (▲), 15 mM NaN_3 (•), and 1 mM NEM (*). Metabolic inhibitors were added 10 minutes following the initiation of gentamicin accumulation as indicated by the arrow. The other strains tested (F991, E1481) demonstrated similar results. Experiments were performed in duplicate with each strain.

A.

DCCD Concentration	pmf
0 uM	-156 ± 3 mV
100 uM	-164 ± 5 mV
200 uM	-171 ± 6 mV
300 uM	-163 ± 5 mV

B.

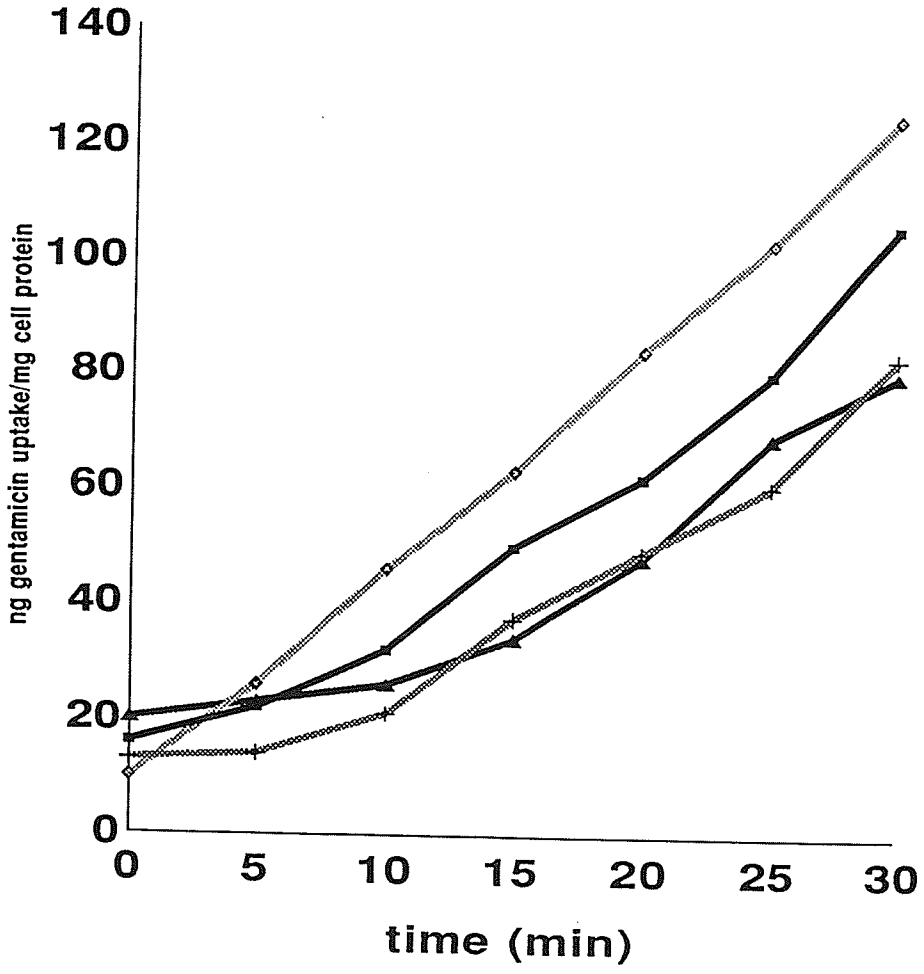


Figure 24. **Effect of DCCD on gentamicin accumulation in exponentially growing *Pseudomonas aeruginosa* ATCC 27853.** (A) The effect of DCCD concentration on pmf measured in *P. aeruginosa.* (B) Gentamicin accumulation in exponentially growing *P. aeruginosa* ATCC 27853 (▲), and in *P. aeruginosa* ATCC 27853 treated with 100 µM DCCD (■), 200 µM DCCD (♦), and 300 µM DCCD (+). The other strains tested (F991, E1481) demonstrated similar results. Experiments were performed in duplicate with each strain.

A.

DCCD Concentration	pmf
0 uM	-148 ± 3 mV
100 uM	-159 ± 5 mV
200 uM	-168 ± 4 mV
300 uM	-162 ± 4 mV

B.

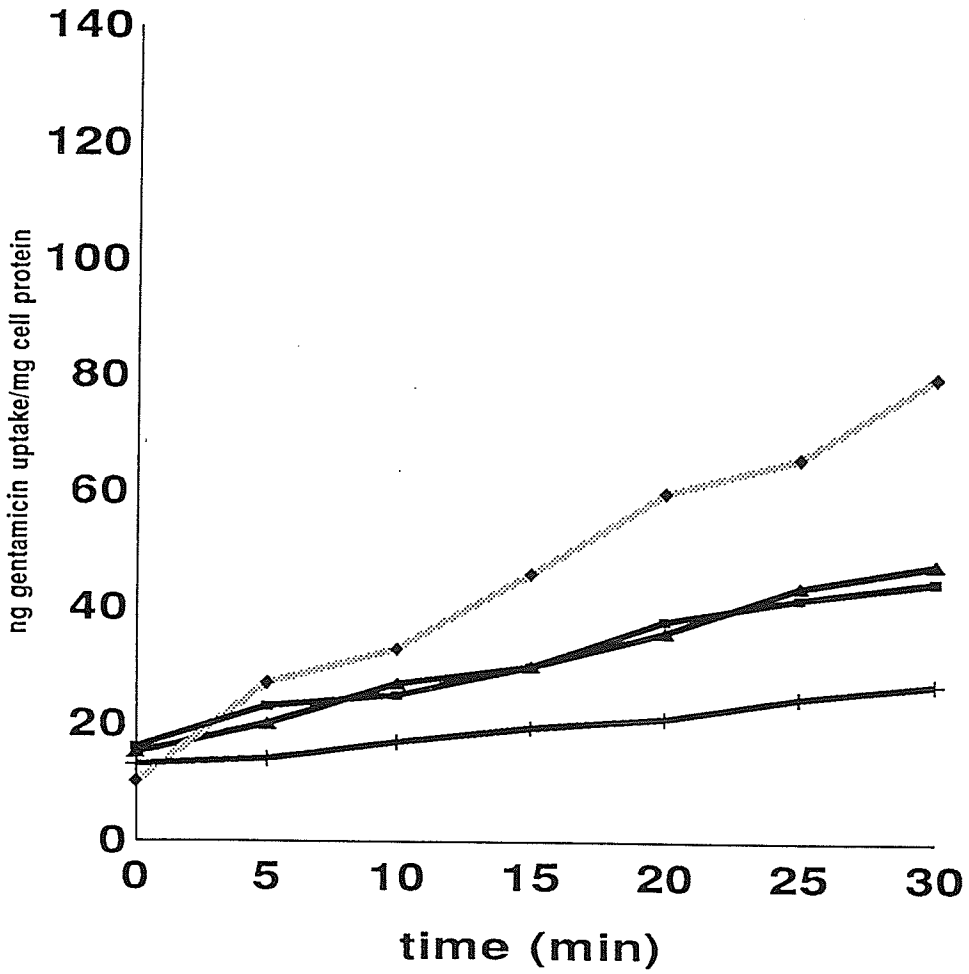
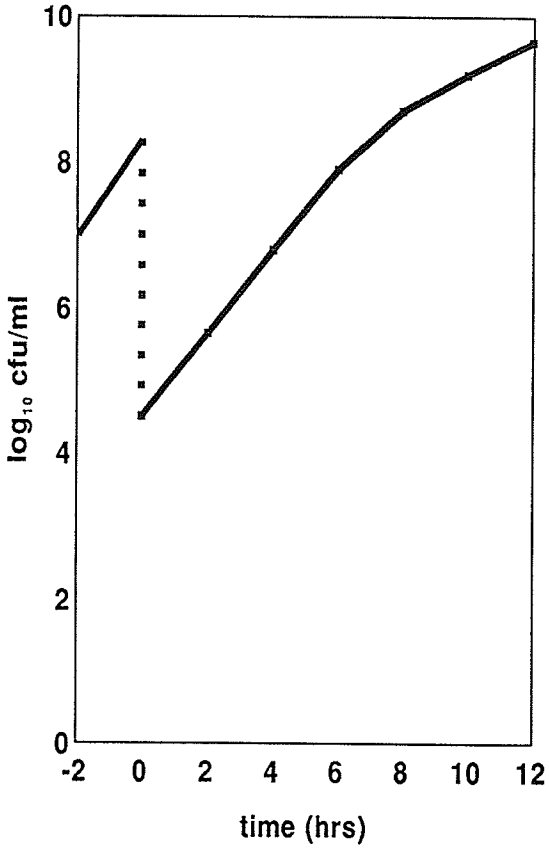
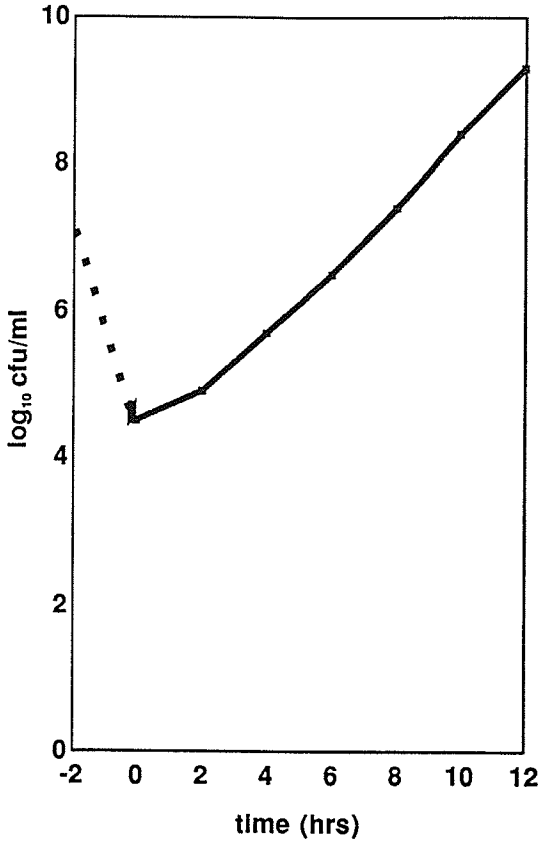
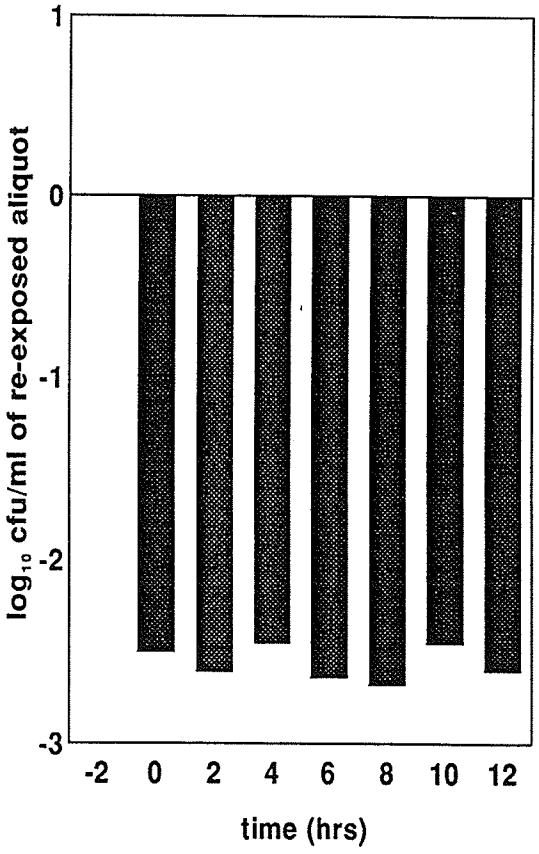


Figure 25. Effect of DCCD on gentamicin accumulation in adaptively resistant *Pseudomonas aeruginosa* ATCC 27853. (A) The effect of DCCD concentration on pmf measured in adaptively resistant *P. aeruginosa*. (B) Gentamicin accumulation in adaptively resistant *P. aeruginosa* ATCC 27853 (▲), and in adaptively resistant *P. aeruginosa* ATCC 27853 treated with 100 µM DCCD (■), 200 µM DCCD (♦), and 300 µM DCCD (+). The other strains tested (F991, E1481) demonstrated similar results. Experiments were performed in duplicate with each strain.


10
8
6
4
2
0
log10 cfu/ml
-2 0 2 4 6 8 10 12
time (hrs)


10
8
6
4
2
0
log10 cfu/ml
-2 0 2 4 6 8 10 12
time (hrs)


1
0
-1
-2
-3
log10 cfu/ml of re-exposed aliquot
-2 0 2 4 6 8 10 12
time (hrs)

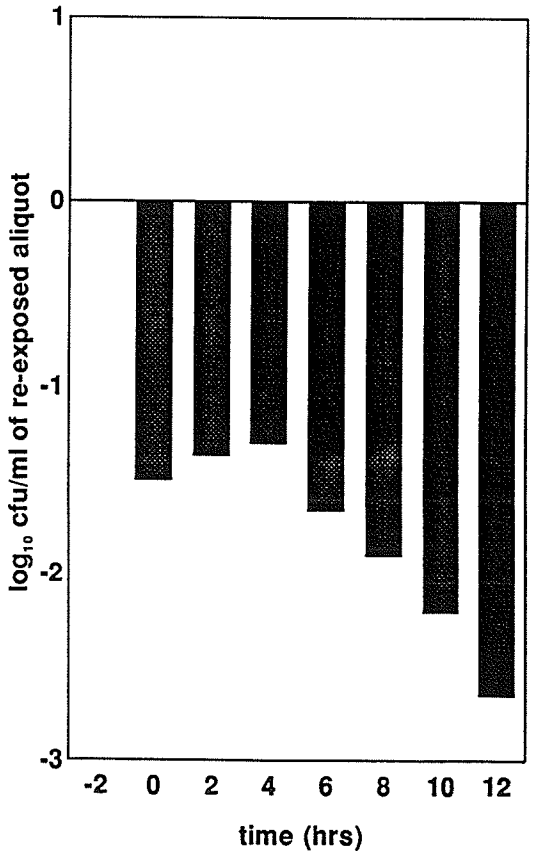

1
0
-1
-2
-3
log10 cfu/ml of re-exposed aliquot
-2 0 2 4 6 8 10 12
time (hrs)

Figure 26. Effect of DCCD on gentamicin adaptive resistance *Pseudomonas aeruginosa* ATCC 27853. The two lefthand panels represent *P. aeruginosa* ATCC 27853 control cultures which did not receive a 2 hour gentamicin pre-exposure. The upper left panel depicts the number of viable bacteria versus time and the lower left panel the bacterial killing of gentamicin at 1X MIC in the presence of 200 μM DCCD at increasing bacterial concentrations. Control cultures were adjusted to approximate cell counts in test cultures following initial gentamicin exposure. The two righthand panels represent *P. aeruginosa* ATCC 27853 test cultures that received 2 hour gentamicin pre-exposure. The upper right panel depicts the number of viable bacteria versus time and the lower right the bacterial killing following a second 2 hour gentamicin exposure at 1X MIC in the presence of 200 μM DCCD. The other strains tested (F991, E1481) demonstrated similar results. Experiments were performed in triplicate with each strain.

Part 3. Cell Envelope Analysis

1. Morphology of Adaptively Resistant *Pseudomonas aeruginosa*

Following a 2 hour aminoglycoside exposure at 1X MIC cultures were plated onto blood agar for viable counting. Following 18 to 24 hours of incubation at 37°C, plates consistently demonstrated several smaller or malformed colonies among a majority of regularly sized colonies. However, differences in bacterial size or shape were not detected with phase contrast or electron microscopy when unexposed control, adaptively resistant, and post-adaptively resistant cultures were compared (Figure 27).

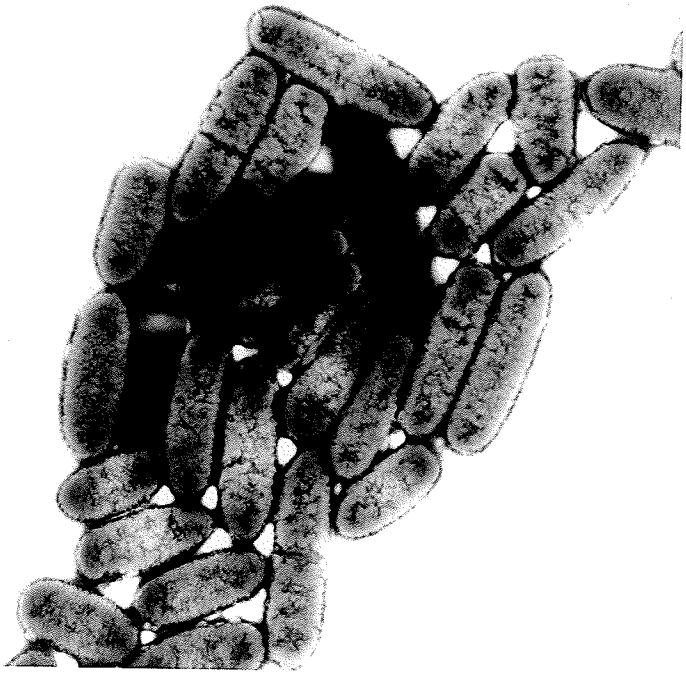
2. Lipopolysaccharide (LPS) Analysis

No major changes in LPS banding patterns were noted between unexposed, wild-type controls, adaptively resistant, and post-adaptively resistant forms of the *P. aeruginosa* strains tested (ATCC 27853, F327, F443, F991, E1481) (Figure 28). No conversion from smooth to rough phenotype was noted (Figure 28).

3. LPS Immunoblots

No major changes in A-band (Figure 29) or B-band (Figure 30) monoclonal antibody binding to LPS were noted between unexposed, wild-type controls, adaptively resistant, and post-adaptively resistant forms of the *P. aeruginosa* strains tested (ATCC 27853, F327, F443, F991, E1481).

B.



C.

Figure 27. Electron micrographs of wild-type control (A), adaptively resistant (B), and post-adaptively resistant (C) *Pseudomonas aeruginosa* ATCC 27853. *P. aeruginosa* cells were negatively stained with 2.5 mM phosphotungstic acid (PTA), adjusted to pH 7.0 with sodium hydroxide, and viewed on a Philips model 201 electron microscope. Clinical isolates F327, F443, F991 and E1481 also show no morphological change when wild-type control, adaptively resistant, and post-adaptively resistant forms were compared.

A.

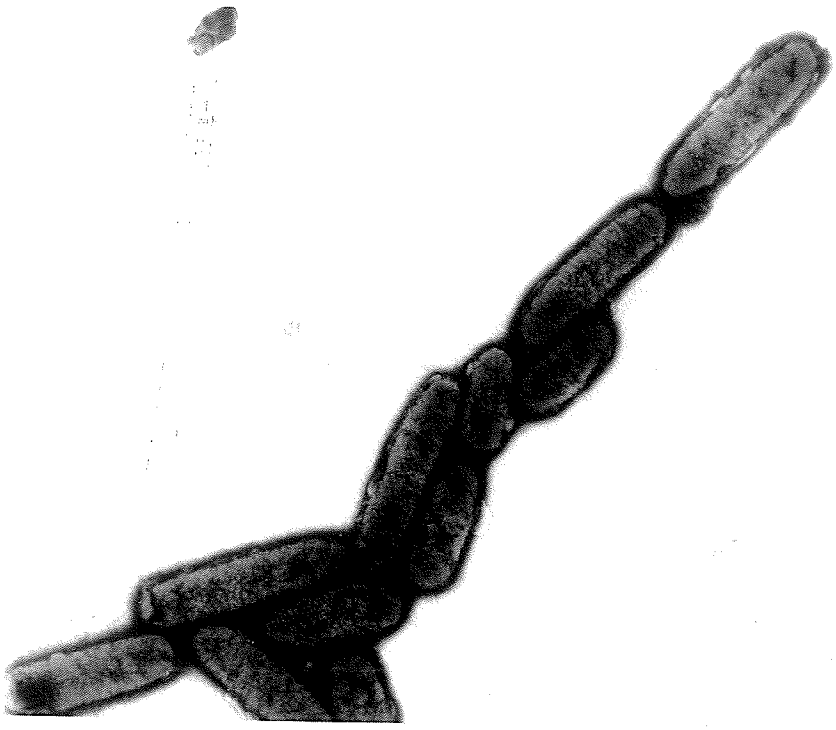


Figure 28. **LPS profiles of wild-type control, adaptively resistant, and post-adaptively resistant *Pseudomonas aeruginosa.*** LPS was prepared and electrophoresed as described in Materials and Methods. Approximately 20 µg of LPS was loaded per lane. Lane 1, wild-type control ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853. Similarly, lanes 2, 2A, 2B contain F327; lanes 3, 3A, 3B contain F443; lanes 4, 4A, 4B contain F991; and lanes 5, 5A, 5B contain E1481.

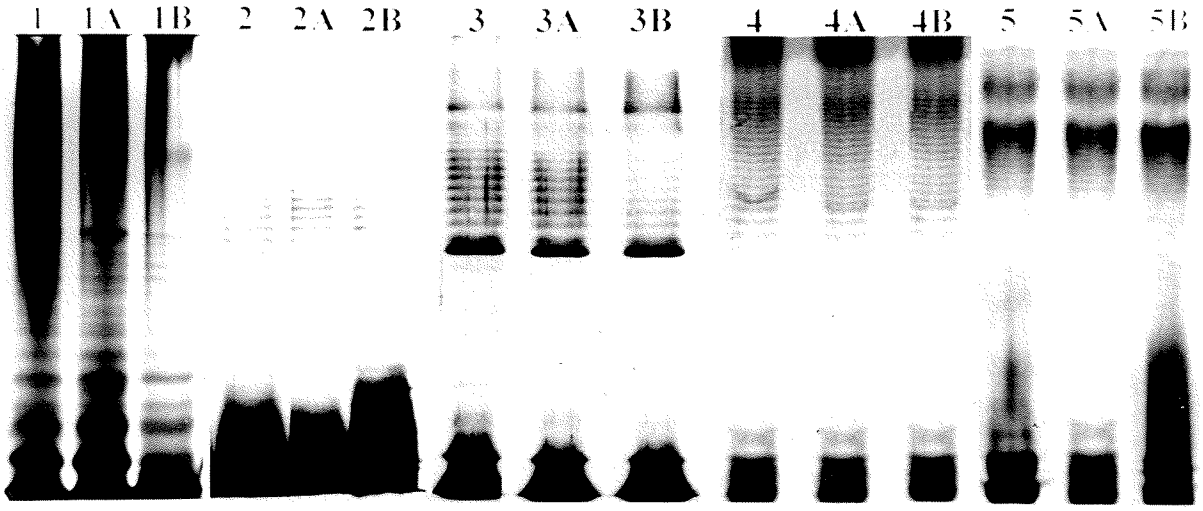


Figure 29. ***Pseudomonas aeruginosa*** **LPS immunoblot with monoclonal antibody N1F 10 (A-band).** LPS was prepared, electrophoresed, transfered to nitrocellulose, and immunoblotted as described in Materials and Methods. Approximately 20 µg of LPS was loaded per lane. Lane 1, wild-type control ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853. Similarly, lanes 2, 2A, 2B contain F327; lanes 3, 3A, 3B contain F443; lanes 4, 4A, 4B contain F991; and lanes 5, 5A, 5B contain E1481. Lane 6 contains *P. aeruginosa* PAO1 LPS as a positive control.

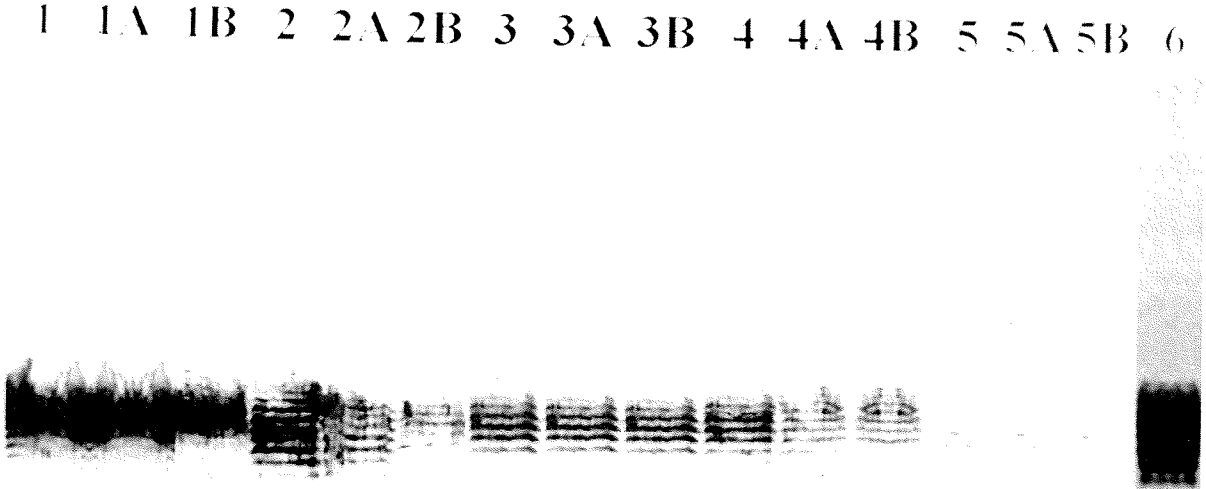
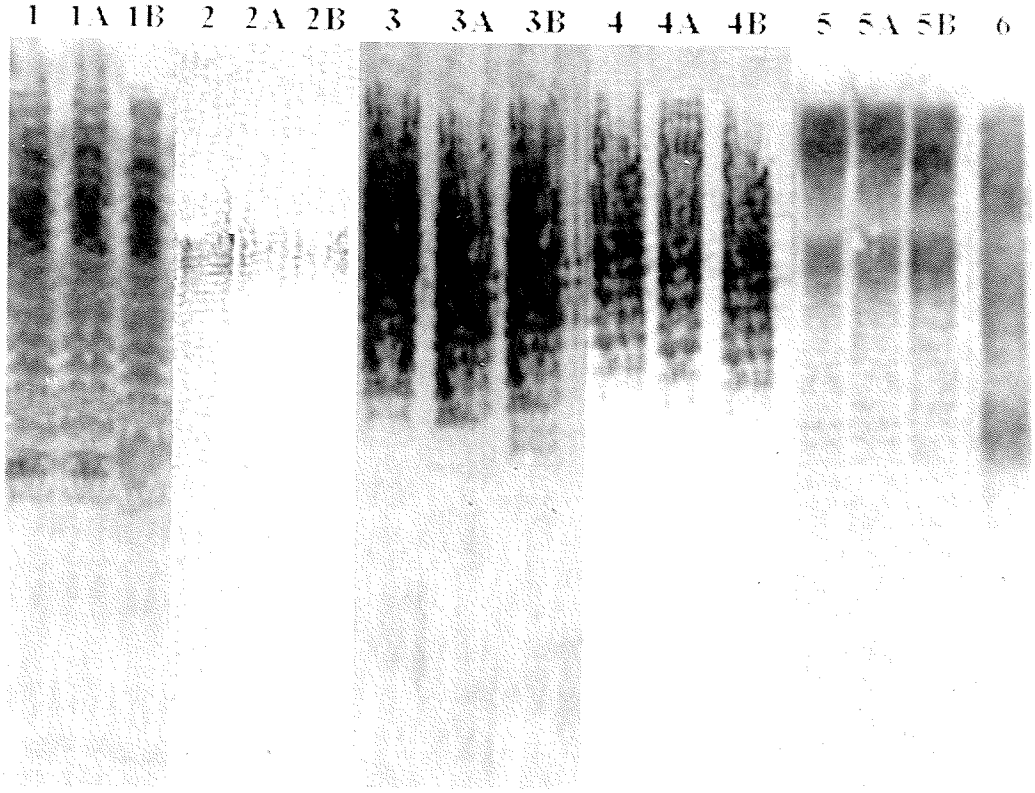


Figure 30. ***Pseudomonas aeruginosa*** **LPS immunoblot with monoclonal antibody MF 15-4 (B-band).** LPS was prepared, electrophoresed, transfered to nitrocellulose, and immunoblotted as described in Materials and Methods. Approximately 20 µg of LPS was loaded per lane. Lane 1, wild-type control ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853. Similarly, lanes 2, 2A, 2B contain F327; lanes 3, 3A, 3B contain F443; lanes 4, 4A, 4B contain F991; and lanes 5, 5A, 5B contain E1481. Lane 6 contains *P. aeruginosa* PAO1 LPS as a positive control.



4. Aminoglycoside Susceptibility of LPS Mutants

A series of *P. aeruginosa* LPS mutants (PAO1 [A-band LPS present, B-band LPS present]; AK1401 [A-band LPS present, B-band LPS absent]; dps89 [A-band LPS absent, B-band LPS present]; rd7513 [A-band LPS absent, B-band LPS absent]) were obtained from Dr. Jag Kadurugamuwa, University of Guelph, Guelph, Ontario, Canada (Kadurugamuwa *et al.*, 1993). The gentamicin and tobramycin MICs of this series of *P. aeruginosa* LPS mutants are presented in Table 4 (p. 88). Aminoglycoside MICs for strain rd7513 differed modestly, but significantly (lower) from the PAO1 control, while AK1401 and dps89 did not. Doubling dilution MICs are considered significantly different if their values differ by at least four fold (2 tubes) (NCCLS, 1990). These results suggest that major LPS alterations (loss of A-band LPS, loss of B-band LPS, or both) have very little, to no impact on aminoglycoside susceptibility in *P. aeruginosa.*

5. Outer Membrane Protein (OMP) Analysis

No changes in outer membrane protein profiles were noted between unexposed, wild-type controls, adaptively resistant, and post-adaptively resistant forms of the *P. aeruginosa* strains tested (ATCC 27853, F327, F443, F991, E1481) (Figure 31).

6. Cytoplasmic Membrane Protein Analysis

The cytoplasmic membrane profiles of adaptively resistant *P. aeruginosa* demonstrated an increase in intensity of three bands, of 35, 41, and 60 kDa respectively, as well as new bands at 28 and 45 kDa when compared to wild-type control and post-adaptively resistant profiles (Figure 32). The cytoplasmic membrane profiles of adaptively resistant *P. aeruginosa* also showed a decrease in intensity of a 36 kDa band

when compared to wild-type control and post-adaptively resistant profiles (Figure 32).

The cytoplasmic membrane profile of anaerobically grown *P. aeruginosa* ATCC 27853 demonstrated an increase in intensity of a 60 kDa band, new bands at 45, 53, and >100 kDa, as well as a decrease in intensity of a 36 kDa band when compared to wild-type control and post-adaptively resistant profiles (Figure 33). Three band changes in adaptively resistant *P. aeruginosa* appeared to coincide with those demonstrated in anaerobically grown *P. aeruginosa* (Figures 32 and 33). They were an increase in intensity of a 60 kDa band, a new band at 45 kDa, and a decrease in intensity of a 36 kDa band.

The anaerobic (nitrite reductase) respiratory and denitrification pathways of *P. aeruginosa* have been characterized (Palleroni, 1984; Zannoni, 1989), and were discussed in the introduction. As a brief review, nitrate reductase of *P. aeruginosa* is an integral, cytoplasmic membrane protein that consists of 2 polypeptides: α of approximately 150 kDa and ß of approximately 60 kDa (Carlson *et al.*, 1982). Nitrite reductase is a periplasmic, cytoplasmic membrane associated, non-covalently linked homodimer of 120 kDa (i.e. two identical 60 kDa subunits). Nitrous oxide reductase is a periplasmic, membrane associated protein with a mass of approximately 47 kDa (Zannoni, 1989). Although specific bands were not identified by immunological means in any instance, it appeared that the cytoplasmic membrane profile changes, demonstrated by adaptively resistant cells, coincided with several changes also seen in anaerobically grown cells. It also appeared that the coincidental band changes were similar in size to known anaerobic (monomeric nitrite reductase [60 kDa], ß subunit of nitrate reductase [60 kDa], and nitrous oxide reductase [47 kDa]) respiratory and denitrification pathway components. For these reasons, it was hypothesized that *P. aeruginosa* adaptive resistance to

aminoglycoside antimicrobial agents may arise, at least in part, by increased cellular reliance upon the anaerobic respiratory pathway, in which nitrite reductase serves as the facilitator of terminal electron acceptance.

Figure 31. **Outer membrane protein profiles of wild-type control, adaptively resistant, and post-adaptively resistant *Pseudomonas aeruginosa*.** Proteins were prepared and analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The proteins were stained with Coomassie blue. Approximately 10 µg of protein was loaded per lane. Lane 1, wild-type control ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853. Similarly, lanes 2, 2A, 2B contain F327; lanes 3, 3A, 3B contain F443; lanes 4, 4A, 4B contain F991; and lanes 5, 5A, 5B contain E1481. The molecular mass standards (left lane) are given in kDa.

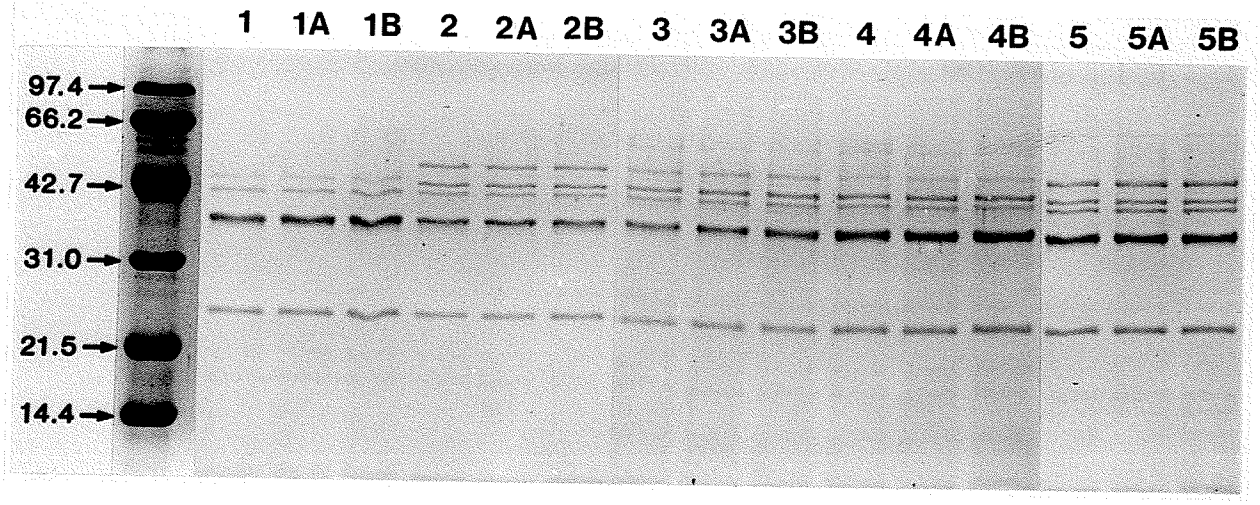


Figure 32. **Cytoplasmic membrane protein profiles of wild-type control, adaptively resistant, and post-adaptively resistant *Pseudomonas aeruginosa*.** Proteins were prepared and analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Approximately 15 µg of protein was loaded per lane. Proteins were stained with Coomassie blue. Black circles identify protein changes in adaptively resistant cultures compared with wild-type control and post-adaptively resistant cultures. Lane 1, unexposed control ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853. Similarly, lanes 2, 2A, 2B contain F327; lanes 3, 3A, 3B contain F443; lanes 4, 4A, 4B contain F991; and lanes 5, 5A, 5B contain E1481. The molecular mass standards (left lane) are given in kDa.

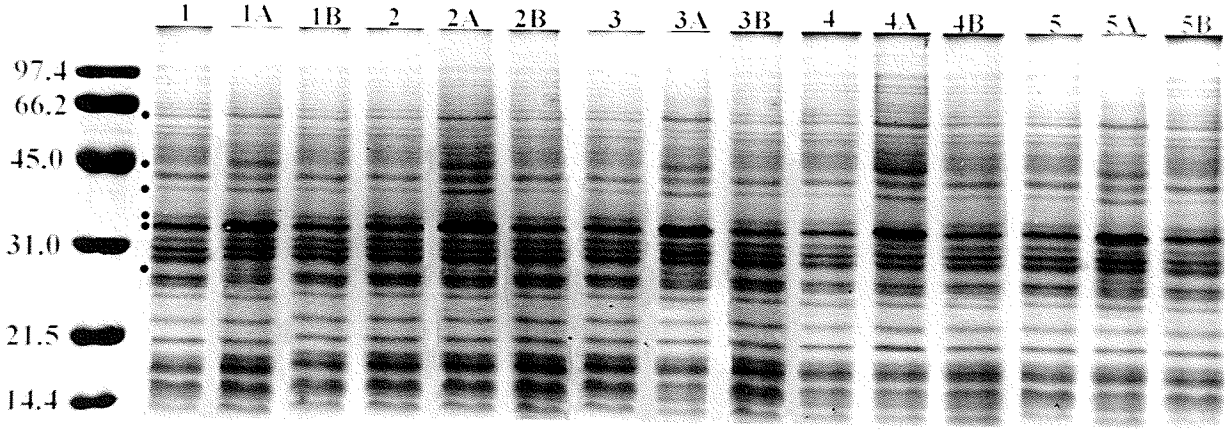
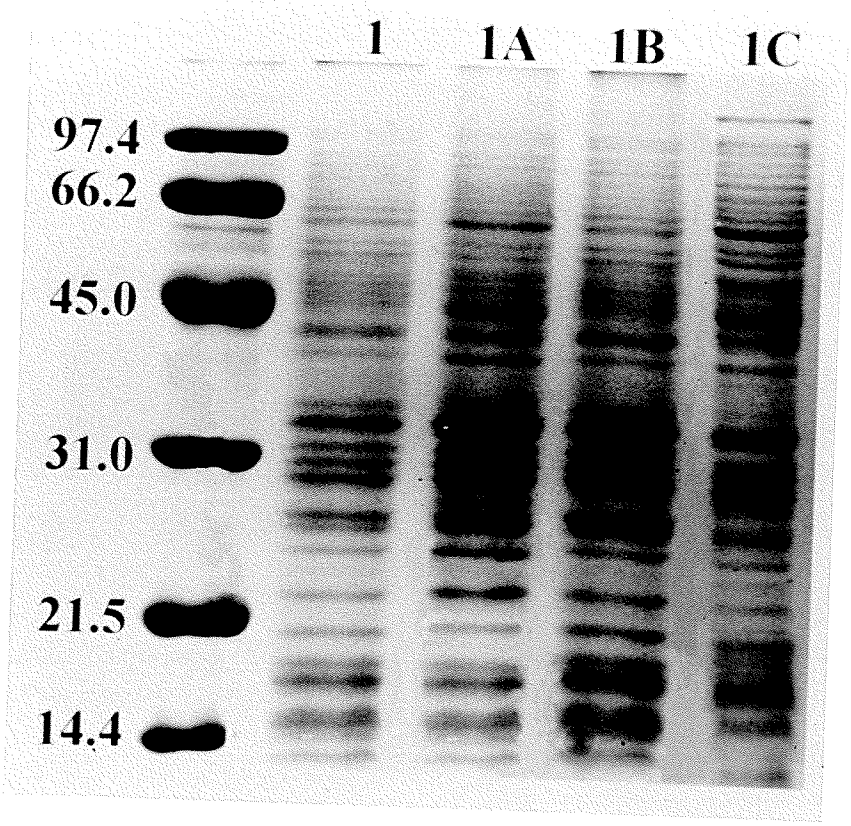


Figure 33. **Cytoplasmic membrane protein profile of anaerobically grown *Pseudomonas aeruginosa* ATCC 27853.** Proteins were prepared and analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Approximately 15 µg of protein was loaded per lane. Proteins were stained with Coomassie blue. Lane 1, wild-type control ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853; lane 1C anaerobically grown ATCC 27853. The molecular mass standards (left lane) are given in kDa.



Part 4. Genetic Analysis

1. *Pseudomonas aeruginosa* Nitrite Reductase Transcription

Research by other investigators has shown low level basal expression of the protein nitrite reductase (DENA) in aerobically maintained *P. aeruginosa* in the absence of nitrate or nitrite supplementation (Zennaro *et al.*, 1993). Expression of nitrite reductase is greatly enhanced by nitrate or nitrite supplementation under both anaerobic and aerobic conditions (Zennaro *et al.*, 1993). Anaerobiosis is not required for high levels of DENA production (Zennaro *et al.*, 1993). To test the hypothesis that adaptive resistance to aminoglycoside antimicrobial agents may be due in part to increased cellular reliance upon the anaerobic respiratory pathway, in which nitrite reductase serves as the facilitator of terminal electron acceptance, *denA* mRNA levels during aminoglycoside adaptive resistance were determined and compared with mRNA levels in wild-type, control and post-adaptively resistant cells. A 2 hour aminoglycoside exposure transiently increased *denA* mRNA levels in the ATCC 27853 reference strain and 2 clinical isolates (F991, E1481) of *P. aeruginosa* (Figure 34). *denA* mRNA levels were discernably higher in adaptively resistant cells when compared with wild-type control and post-adaptively resistant cells (Figure 34). These experiments were conducted in the absence of nitrate or nitrite media supplementation. Lane 4 in Figure 34 depicts *denA* expression in anaerobically grown *P. aeruginosa* ATCC 27853 which served as a positive control. Anaerobic cultures were supplemented with 50 mM KNO_3.

RNA standards (16S [1766 bases] and 23S [3566 bases] ribosomal RNA) were run concurrently with total RNA samples identifying the band in Figure 34 as approximately 2 kb in length. This suggests that the *denAB* operon transcript was being identified (*denA* 1707 bases; *denB* 312 bases) (Silvestrini *et al.*, 1989; Arai *et al.*, 1990; Arai *et al.*, 1991).

2. *Pseudomonas aeruginosa* Nitrite Reductase Promoter Sequencing

To determine if increased *denA* expression was arising because of one or more promoter mutations, the *denA* promoter region, and flanking sequences, of *P. aeruginosa* ATCC 27853, F991 and E1481 were sequenced to look for nucleotide changes. No changes were noted in the *denA* promoter region with any strain sequenced, when adaptively resistant cells were compared with unexposed, wild-type controls and post-adaptively resistant cells (Figure 35). *P. aeruginosa* PAO1, whose *denA* promoter sequence was previously published (Silvestrini *et al.*, 1989), was also sequenced, and served as a positive control.

3. *Pseudomonas aeruginosa* ANR Transcription

ANR has been shown to be a regulatory protein that is essential for nitrate respiration, anaerobic arginine degradation, and KCN production in *P. aeruginosa* (Galimand *et al.*, 1991; Ye *et al.*, 1995). A 2 hour aminoglycoside exposure transiently increased *anr* mRNA levels in the ATCC 27853 reference strain and 2 clinical isolates (F991, E1481) of *P. aeruginosa* (Figure 36). *anr* mRNA levels were discernably higher in adaptively resistant cells when compared with wild-type control and post-adaptively resistant cells (Figure 36). These experiments were conducted in the absence of nitrate or nitrite media supplementation. Lane 4 in Figure 36 depicts *anr* expression in anaerobically grown *P. aeruginosa* ATCC 27853 and served as a positive control. Anaerobic cultures were supplemented with 50 mM KNO_3.

RNA standards (16S [1766 bases] and 23S [3566 bases] ribosomal RNA) were run concurrently with total RNA samples identifying the band in Figure 36 as being considerably smaller than 1.8 kb in length. The *anr* open reading frame contains 732

nucleotides (Zimmermann *et al.*, 1991).

4. *Pseudomonas aeruginosa anr* Promoter Sequence

To determine if increased *anr* expression was arising because of one or more promoter mutations, the *anr* promoter region of *P. aeruginosa* ATCC 27853, F991 and E1481 were sequenced to look for nucleotide changes. No changes were noted in the *anr* promoter region with any strain sequenced, when adaptively resistant cells were compared with unexposed, wild-type controls and post-adaptively resistant cells (Figure 37). *P. aeruginosa* PAO1, whose *anr* promoter region sequence was previously published (Zimmermann *et al.*, 1991), was also sequenced, and served as a positive control.

Figure 34. **Northern analysis of *denA*.** RNA was prepared, electrophoresed, transferred to nylon membrane and probed as described in Materials and Methods. Approximately 20 µg of total RNA was loaded per lane. Lane 1, wild-type, control ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853. Similarly, lanes 2, 2A, 2B contain F991 and lanes 3, 3A, 3B contain E1481. Lane 4 depicts *denA* mRNA levels in anaerobically grown *P. aeruginosa* ATCC 27853. Anaerobically grown *P. aeruginosa* ATCC 27853 cultures were supplemented with 50 mM KNO_3. All other cultures (lanes 1-3B) were not supplemented with KNO_3. RNA standards (16S [1766 bases] and 23S [3566 bases] ribosomal RNA) were run, not shown, identifying the band as approximately 2 kb in length.

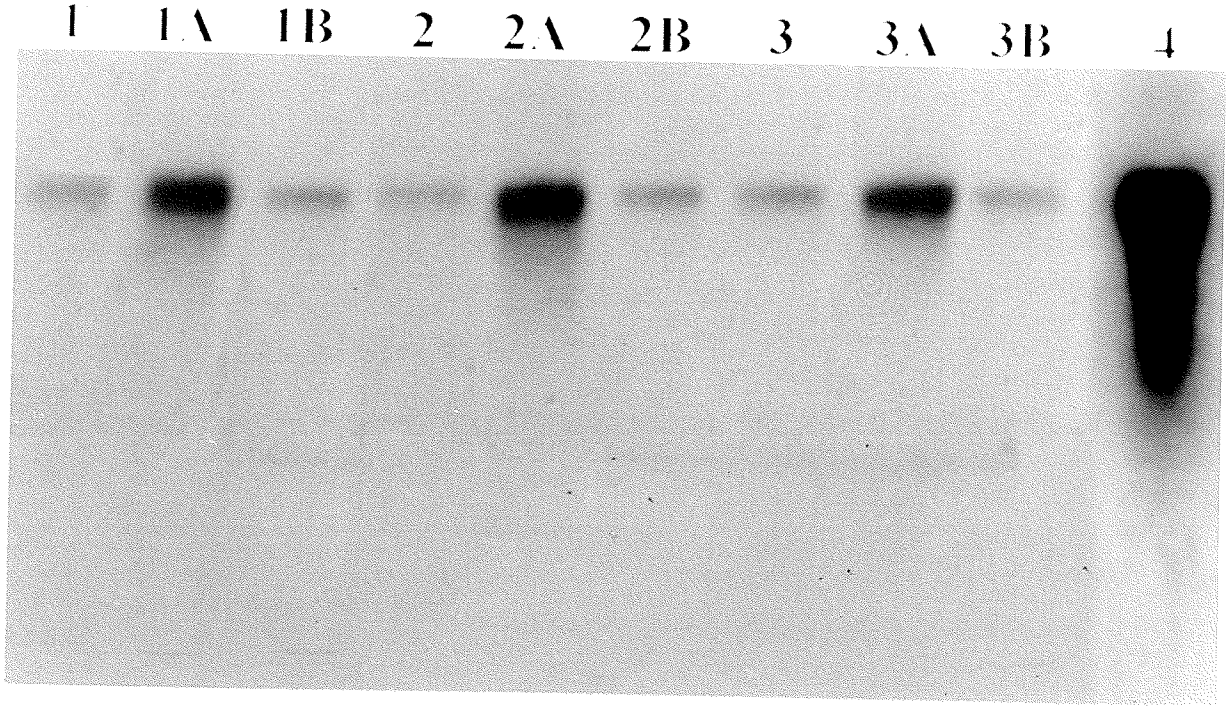


Figure 35. ***denAB* promotor region DNA sequence.** The nucleotide sequence is written in the 5'-3' direction of the coding strand. The ATG start codon for the *denAB* operon is at nucleotide position 285-287 and is underlined. The CAT start codon for an unidentified open reading frame, in the opposite direction, is at nucleotide position 67-69 and is underlined. The putative ANR regulatory protein binding site is at nucleotide position 148-161 and is underlined. *P. aeruginosa* PAO1 was sequenced as a control. *P. aeruginosa* ATCC 27853, F991, and E1481 sequences are presented for wild-type control (WT), adaptively resistant (AR), and post-adaptively resistant (R) forms of each isolate.

```
Strain
PAO1      CGCCAGGCGC GCTCGAAGAC TTCGATCTCA ATGGCCGGTG   40
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

PAO1      GCCTCGTAGA AGGGTGTCGC GTCCCGCATG TCCTACTCCT   80
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
991R      ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

PAO1      GCGCTAGGGA TTAGGACCGC ACGCTATTCA CAGTTGGAAG  120
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
991WT     ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------
```

```
PA01      GTGCCACAAG CGCAAAGCAA CGCAATCTTG ATTCCGGTCA 160
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

          AGCAAGGGTA AAGACCCTGC TTTCTATGAT CCTTTCGCGC 200
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

PA01      CATGAATTCC CGGGAGTTCC CGACGCAGCC ACCCCCAAAA 240
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

PAO1      CACTGCTAAG GGAGCGCCTC GCAGGGCTCC TGAGGAGATA 280
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
1481AR    ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

PAO1      GACCATGCCA TTTGGCAAGC CACTGGTGGG CACCTTGCTC 320
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------
```

```
PAO1       GCCTCGCTGA CGCTGCTGGG CCTGGCCAC                    349
ATCCWT     ---------- ---------- ---------
ATCCAR     ---------- ---------- ---------
ATCCR      ---------- ---------- ---------
F991WT     ---------- ---------- ---------
F991AR     ---------- ---------- ---------
F991R      ---------- ---------- ---------
E1481WT    ---------- ---------- ---------
E1481AR    ---------- ---------- ---------
E1481R     ---------- ---------- ---------
```

Figure 36. **Northern analysis of *anr*.** RNA was prepared, electrophoresed, transferred to nylon membrane and probed as described in Materials and Methods. Approximately 20 µg of total RNA was loaded per lane. Lane 1, wild-type, control ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853. Similarly, lanes 2, 2A, 2B contain F991 and lanes 3, 3A, 3B contain E1481. Lane 4 depicts *anr* mRNA levels in anaerobically grown *P. aeruginosa* ATCC 27853. Anaerobically grown *P. aeruginosa* ATCC 27853 cultures were supplemented with 50 mM KNO_3. All other cultures (lanes 1-3B) were not supplemented with KNO_3. RNA standards (16S [1766 bases] and 23S [3566 bases] ribosomal RNA) were run, not shown. The band was considerably smaller than 1.8 kb in length. The *anr* open reading frame contains 732 nucleotides (Zimmermann *et al.*, 1991).

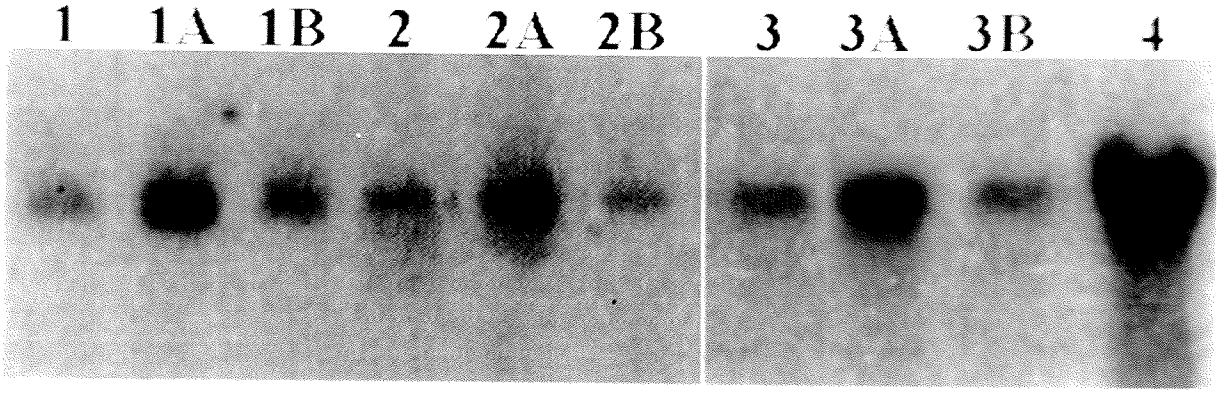


Figure 37. ***anr* promoter region sequence.** The nucleotide sequence is written in the 5'-3' direction of the coding strand. The ATG start codon for *anr* is at nucleotide position 230-232 and is underlined. *P. aeruginosa* PAO1 was sequenced as a control. *P. aeruginosa* ATCC 27853, F991, and E1481 sequences are presented for wild-type control (WT), adaptively resistant (AR), and post-adaptively resistant (R) forms of each isolate.

```
Strain
PAO1      AAAATGGAAT TCTTCCATTG GATCGGCCCA CGCGTCGCGA    40
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

PA01      ACTTGAGCCC CCTTTTCGTC GCCCCTTGAC AGGGTGCGAC    80
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

PAO1      AGGTAGTCGC AGTTGTTTGA CGCAAGTCAC TGATTGGAAA   120
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------
```

```
PA01      CGCCATCGGC CTGTCAGAAA TGGTCGTTGC CAGACCTATG    160
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
991AR     ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

PA01      GCTGGCACCC GCATCGCGGC TGCGTTACCC TTACTCCTGT    200
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

PA01      TGTGCCTTTA ACCTAGCAAG GACCCCTCAA TGGCCGAAAC    240
ATCCWT    ---------- ---------- ---------- ----------
ATCCAR    ---------- ---------- ---------- ----------
ATCCR     ---------- ---------- ---------- ----------
F991WT    ---------- ---------- ---------- ----------
F991AR    ---------- ---------- ---------- ----------
F991R     ---------- ---------- ---------- ----------
E1481WT   ---------- ---------- ---------- ----------
E1481AR   ---------- ---------- ---------- ----------
E1481R    ---------- ---------- ---------- ----------

PA01      CATCAAGGTG CGCGCACTGC CC                      262
ATCCWT    ---------- ---------- --
ATCCAR    ---------- ---------- --
ATCCR     ---------- ---------- --
F991WT    ---------- ---------- --
F991AR    ---------- ---------- --
F991R     ---------- ---------- --
E1481WT   ---------- ---------- --
E1481AR   ---------- ---------- --
E1481R    ---------- ---------- --
```

5. Complementation Analysis

P. aeruginosa PAO1, like other strains of *P. aeruginosa*, has been demonstrated to grow under anaerobic conditions with nitrate, nitrite, and nitrous oxide as alternative electron acceptors (Ye *et al.*, 1995). *P. aeruginosa* PAO6261, an *anr* deficient mutant of PAO1, has been shown to be unable to grow under anaerobic conditions with nitrate, nitrite, or nitrous oxide supplementation (Ye *et al.*, 1995). Anaerobic growth of *P. aeruginosa* PAO6261 can be restored upon complementation with the wild-type *anr* gene (pME3580) (Ye *et al.*, 1995). These results were confirmed in the present study using *P. aeruginosa* PAO1 and PAO6261, supplied by Dr. Dieter Haas (Ye *et al.*, 1995), and pME3580 (*anr*$^+$) obtained from Dr. Marc Galimand (Galimand *et al.*, 1991). Ye and coworkers (1995) also demonstrated that in *P. aeruginosa* PAO6261 the activities of nitrite reductase and nitric oxide reductase were not detectable with growth under anaerobic conditions but were restored when complemented with the *anr* gene (pME3580) (Ye *et al.*, 1995).

The purpose of using *P. aeruginosa* PAO6261, an *anr* deficient *P. aeruginosa* isolate, was to determine its response to aminoglycoside insult and its ability to become adaptively resistant. Testing this strain may give some clue as to the nature of aminoglycoside adaptive resistance induction in *P. aeruginosa*. Gentamicin accumulation experiments and pmf determinations were also conducted to identify differences, if any, between the *anr* deficient mutant (PAO6261) and its wild-type parent (PAO1) and complemented form (PAO6261(*anr*$^+$)).

a. Adaptive Resistance Determinations in *Pseudomonas aeruginosa* PAO1, PAO6261, and PAO6261(*anr*$^+$)

Figure 38 depicts aminoglycoside adaptive resistance following a single, 2 hour

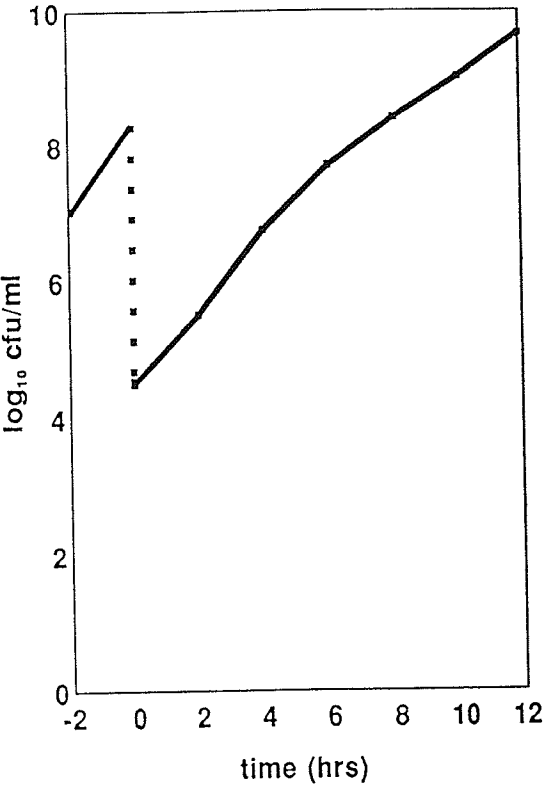
gentamicin exposure at 1X MIC (2 mg/l) against *P. aeruginosa* PAO1. The results presented in Figure 38 are similar to those of other *P. aeruginosa* strains discussed earlier (Figure 7). *P. aeruginosa* PAO1 was tested on 5 separate occasions. Similar observations can be made from both Figures 7 and 39. In the control culture, the addition of 1X MIC consistently produced similar ($P>0.05$) bacterial killing of approximately 2 $\log_{10}$ cfu/ml in 2 hours, during 7 consecutive, 2 hour, intervals (Figure 38, lower left panel). The test culture (2 hour gentamicin pre-exposure at 1X MIC) showed the appearance and disappearance of unstable adaptive resistance (reduced bacterial killing) to the readdition of gentamicin, at 1X MIC, during growth for 12 hours in antimicrobial free medium (Figure 38, lower right panel). From 0 to 4 hours after aminoglycoside removal and growth in drug free medium, the readdition of 1X MIC of gentamicin had decreasing bactericidal action. From 4 to 10 hours the culture gradually regained its aminoglycoside susceptibility. Ten hours after the culture was removed from the first aminoglycoside exposure, when the bacterial population had increased approximately 3 $\log_{10}$ cfu/ml (99.9% of the culture are new progeny in aminoglycoside free media) in aminoglycoside free medium, the culture again was susceptible to the bactericidal action of gentamicin with a bacterial killing of approximately 2 $\log_{10}$ cfu/ml in 2 hours. Analysis of variance, followed by Tukey's multiple comparison test, indicated that bacterial killing was significantly different from wild-type controls from 0 to 8 hours following aminoglycoside removal ($P<0.05$). Adaptive resistance was greatest (least bacterial killing) ($P<0.05$) when logarithmic growth of the pre-exposed culture had just resumed (4 hours following aminoglycoside removal) (compared with bacterial killing over other 2 hour intervals).

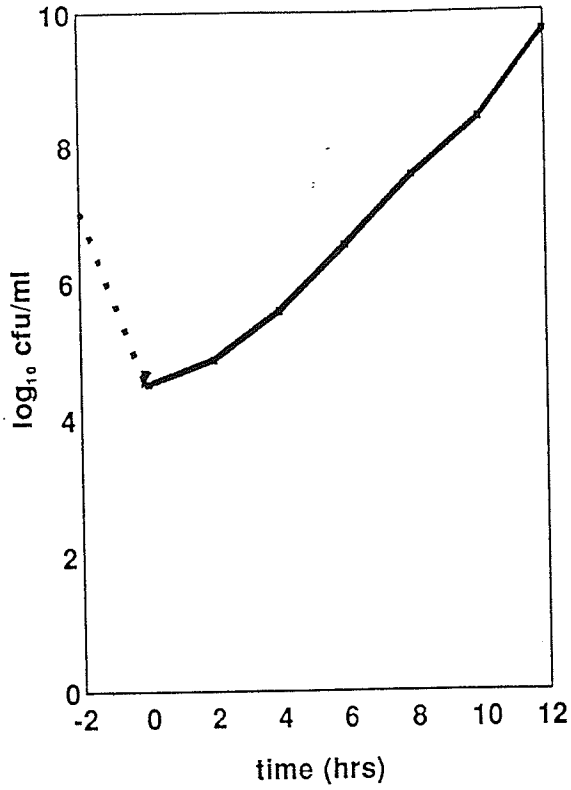
Figure 39 depicts aminoglycoside adaptive resistance following a single, 2 hour

gentamicin exposure at 1X MIC (1 mg/l) against *P. aeruginosa* PAO6261. In the control culture, the addition of 1X MIC consistently produced similar ($P>0.05$) bacterial killing of approximately 2 $\log_{10}$ cfu/ml in 2 hours, during 7 consecutive, 2 hour, intervals (Figure 39, lower left panel). The test culture (2 hour gentamicin pre-exposure at 1X MIC) did not show an apparent induction and reduction pattern of unstable adaptive resistance to the re-addition of gentamicin, at 1X MIC (Figure 39, lower right panel), as had been seen in PAO1 (Figure 38) and the other strains of *P. aeruginosa* tested (Figure 7). Instead the test culture demonstrated approximately 1 $\log_{10}$ cfu/ml of bacterial killing immediately following aminoglycoside removal which lasted for the following 4 hours and then returned, over the following 4 hours, to wild-type control levels (Figure 39, lower right panel). Analysis of variance, followed by Tukey's multiple comparison test, indicated that bacterial killing in the test cultures was significantly different from that in wild-type controls, for 0 to 6 hours following aminoglycoside removal ($P<0.05$).

The bacterial kill results presented in Figures 38 and 39, when compared by multi-way analysis of variance, followed by Tukey's multiple comparison test, indicated that bacterial killing was significantly less ($P<0.05$) in adaptively resistant PAO6261 (compared with adaptively resistant PAO1) at 2, 4, 6, and 8 hours following aminoglycoside removal (following the initial 2 hour aminoglycoside exposure at 1X MIC). These results suggest that ANR may be involved in the regulation of aminoglycoside adaptive resistance. However, this finding does not preclude the possible involvement of other factors, as bacterial killing in adaptively resistant *P. aeruginosa* PAO6261 remains significantly ($P>0.05$) lower than wild-type PAO1 at 0, 2, 4, and 6 hours following aminoglycoside removal (multi-way analysis of variance, followed by Tukey's multiple comparison test).

Adaptive resistance results with *P. aeruginosa* PAO6261(*anr*$^+$) (Figure 40) were similar to PAO1 (Figure 38). Complementation with p3580 (*anr*$^+$) restored the appearance and disappearance of aminoglycoside adaptive resistance (Figure 40). Multi-way analysis of variance, comparing bacterial kill data in Figures 38 and 40, followed by Tukey's multiple comparison test, indicated that no differences ($P>0.05$) in bacterial killing existed, with a second gentamicin exposure at 1X MIC, between identical individual time points (i.e. no differences between Figures 38 and 40 at 0, 2, 4, 6, 8, 10, and 12 hours) following an initial 2 hour gentamicin exposure (1X MIC) and removal.


10
8
6
4
2
0
log10 cfu/ml
-2 0 2 4 6 8 10 12
time (hrs)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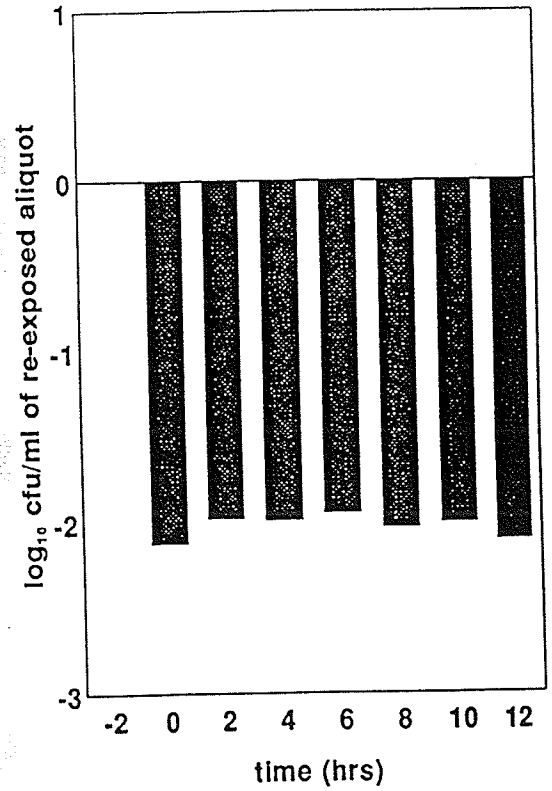
2
0
log10 cfu/ml
-2 0 2 4 6 8 10 12
time (hrs)


1
0
-1
-2
-3
log10 cfu/ml of re-exposed aliquot
-2 0 2 4 6 8 10 12
time (hrs)

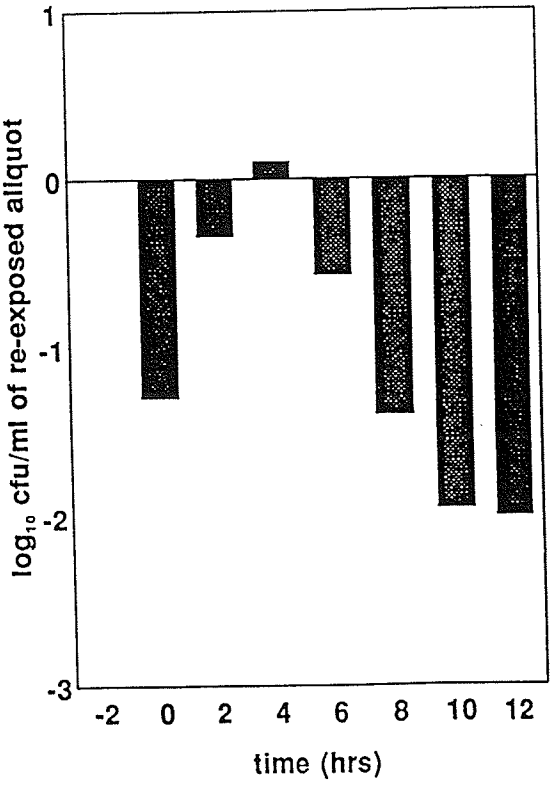
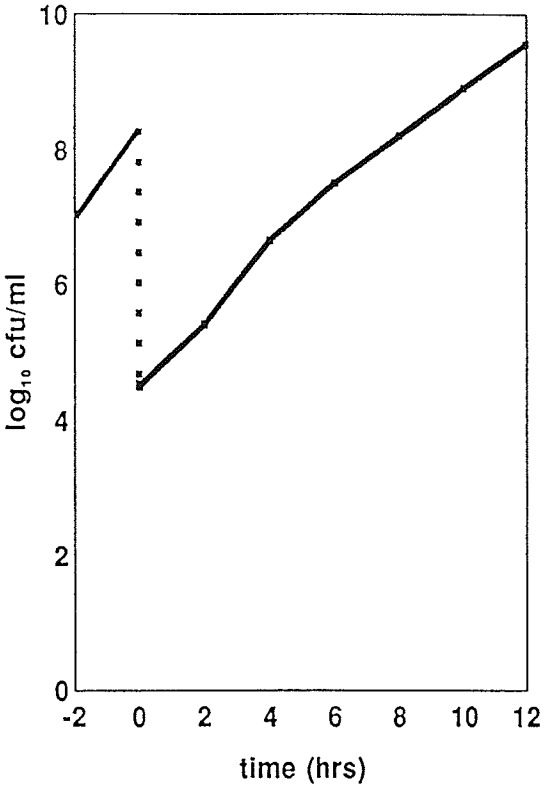

1
0
-1
-2
-3
log10 cfu/ml of re-exposed aliquot
-2 0 2 4 6 8 10 12
time (hrs)

Figure 38. **Adaptive resistance following a single, 2 hour gentamicin exposure at 1X MIC against *Pseudomonas aeruginosa* PAO1.** Upper left panel shows bacterial growth of control culture with no prior gentamicin exposure. Upper right panel shows bacterial regrowth following a 2 hour gentamicin pre-exposure, at 1X MIC. Extracellular gentamicin was removed by centrifugation following 2 hour pre-exposure. Control (upper and lower left panels) and test (upper and lower right panels) cultures were diluted to approximately 5 x 10^4 cfu/ml at time zero. Lower left panel shows bacterial killing, at successive 2 hour intervals, in control cultures (no previous gentamicin exposure) 2 hours after the addition of gentamicin at 1X MIC. Lower right panel shows bacterial killing, at successive 2 hour intervals, in test cultures (2 hour gentamicin pre-exposure at 1X MIC) 2 hours after the addition of gentamicin at 1X MIC. *P. aeruginosa* PAO1 was tested on 5 separate occasions.


10
8
6
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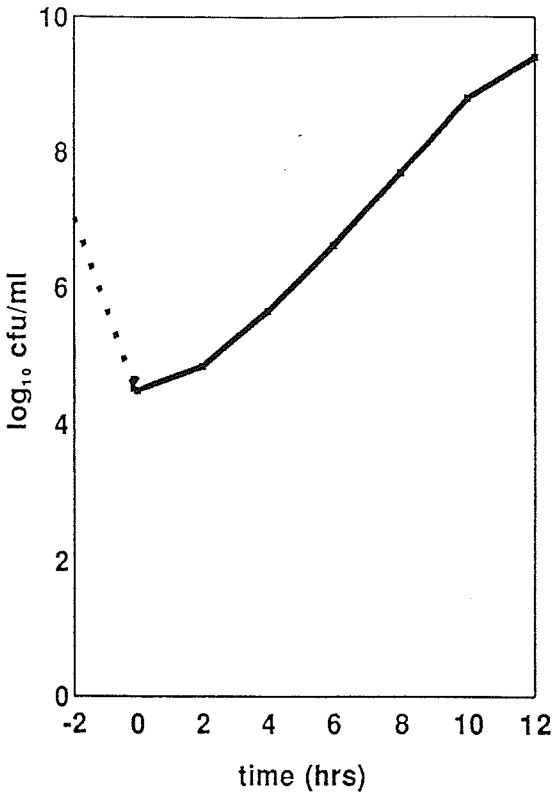
0
-2
0
2
4
6
8
10
12
log10 cfu/ml
time (hrs)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4
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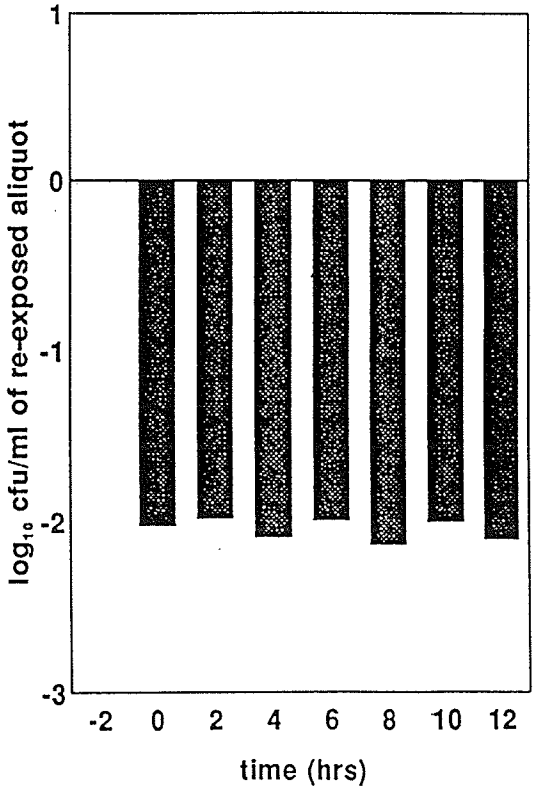
0
-2
0
2
4
6
8
10
12
log10 cfu/ml
time (hrs)


1
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-1
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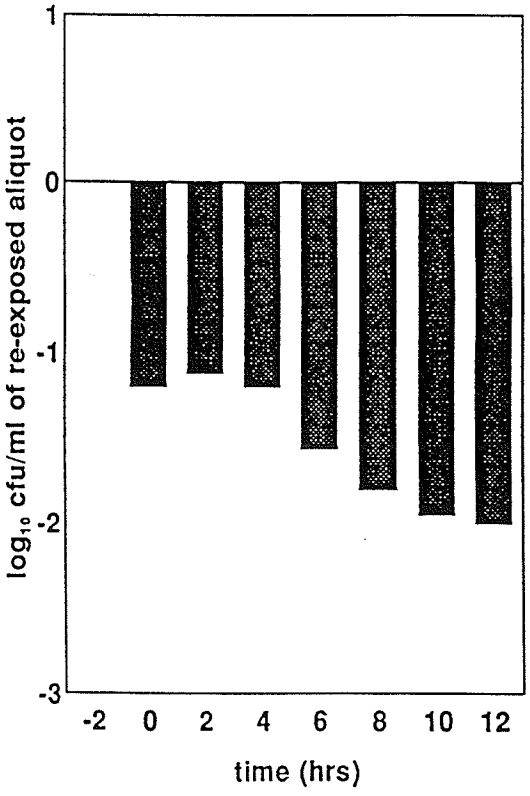
2
4
6
8
10
12
log10 cfu/ml of re-exposed aliquot
time (hrs)


1
0
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-3
-2
0
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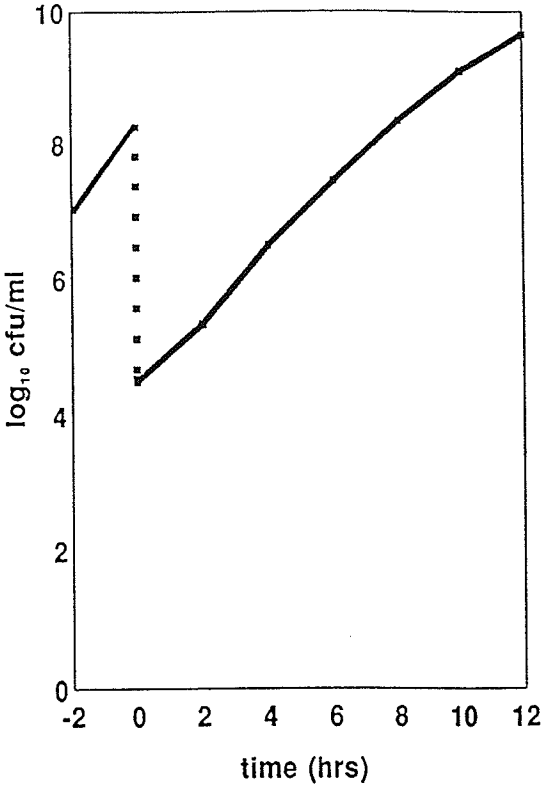
4
6
8
10
12
log10 cfu/ml of re-exposed aliquot
time (hrs)

Figure 39. Adaptive resistance following a single, 2 hour gentamicin exposure at 1X MIC against *Pseudomonas aeruginosa* PAO6261. Upper left panel shows bacterial growth of control culture with no prior gentamicin exposure. Upper right panel shows bacterial regrowth following a 2 hour gentamicin pre-exposure, at 1X MIC. Extracellular gentamicin was removed by centrifugation following 2 hour pre-exposure. Control (upper and lower left panels) and test (upper and lower right panels) cultures were diluted to approximately 5 x 10^4 cfu/ml at time zero. Lower left panel shows bacterial killing, at successive 2 hour intervals, in control cultures (no previous gentamicin exposure) 2 hours after the addition of gentamicin at 1X MIC. Lower right panel shows bacterial killing, at successive 2 hour intervals, in test cultures (2 hour gentamicin pre-exposure at 1X MIC) 2 hours after the addition of gentamicin at 1X MIC. *P. aeruginosa* PAO6261 was tested on 5 separate occasions.


10
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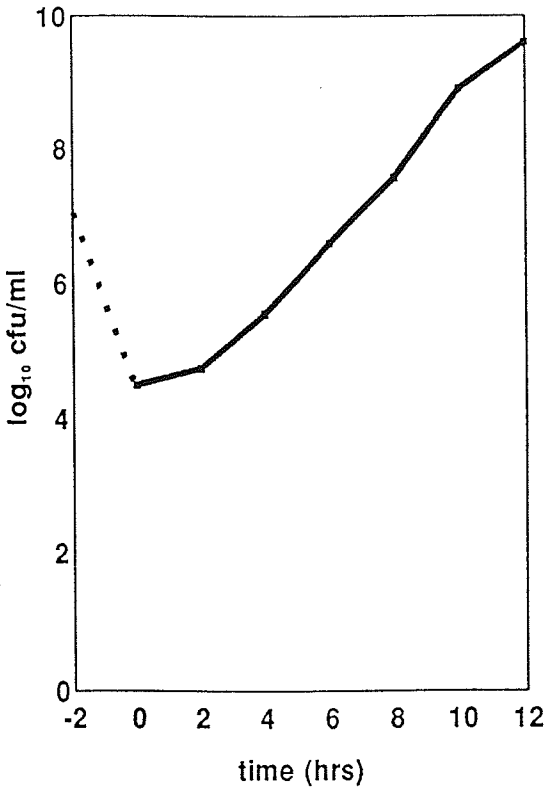
6
4
2
0
log10 cfu/ml
-2 0 2 4 6 8 10 12
time (hrs)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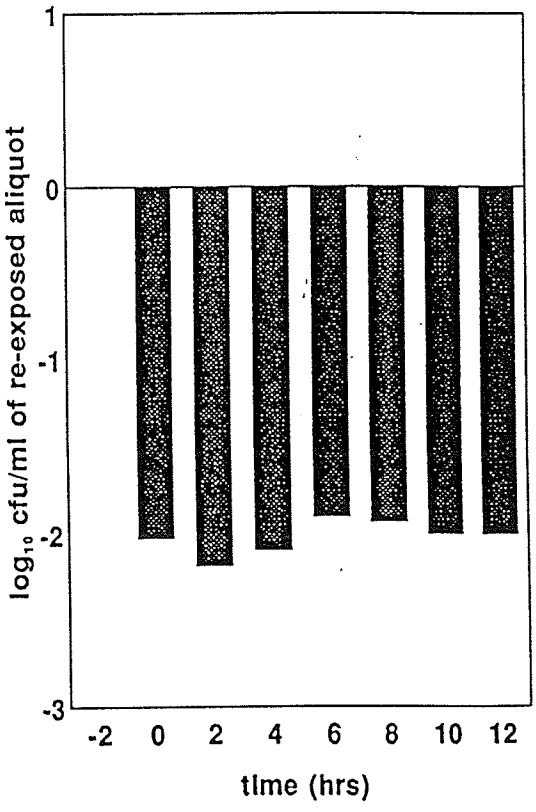
8
6
4
2
0
log10 cfu/ml
-2 0 2 4 6 8 10 12
time (hrs)


1
0
-1
-2
-3
log10 cfu/ml of re-exposed aliquot
-2 0 2 4 6 8 10 12
time (hrs)

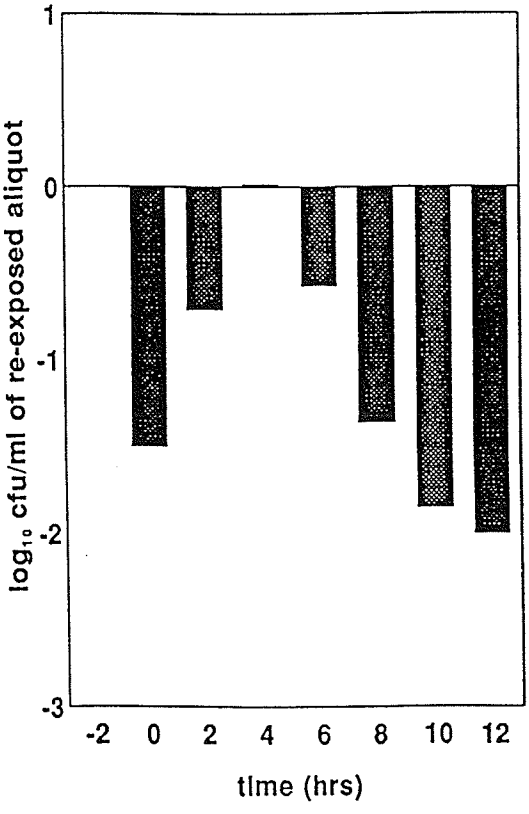

1
0
-1
-2
-3
log10 cfu/ml of re-exposed aliquot
-2 0 2 4 6 8 10 12
time (hrs)

Figure 40. **Adaptive resistance following a single, 2 hour gentamicin exposure at 1X MIC against *Pseudomonas aeruginosa* PAO6261 (*anr*$^+$).** Upper left panel shows bacterial growth of control culture with no prior gentamicin exposure. Upper right panel shows bacterial regrowth following a 2 hour gentamicin pre-exposure, at 1X MIC. Extracellular gentamicin was removed by centrifugation following 2 hour pre-exposure. Control (upper and lower left panels) and test (upper and lower right panels) cultures were diluted to approximately 5 x 10^4 cfu/ml at time zero. Lower left panel shows bacterial killing, at successive 2 hour intervals, in control cultures (no previous gentamicin exposure) 2 hours after the addition of gentamicin at 1X MIC. Lower right panel shows bacterial killing, at successive 2 hour intervals, in test cultures (previous 2 hour gentamicin exposure at 1X MIC) 2 hours after the addition of gentamicin at 1X MIC. *P. aeruginosa* PAO6261 (*anr*$^+$) was tested on 5 separate occasions.

b. Gentamicin Accumulation in *Pseudomonas aeruginosa* PAO1, PAO6261, and PAO6261(*anr*$^+$)

Gentamicin accumulation in adaptively resistant *P. aeruginosa* was measured in the reference strain PAO1, its *anr* deficient mutant PAO6261, and *anr* complemented PAO6261 (PAO6261(*anr*$^+$)) (Figures 41-43 respectively). Gentamicin accumulation in each was measured at 1X MIC in an unexposed control culture, immediately following 2 hour gentamicin exposure (1X MIC) and removal, during peak adaptive resistance (4 hours following aminoglycoside removal), and at 8 and 12 hours following aminoglycoside removal. *P. aeruginosa* PAO1 (Figure 41) and PAO6261(*anr*$^+$) (Figure 43) demonstrated accumulation kinetics similar to those of the other *P. aeruginosa* strains tested earlier (Figure 17). Aminoglycoside accumulation was significantly reduced ($P<0.05$) immediately following the initial 2 hour gentamicin exposure and removal, and at 4 (peak adaptive resistance) and 8 hours thereafter (Figures 41 and 43). Peak adaptively resistant cultures demonstrated the lowest levels of gentamicin accumulation ($P<0.05$) when compared with the other times measured (Figures 41 and 43). Aminoglycoside accumulation normalized 12 hours following aminoglycoside removal.

P. aeruginosa PAO6261 demonstrated higher levels of gentamicin accumulation during the adaptive resistance interval (Figure 42) when compared with PAO1 (Figure 41) and PAO6261(*anr*$^+$) (Figure 43). The increased gentamicin accumulation in *P. aeruginosa* PAO6261 coincided with the increased bacterial killing demonstrated in adaptive resistance experiments (Figure 39).

Proton motive force determinations were performed, in triplicate, in control cultures of *P. aeruginosa* PAO1, PAO6261, and PAO6261(*anr*$^+$). Statistically significant differences ($P>0.05$) were not detected between the three control cultures (PAO1 -153 ±

6 mV; PAO6261 -155 ± 5 mV; PAO6261(*anr*$^+$) -155 ± 5 mV). Proton motive force determinations were also performed in cultures during peak adaptive resistance (4 hours following gentamicin removal). Again, statistically significant differences (P>0.05) were not detected (PAO1 -149 ± 5 mV, PAO6261 -154 ± 5 mV, PAO6261(*anr*$^+$) -151 ± 6 mV). Significant differences (P>0.05) were also not detected between wild-type, control and adaptively resistant cultures for any of the 3 strains. These results suggest the pmf likely does not play a critical role in reduced aminoglycoside accumulation in adaptively resistant *P. aeruginosa.*

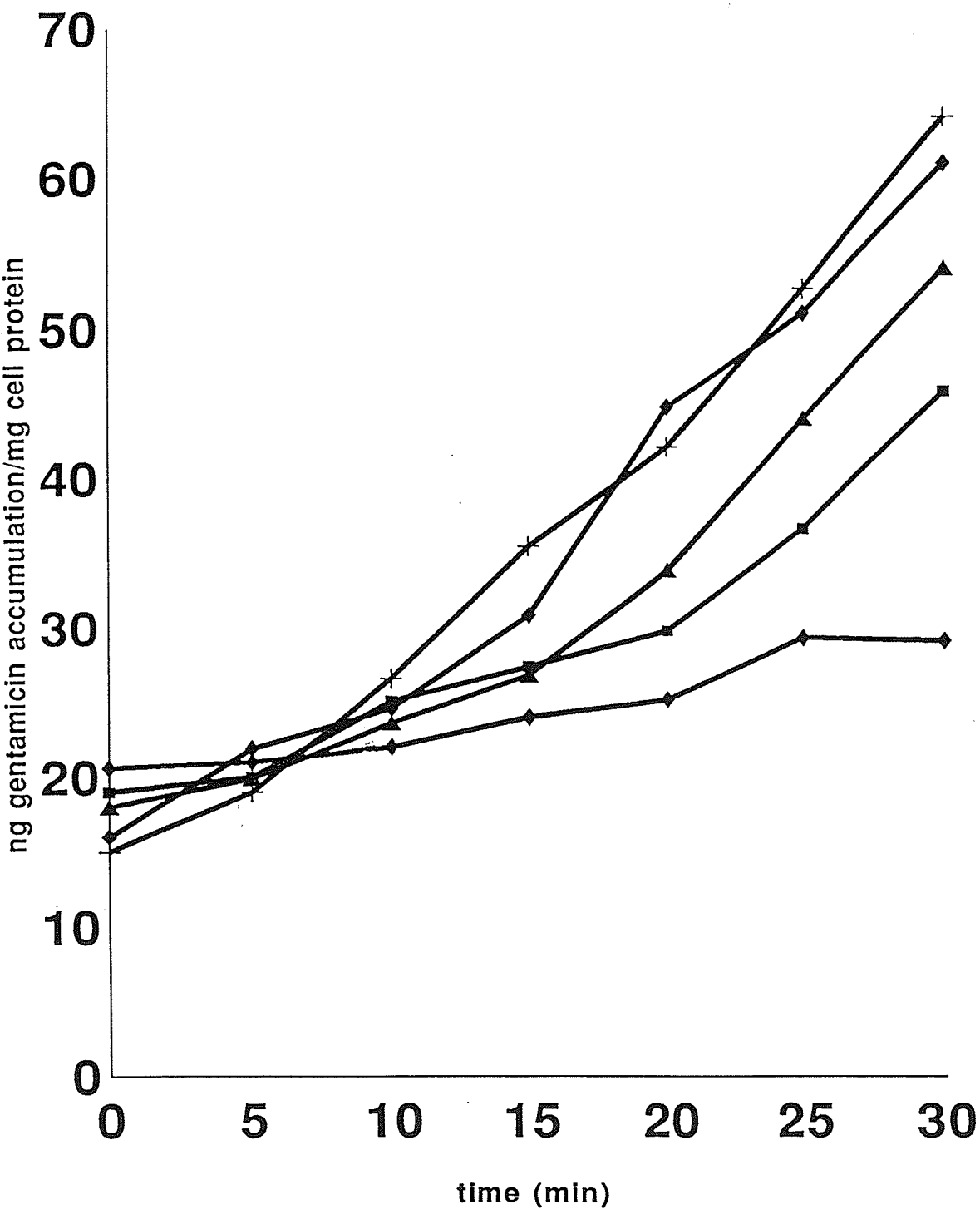

70
60
50
40
30
20
10
0
ng gentamicin accumulation/mg cell protein
0
5
10
15
20
25
30
time (min)

Figure 41. **^{3}H-Gentamicin accumulation in *Pseudomonas aeruginosa* PAO1 following a 2 hour gentamicin exposure at 1X MIC.** Gentamicin accumulation was determined in unexposed controls (+) and compared with accumulation in cultures following a 2 hour exposure to gentamicin at 1X MIC. Gentamicin accumulation was measured in gentamicin pre-exposed cultures immediately following initial gentamicin removal (0 hour, ■), and then following 4 (♦), 8 (▲) and 12 (•) hours of growth in aminoglycoside free MHB at 37°C. Experiments were repeated in triplicate and mean values plotted.

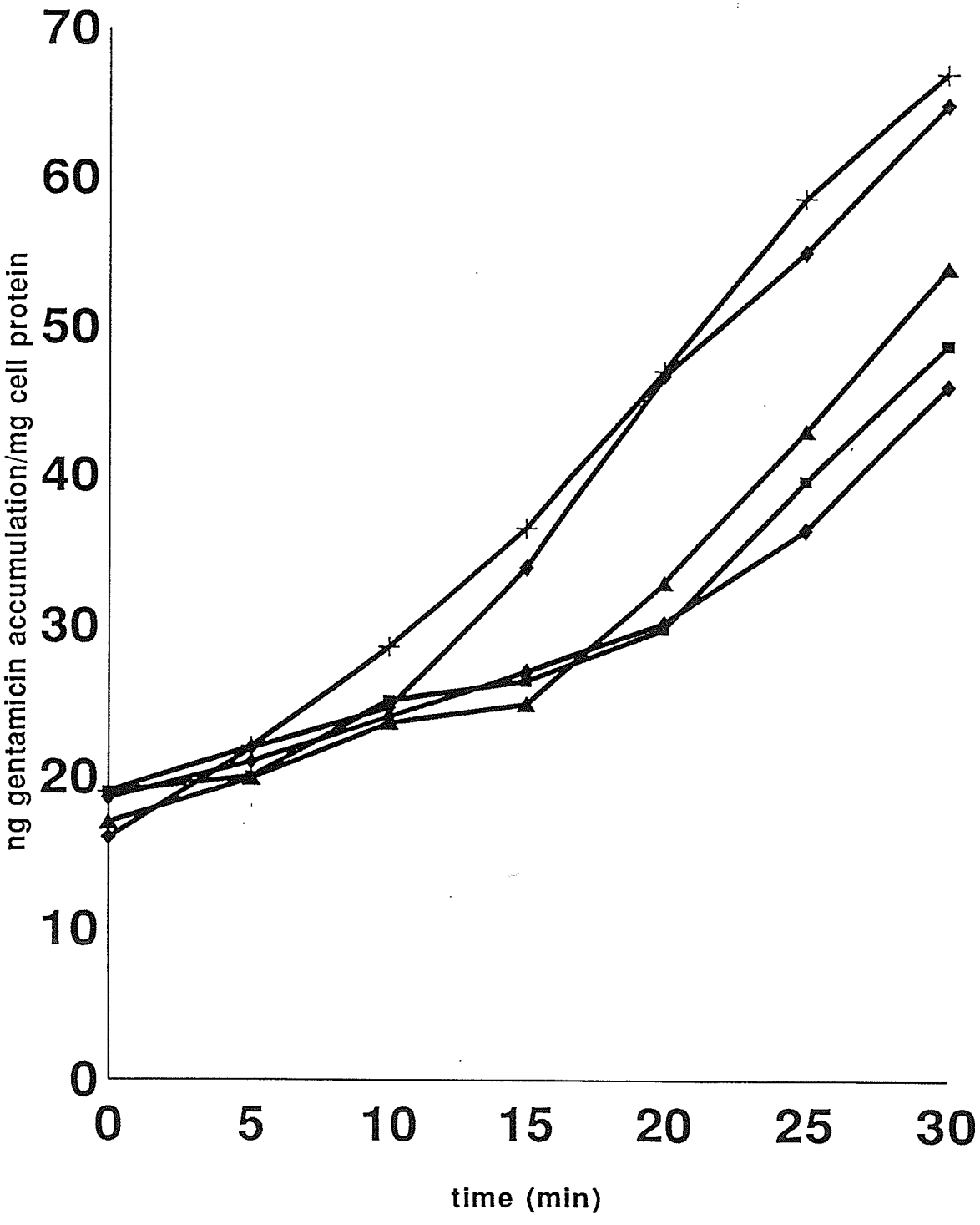

70
60
50
40
30
20
10
0
ng gentamicin accumulation/mg cell protein
0
5
10
15
20
25
30
time (min)

Figure 42. **^{3}H-Gentamicin accumulation in *Pseudomonas aeruginosa* PAO6261 following a 2 hour gentamicin exposure at 1X MIC.** Gentamicin accumulation was determined in unexposed controls (+) and compared with accumulation in cultures following a 2 hour exposure to gentamicin at 1X MIC. Gentamicin accumulation was measured in gentamicin pre-exposed cultures immediately following initial gentamicin removal (0 hour, ■), and then following 4 (♦), 8 (▲) and 12 (•) hours of growth in aminoglycoside free MHB at 37°C. Experiments were repeated in triplicate and mean values plotted.

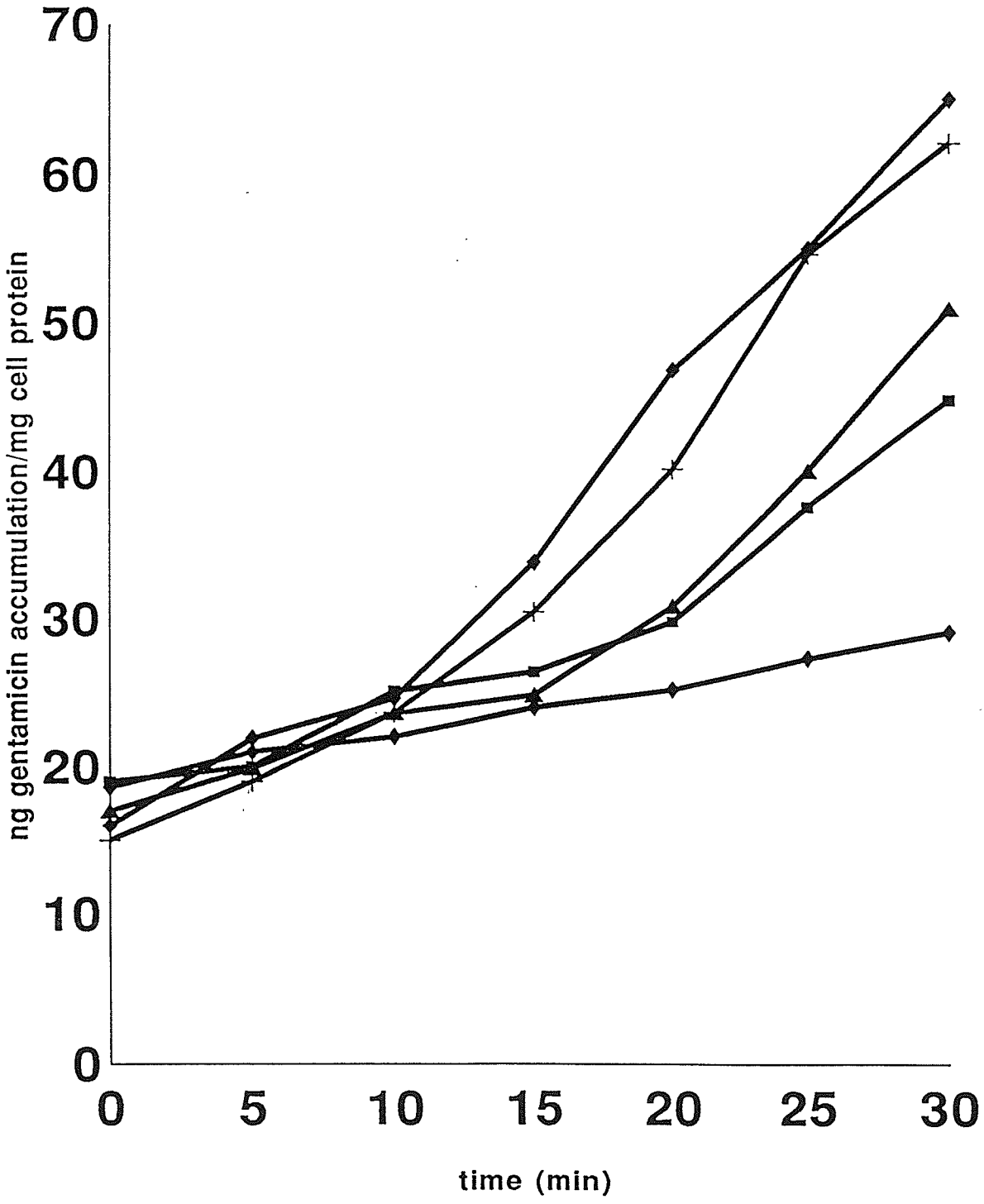

70
60
50
40
30
20
10
0
ng gentamicin accumulation/mg cell protein
0
5
10
15
20
25
30
time (min)

Figure 43. **^{3}H-Gentamicin accumulation in *Pseudomonas aeruginosa* PAO6261(*anr*$^{+}$) following a 2 hour gentamicin exposure at 1X MIC.** Gentamicin accumulation was determined in unexposed controls (+) and compared with accumulation in cultures following a 2 hour exposure to gentamicin at 1X MIC. Gentamicin accumulation was measured in gentamicin pre-exposed cultures immediately following initial gentamicin removal (0 hour, ■), and then following 4 (♦), 8 (▲) and 12 (•) hours of growth in aminoglycoside free MHB at 37°C. Experiments were repeated in triplicate and mean values plotted.

6. *denA* and *anr* mRNA Levels in Transiently, Highly Resistant *Pseudomonas aeruginosa* ATCC 27853

P. aeruginosa ATCC 27853 was serially passaged against doubling gentamicin concentrations (initial gentamicin concentration 4 mg/l) to a final concentration of 64 mg/l. mRNA levels of *denA* and *anr* were then determined in these cells and compared with wild-type control, adaptively resistant (1X MIC), and post-adaptively resistant cells (Figures 44 and 45 respectively). Both *denA* (Figure 44, lane 1C) and *anr* (Figure 45, lane 1C and 1D) mRNA levels were discernably higher in cells growing in 64 mg/l of gentamicin compared with cells adaptively resistant to 3.6 mg/l of gentamicin (Figures 44 and 45, lane 1A in both). These results suggest that increasing aminoglycoside concentration may upregulate *anr*, and subsequently *denA*, mRNA levels.

Figure 44. **Northern analysis of *denA* in transient, highly aminoglycoside resistant *Pseudomonas aeruginosa* ATCC 27853.** *P. aeruginosa* ATCC 27853 was serially passaged with doubling gentamicin concentrations to a final concentration of 64 mg/l. RNA was prepared, electrophoresed, transferred to nylon membrane and probed as described in Materials and Methods. Lane 1, wild-type, control ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853; lane 1C, highly aminoglycoside resistant ATCC 27853; lane 1D, anaerobically grown ATCC 27853. RNA standards (16S [1766 bases] and 23S [3566 bases] ribosomal RNA) were run, not shown, identifying the band as approximately 2 kb in length.

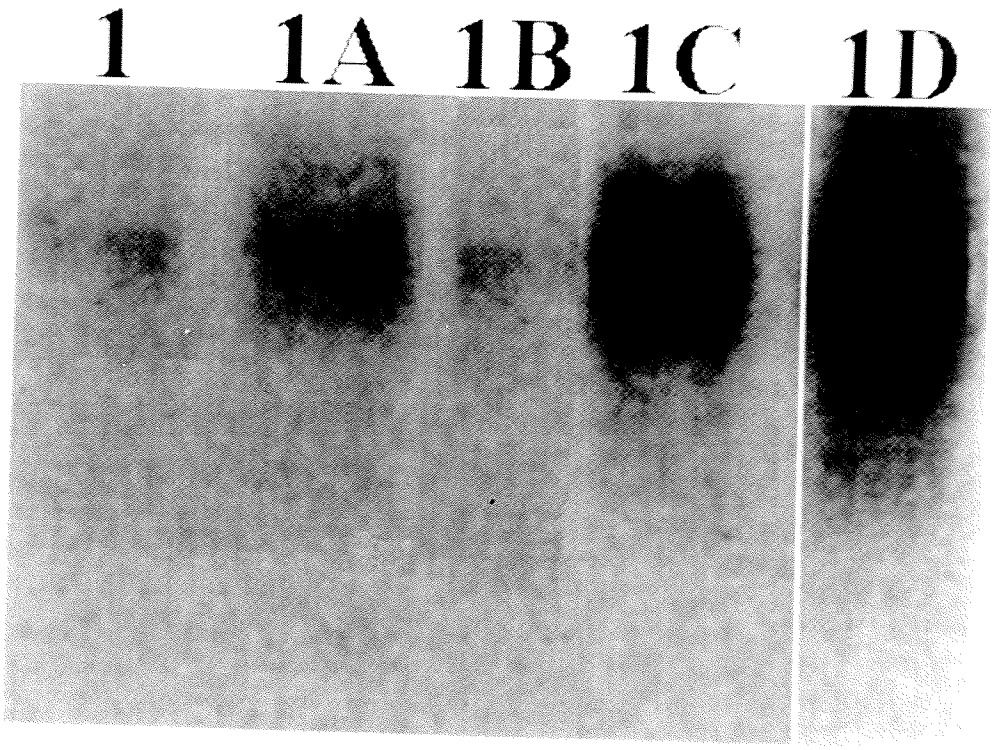
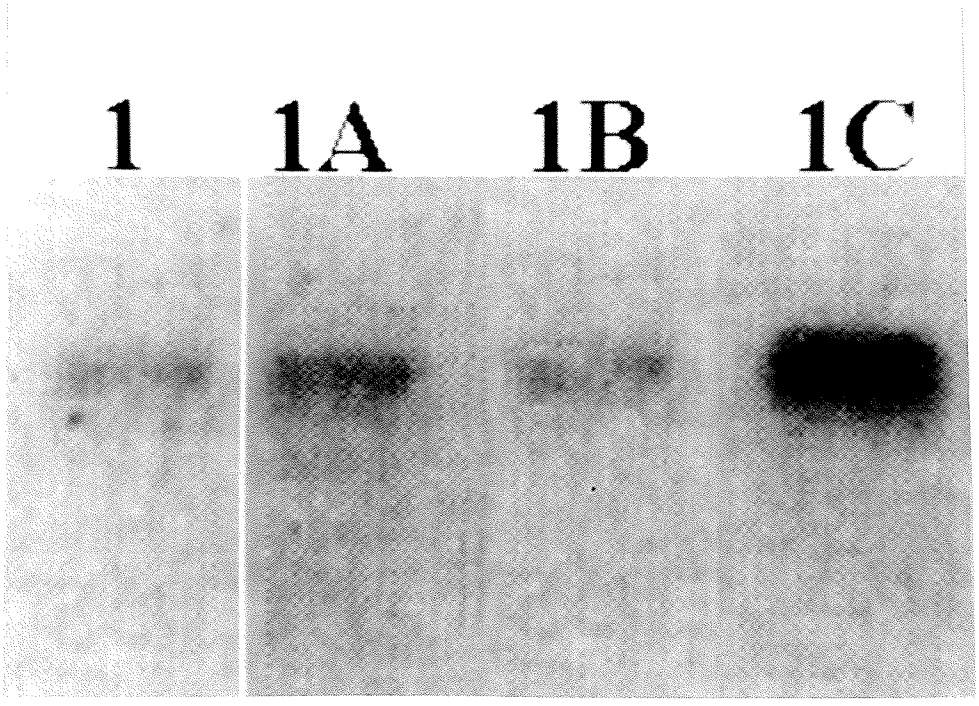


Figure 45. Northern analysis of *anr* in transient, highly aminoglycoside resistant *Pseudomonas aeruginosa* ATCC 27853. *P. aeruginosa* ATCC 27853 was serially passaged with doubling gentamicin concentrations to a final concentration of 64 mg/l. RNA was prepared, electrophoresed, transferred to nylon membrane and probed as described in Materials and Methods. Lane 1, wild-type, control ATCC 27853; lane 1A, adaptively resistant ATCC 27853; lane 1B, post-adaptively resistant ATCC 27853; lane 1C, highly aminoglycoside resistant ATCC 27853. RNA standards (16S [1766 bases] and 23S [3566 bases] ribosomal RNA) were run, not shown. The band was considerably smaller than 1.8 kb in length. The *anr* open reading frame contains 732 nucleotides (Zimmerman *et al.*, 1991).



D. DISCUSSION

1. Why Study *Pseudomonas aeruginosa* Adaptive Resistance to Aminoglycoside Antimicrobial Agents

One important reason underlying the continued clinical use of aminoglycosides, despite their well documented toxicity and the development of new, highly active fluoroquinolone, carbapenem and monobactam antimicrobials, is their limited tendency towards detectable resistance development during therapy (Gilbert, 1995). Despite favorable clinical outcomes, epidemiological surveys suggest that unstable, aminoglycoside resistant, bacterial subpopulations are commonly present at sites of colonization and infection in hospitalized patients receiving aminoglycoside therapy (Weinstein *et al.*, 1980; Price *et al.*, 1981; Young, 1984). These variants appear to be of little concern in immunocompetent patients (Weinstein *et al.*, 1980; Price *et al.*, 1981; Young, 1984) and may very well be going undetected in the clinical microbiology laboratory, as isolates may revert to susceptibility at initial isolation (Daikos *et al.*, 1990b; Gilbert, 1995). However, aminoglycoside resistant subpopulations may have definite clinical relevance to cases of treatment failure in conditions requiring prolonged or repeated aminoglycoside therapy, such as in immunocompromised patients, or patients with endocarditis or cystic fibrosis (Gilbert, 1995).

An understanding of the process of *P. aeruginosa* aminoglycoside adaptive resistance is of particular importance, principally because this bacterium is a common opportunistic pathogen in immunocompromised individuals. The effect of adaptive resistance may be to slow therapeutic response, promote colonization, or produce outright clinical failure. *P. aeruginosa* commonly causes difficult to treat nosocomial infections and has the highest mortality rate of any bacterium (40 to 93% for *P. aeruginosa*

bacteremia) (Korvick and Yu, 1991). Aminoglycoside antibiotics are a cornerstone of therapy in treating infections caused by *P. aeruginosa*, and therefore, understanding the development and control of aminoglycoside adaptive resistance is important to maximize the clinical effectiveness of these agents. Adaptive resistance development suggests that longer dosing intervals for aminoglycosides may improve clinical efficacy by allowing time for adaptive resistance to resolve between doses (Figure 7, Tables 9, 16, 17) (Daikos *et al.*, 1990b; Karlowsky *et al.*, 1994a; Karlowsky *et al.*, 1994b).

2. *Pseudomonas aeruginosa* Adaptive Resistance to Aminoglycoside Antimicrobial Agents Defined

Antimicrobial chemotherapy is usually, but not always, administered as identical, repeated doses. Experiments performed in this thesis, which confirmed the work of others (Daikos *et al.*, 1990; Barclay *et al.*, 1992), demonstrated that following an initial aminoglycoside exposure, surviving *P. aeruginosa* possess, or develop, transient resistance to subsequent aminoglycoside killing (Figure 7). The development of transient aminoglycoside adaptive resistance (i.e. reduced bacterial killing) was phenotypically detected by 3 related pharmacodynamic methods: minimum inhibitory concentration (MIC) reversion times (MRTs), multiple exposure postantibiotic effect (PAE) determinations, and adaptive resistance determinations (Karlowsky *et al.*, 1994a; Karlowsky *et al.*, 1994b).

MRTs were determined to identify the occurence and duration of elevated *P. aeruginosa* culture MICs (i.e. reduced aminoglycoside susceptibility) following aminoglycoside exposure (Karlowsky *et al.*, 1994b). These experiments compared transient resistance development in simulated traditional aminoglycoside dosing, (2 mg/kg

q8h, attaining an approximate Cp_{max} [peak serum concentration] of 8mg/l) with once-daily aminoglycoside dosing (6 mg/kg q24h, attaining an approximate Cp_{max} of 24 mg/l), with regard to the time required for the MICs of *P. aeruginosa* cultures to revert to their original values. In addition, the times required for the MICs for the cultures to fall below or to the aminoglycoside dosing concentration and the aminoglycoside concentration/MIC ratios were also noted.

MRTs of significant duration followed all single and multiple 2 hour aminoglycoside exposures at 8 and 24 mg/l (Tables 8 and 9). Single 2 hour aminoglycoside exposures to 8 mg/l produced culture MRTs (gentamicin, 21.5 ± 4.0 hours; tobramycin, 22.3 ± 2.8 hours) that were significantly ($P<0.05$) shorter than those measured following identical exposures to 24 mg/l (gentamicin, 28.9 ± 3.8 hours; tobramycin, 26.8 ± 3.1 hours). However, 3 sequential 2 hour exposures to 8 mg/l, one exposure every 8 hours, produced MRTs following the third exposure (gentamicin, 68.1 ± 5.2 hours; tobramycin, 77.8 ± 7.8 hours) that were significantly longer ($P<0.005$) than those determined following three 2 hour exposures to 24 mg/l, one exposure every 24 hours (gentamicin, 36.1 ± 3.0 hours; tobramycin, 34.5 ± 3.0 hours). The observed difference between the 2 multiple exposure regimens suggested that cultures exposed to 8 mg/l every 8 hours never returned to their initial susceptibilities between exposures. This implies that subsequent exposures selected and maintained cultures for which the MICs were increasing. In comparison, cultures exposed to 24 mg/l, every 24 hours, returned to, or very close to, their initial susceptibilities between doses. Furthermore, the mean time for the MICs to fall below or to the aminoglycoside dosing concentration was significantly ($P<0.005$) shorter for the once-daily aminoglycoside dosing regimen, than for traditional dosing regimen (Table 9).

From a clinical perspective, the MRT data collected lends support to the concept of once-daily aminoglycoside dosing (i.e. higher aminoglycoside doses administered less frequently). Support for the concept of once daily aminoglycoside dosing may also be garnered from the aminoglycoside concentration/MIC ratios that were calculated (Table 10). Recent work on the pharmacodynamics of aminoglycosides has shown that the larger the aminoglycoside concentration/MIC ratio, the greater the rate and extent of bacterial killing (Drusano, 1991). Significantly ($P < 0.005$) higher aminoglycoside concentration/MIC ratios were demonstrated with simulated once-daily dosing (24 mg/l, every 24 hours) compared with traditional dosing (8 mg/l, every 8 hours) (Table 10). The rationale for using once-daily aminoglycoside dosing from a clinical efficacy point of view relates to the observation that larger Cp_{max}/MIC ratios correlate with clinical cure (Drusano, 1991).

PAE is the recovery period, or persistent suppression of bacterial growth, following a short one or two hour antimicrobial exposure. In the past it had been assumed that because post-PAE cultures resume logarithmic phase growth, they had returned to their wild-type susceptibility (Craig and Gudmundsson, 1991). Experiments performed in this thesis show that this assumption may be incorrect for *P. aeruginosa* and aminoglycosides (Tables 15, 17, and 18). Repeated aminoglycoside exposures, against *P. aeruginosa*, at times well after the conventional PAE interval was known to have passed, produced significant reductions in bacterial killing and PAE, implying that post-PAE-phase cultures have not returned to their initial susceptibilities (Tables 15, 17 and 18) (Karlowsky *et al.*, 1994a). This suggests that the mechanism of the *P. aeruginosa* PAE with aminoglycosides may be independent of the mechanism of adaptive resistance.

To gain insight into the reduction in PAE and bacterial killing noted for re-

exposures at the original MIC (Tables 15, 17 and 18), cultures were re-exposed to their induced MICs, which ranged from 2 to 4 times the original MIC following first exposure, and 4 to 16 times the original MIC following the second exposure (Table 16). A significant increase in PAE ($P<0.05$) was demonstrated between first and second exposures, but not between second and third exposures, for all aminoglycosides tested. Conversely, statistically significant ($P<0.05$) decreases in bacterial killing were only seen between first and second exposures, but not between second and third exposures, for all aminoglycosides tested. The results of these experiments, once again, suggest that transient adaptive resistance (reductions in bacterial killing) to aminoglycosides arises by a mechanism that is independent of the PAE.

In contrast to experiments performed with aminoglycosides, *P. aeruginosa* did not demonstrate a PAE, or any change in bacterial killing, with multiple ceftazidime exposures (Table 19). Ciprofloxacin exposures against *P. aeruginosa*, at 1X MIC, demonstrated a trend towards increased MICs, decreased PAEs and reduced bacterial killing, similar to that of aminoglycosides. This trend, however, was much less discernable and reproducible. For comparison, similar multiple antibiotic exposure experiments were conducted using *E. coli* (Table 20). *E. coli* did not demonstrate a significant PAE with cefotaxime. No changes in bacterial killing or MIC with multiple exposures were observed (Table 20). With *E. coli* both ciprofloxacin and gentamicin pre-exposures at 2X MIC increased MICs, reduced PAE, and reduced bacterial kill with subsequent exposures, but not to the extent demonstrated with aminoglycosides and *P. aeruginosa* (Table 20).

Adaptive resistance determinations were made using a previously described method (Daikos *et al*, 1990). Adaptive resistance was initiated, in most instances, with a 2 hour

gentamicin or tobramycin exposure at 1X MIC. Each *P. aeruginosa* strain tested at 1X MIC demonstrated an adaptive resistance period of between 8 and 12 hours with both aminoglycosides tested (Figure 7). Aminoglycoside adaptive resistance developed and receded in a stepwise fashion (Figure 7). Maximum reductions in bacterial killing were not present immediately following a 1 or 2 hour static aminoglycoside exposure but occurred between 3 to 5 hours later (Figure 7). However, longer times to achieve maximum reductions in bacterial killing have been previously reported using an in vitro pharmacodynamic model (Barclay *et al.*, 1992).

Adaptive resistance was associated with significant bacterial replication (Figure 7) (Daikos *et al.*, 1990; Barclay *et al.*, 1992). Cultures demonstrating maximum reductions in bacterial killing, that is, peak adaptive resistance (maximum reduction in bacterial killing), possess mean generation times similar to growth controls (Table 20). A 1 to 2 $\log_{10}$ cfu/ml (i.e. 90 to 99% new progeny [approximately 4 to 7 net cell divisions]) increase in cell number correlated with maximum reduction in bacterial killing (Figure 7). Continued bacterial replication, totalling between 3 and 4 $\log_{10}$ cfu/ml (i.e. when 99.9 and 99.99% of the cell cutlure are new progeny [approximately 10 to 14 net cell divisions]), yielded cells fully susceptible to aminoglycoside bactericidal action and once again subject to repetition of first exposure effects. Following the abrupt removal of extracellular aminoglycoside, and the gradual reduction in intracellular aminoglycoside concentration, (assumed, not proven experimentally) there appear to be strong selective forces promoting the return of the parental form of *P. aeruginosa.* The phenotypic or regulatory changes which occur do so because the aminoglycoside free growth conditions must favour them.

Following an aminoglycoside exposure, maximum reductions in bacterial killing

may occur in the presence or absence of aminoglycoside (Daikos *et al.*, 1990). The continued presence of aminoglycoside prolongs adaptive resistance (Daikos *et al.*, 1990). The extent of adaptive resistance induction is also concentration dependent (Daikos *et al.*, 1990; Barclay *et al.*, 1992). The degree of transient resistance which develops correlates approximately with the concentration of aminoglycoside used (Daikos *et al.*, 1990).

To serve as a comparison to aminoglycoside adaptive resistance, adaptive resistance determinations were performed with *P. aeruginosa* and ceftazidime and ciprofloxacin (Figures 10 and 11). Figure 10 demonstrates that ceftazidime does not produce an adaptive resistance interval and that bacterial killing depends exclusively upon initial inoculum. Ciprofloxacin produced transient reductions in bacterial killing, but not to the extent that aminoglycosides do at 1X MIC. Adaptive resistance determinations in *E. coli*, exposed to ciprofloxacin or gentamicin at 1X MIC, did not produce adaptive resistance intervals of significant duration.

Debate remains concerning the nature of the shape of adaptive resistance data curves (e.g. Figure 7) (Daikos *et al.*, 1990). Do they reflect the selection of a specific subpopulation from within a bacterial culture, or do they suggest that adaptive resistance is a regulatory event with specific induction and recession phases? Aminoglycoside kill curves (Figures 13, 14, and 15) demonstrate that bacterial killing, at 1X MIC, is not complete within the 1 or 2 hour exposure intervals traditionally used by investigators in this area. Experiments performed in this thesis demonstrated that aminoglycoside killing at 1X MIC reached a maximum after approximately 3 hours of exposure (Figures 13, 14 and 15). An interesting observation can be made if Figures 7 and 13 through 15 are studied together. The residual, susceptible cells present following a 2 hour aminoglycoside exposure at 1X MIC (approximately 1 $\log_{10}$ cfu/ml) appear to be lost with

incubation in aminoglycoside free media. Even though this subpopulation of cells should be present during every subsequent 2 hour exposure of the pre-exposed culture, and its kill noted, it is not. The susceptibility of the bacterial culture as a whole appears to shift (Figure 7). This observation argues strongly against the notion of aminoglycoside adaptive resistance arising as the result of subpopulation selection. The character of a selected subpopulation of cells would be expected to be far more stable. Experiments using longer initial exposure times show no induction phase (Daikos *et al.*, 1990). However, if aminoglycoside exposure simply unmasks a resistant subpopulation, it is unclear why this subpopulation, which is capable of logarithmic phase growth, should gradually revert to aminoglycoside sensitivity. The data, when considered from this perspective, are more consistent with a regulated induction and recession of aminoglycoside resistance by the majority of the bacterial population of surviving organisms.

However, experimental evidence was also collected supporting the notion of subpopulation selection. The in vitro adaptive resistance methodology used, theoretically, allowed equal access of aminoglycoside to all cells. Why then are not all cells killed, if they are clonally related, and have equal access to aminoglycoside. At 1X MIC, approximately 1% of a *P. aeruginosa* population survives a 2 hour aminoglycoside exposure (Figure 7). This suggests that very few cells within the population have the ability to regulate themselves favorably in response to an aminoglycoside insult. This scenario, as far as being an example of induction, does not compare well to our traditional induction model, that being the lactose operon of *E. coli*. The lactose operon of *E. coli* is induced in the entire bacterial population and requires only a single molecule of lactose, in the absence of glucose, for induction. The poor response to aminoglycoside insult

argues more favorably for subpopulation selection than regulation. Perhaps *P. aeruginosa* aminoglycoside adaptive resistance arises by a combination of both subpopulation selection and regulatory response.

Further experiments were performed to determine the concentration of aminoglycoside required to induce adaptive resistance. Adaptive resistance was still produced with concentrations of 1/2X MIC (Figure 8). However, at 1/4X MIC and 1/8X MIC adaptive resistance was no longer observed (Figure 9). Net bacterial killing during the 2 hour aminoglycoside exposure appears necessary for observable adaptive resistance. This observation implies that 1 or more $\log_{10}$ cfu/ml must be killed for adaptive resistance to appear and suggests selection of a pre-existing subpopulation that is intrinsically more aminoglycoside resistant or that more readily adapts to aminglycoside insult. In conclusion, preliminary pharmacodynamic data were collected that support *P. aeruginosa* aminoglycoside adaptive resistance arising both by subpopulation selection and as the result of one, or more, unknown regulatory responses.

3. Cellular Characterization of *Pseudomonas aeruginosa* Adaptive Resistance to Aminoglycoside Antimicrobial Agents

Adaptive resistance was characterized by transient reductions in aminoglycoside (^{3}H-gentamicin) accumulation which correlated well with the observed reductions in bacterial killing (Figures 7 and 17). Both of these events, in turn, appeared to correlate well with a small (5%), but statistically significant ($P<0.05$) reduction in cellular proton motive force (pmf) seen during peak adaptive resistance (4 hours following aminoglycoside removal) (Table 22). The importance of the observed pmf change, although statistically significant is questionable for several reasons. First, the actual

reduction in pmf during peak adaptive resistance was small, only 5% relative to control (Table 21). Second, no change in bacterial doubling time was noted during peak periods of adaptive resistance (a significantly lower pmf would imply a slower rate of growth). Third, the threshold pmf value conferring intracellular aminoglycoside accumulation was determined to be approximately -130 mV. Therefore *P. aeruginosa* adaptively resistant to 1X MIC of gentamicin remained above the threshold pmf value of -130 mV at all measured time intervals. Fourth, ^{3}H-proline accumulation in *P. aeruginosa* adaptively resistant to 1X MIC of gentamicin, remained unchanged when compared to controls (Figure 19). Proline accumulation in *P. aeruginosa* is an active process directly proportional to the magnitude of the pmf across the cytoplasmic membrane (Fraimow *et al.*, 1991). Fifth, the extent of bacterial killing, relative to control culture, during adaptive resistance determinations was not diminished appreciably by 200 µM DCCD pretreatment, despite similar ($P>0.05$) resulting pmf values (Figures 24 and 25). Sixth, the wild-type, control and adaptive resistance pmf values, determined in *P. aeruginosa* PAO1, its *anr* deletion mutant, PAO6261, and *anr* complemented PAO6261, PAO6261(*anr*$^{+}$), were all similar ($P>0.05$). These results suggest that the mechanism responsible for adaptive resistance is likely not directly pmf dependent.

Another interesting observation was that *P. aeruginosa* adaptively resistant to 1X MIC could be overridden by a higher aminoglycoside concentration (6X MIC) (Figure 18). This result suggests that the transport proteins or gates are present, but inactive. The nature of this inactivity is likely pmf independent (as discussed above) but may reflect an elevated threshold potential in cells surviving an initial aminoglycoside exposure. Other explanations may be decreased aerobic respiratory pathway function, or the possibility that higher aminoglycoside concentrations transiently increase cellular pmf, by

virtue of their polycationic (electrochemical) nature, thereby facilitating their own accumulation (Butko *et al*, 1990). These hypotheses are founded in the work of other researchers who cumulatively have shown that aminoglycoside accumulation depends on both the $\Delta\Psi$ component of the pmf and a functional aerobic electron transport pathway (Taber *et al.*, 1987). However, this observation may also suggest that the magnitude of the "gate" is aminoglycoside concentration dependent. This result may also suggest indirectly that no 30S ribosomal subunit (target site) changes occur during aminoglycoside adaptive resistance as intracellular aminoglycoside accumulation which requires protein synthesis was demonstrable in these cells albeit at a higher aminoglycoside concentration. Aminoglycoside-ribosome binding assays, however, were not performed directly.

To determine the specificity of adaptive resistance in *P. aeruginosa* to other structurally unrelated classes of antimicrobial agents MICs were determined using cells that were adaptively aminoglycoside resistant. Aminoglycoside adaptive resistance in *P. aeruginosa* appears to develop independently of changes in susceptibility to other antimicrobial agents (Figure 12). The data gathered to this point suggested that aminoglycoside adaptive resistance was likely arising because of pmf independent, reduced cytoplasmic aminoglycoside accumulation in *P. aeruginosa.* To test this hypothesis further ^{3}H-gentamicin accumulation was measured in spheroplasts made from wild-type and adaptively resistant cells (Figure 21). Gentamicin accumulation in spheroplasts made from wild-type, control cells was significantly higher ($P<0.001$) than in whole wild-type, control cells (Figure 21). However, gentamicin accumulation in spheroplasts made from adaptively resistant *P. aeruginosa* was very similar ($P>0.05$) to whole adaptively resistant cells suggesting that cytoplasmic membrane changes may be responsible for aminoglycoside adaptive resistance. Recall that a rapid escalation in

cytoplasmic aminoglycoside accumulation is coincident with the lethal event in aminoglycoside action (Hancock, 1981a).

To help localize the structures responsible for reduced intracellular aminoglycoside accumulation during adaptive resistance, cell envelope component profiles were examined in an attempt to identify possible changes. Significant LPS or OMP changes could not be identified when unexposed control, adaptively resistant, and post-adaptively resistant cultures were compared, suggesting that adaptive resistance does not specifically involve these cell envelope components (Figures 28, 29, 30, 31). The absence of OMP changes was anticipated, as aminoglycoside accumulation in *P. aeruginosa* is generally not porin mediated (Hancock and Bell, 1988). Altered LPS structure has been previously described in some clinical isolates of *P. aeruginosa* demonstrating low level, aminoglycoside impermeability resistance (Bryan *et al.*, 1984). The LPS alterations however were chromosomally specified, non-transferable, stable in vitro, and were maintained independent of aminoglycoside exposure. These results were not unexpected, as in vivo experiments have demonstrated that wild-type, control and adaptively resistant *P. aeruginosa* are killed equally well by a healthy immune system (Bayer *et al.*, 1987). This suggests that no changes occur in surface characterisitics (i.e. LPS and OMPs) that would make adaptively resistant *P. aeruginosa* less immunogenic than wild-type, control organisms (Bayer *et al.*, 1987).

Cytoplasmic membrane protein profiles did however demonstrate 6 discernable band changes when adaptively resistant *P. aeruginosa* cultures were compared with unexposed control and post-adaptively resistant cultures (Figure 32). Adaptively resistant cells demonstrated an increase in intensity of three bands, of 35, 41 and 60 kDa respectively, new bands at 28 and 45 kDa, and a decrease in intensity of a 36 kDa band

when compared to control and post-adaptively resistant cells (Figure 32). The nature of these cytoplasmic membrane band changes then became the focus of study.

A line of investigation that proved fruitful in understanding, at least partially, the nature of these cytoplasmic membrane band changes involved following up on the previous observation that aminoglycoside accumulation is significantly reduced in anaerobically grown *P. aeruginosa* (Bryan and Kwan, 1983). These experiments were repeated and showed that anaerobically grown *P. aeruginosa* accumulated reduced amounts of gentamicin, relative to aerobically grown controls, despite both aerobic and anaerobic cultures maintaining similar pmfs (Figure 20, Table 21). Interestingly, the reduction in accumulation demonstrated in anaerobically maintained cells was not as great as that demonstrated in adaptively resistant cells (Figure 20) (discussed later).

Cytoplasmic membrane profiles were run with anaerobically grown *P. aeruginosa* and 5 discernable band changes were noted. There was an increase in intensity of a 60 kDa band, new bands at 45, 53 and >100 kDa, as well as a decrease in intensity of a 36 kDa band when compared to aerobically maintained, wild-type control cells. Three cytoplasmic membrane protein band changes appeared common to anaerobically grown and aminoglycoside adaptively resistant cells. The 3 similar band changes were an increase in intensity of a 60 kDa band, a new band at 45 kDa, and a decrease in intensity of a 36 kDa band. It was hypothesized that the increased 60 kDa band may represent increased amounts of the 60 kDa subunit of nitrite reductase and perhaps also the 60 kDa subunits of nitrate reductase (Zannoni, 1989). However, the actual amount, if any, of nitrite reductase (a periplasmic, cytoplasmic membrane associated, protein) extracted with the cytoplasmic membrane fraction was unknown. The new 45 kDa band may be the 47 kDa nitrous oxide reductase (Zannoni, 1989). Interestingly, adaptively resistant cells did

not display a new >100 kDa band, as anaerobically grown cells did. This band may have been the 150 kDa α subunit of nitrate reductase and suggests, perhaps, that adaptively resistant cells may not elevate nitrate reductase expression. The above suppositions, considered in combination with earlier work, performed by others (Hancock, 1981a; Hancock, 1981b; Taber *et al.*, 1987), demonstrating that intracellular aminoglycoside accumulation requires a functioning aerobic electron transport chain, lead to a further hypothesis. That being, that *P. aeruginosa* adaptive resistant to aminoglycoside antimicrobial agents may arise, at least in part, from increased cellular reliance upon the anaerobic respiratory pathway, in which nitrite reductase serves as the facilitator of terminal electron donation and acceptance.

4. Molecular Characterization of *Pseudomonas aeruginosa* Adaptive Resistance to Aminoglycoside Antimicrobial Agents

A Northern blot of total RNA from wild-type control, adaptively resistant and post-adaptively resistant *P. aeruginosa* cells clearly demonstrated that more *denA* (nitrite reductase) mRNA was present in adaptively resistant cells (Figure 34). The increased mRNA levels were likely due to a cellular regulatory event, and not the result of a mutational event, as DNA sequence changes could not be identified in the *denA* promoter of adaptively resistant cells, when compared with wild-type, control and post-adaptively resistant cells (Figure 35). Nitrite reductase mRNA levels have recently been shown to be controlled by a positive function regulatory protein, ANR (Ye *et al.*, 1995). Adaptively resistant cells demonstrated significant increases in *anr* mRNA levels when compared with wild-type control and post-adaptively resistant cells suggesting that it may be involved in *denA* transcription regulation (Figure 36). DNA sequence changes were not

identified within the *anr* promoter region suggesting cellular regulation of its mRNA levels (Figure 37). The factors regulating *anr* mRNA levels are presently unknown.

To test further the role of ANR in the development of aminoglycoside adaptive resistance in *P. aeruginosa*, an *anr* deficient mutant (*P. aeruginosa* PAO6261) of *P. aeruginosa* PAO1 was procured and tested for adaptive resistance development (Figures 38, 39, 40). *P. aeruginosa* PAO6261 showed significantly increased aminoglycoside killing during the adaptive resistance interval when compared with its parent (PAO1) and its complemented (pME3580) form (PAO6261*anr*$^{+}$) (Figures 38, 39, 40). The increased aminoglycoside killing demonstrated by *P. aeruginosa* PAO6261 correlated with increased intracellular gentamicin accumulation (Figure 42) but not with changes in pmf. *P. aeruginosa* sequentially passed with doubling gentamicin concentrations, to a final concentration of 64 mg/l, showed higher *denA* and *anr* transcription when compared with the same strain adaptively resistant to gentamicin at 1X MIC (3.6 mg/l) (Figures 44 and 45). These observations suggest that *anr* and *denA* mRNA levels may be aminoglycoside concentration dependent.

5. The Mechanism of *Pseudomonas aeruginosa* Adaptive Resistance to Aminoglycoside Antimicrobial Agents

Adaptive resistance is defined as reduced antimicrobial killing in bacterial populations pre-exposed to that agent (Daikos *et al.*, 1990b). The reproducible pattern by which initially susceptible *P. aeruginosa* acquire, or appear to acquire, transient resistance to aminoglycosides, which is lost with growth in antimicrobial free broth (i.e. adaptive resistance) has been demonstrated by several investigators (Daikos *et al.*, 1990b; Gould *et al.*, 1991; Barclay *et al.*, 1992). However, the mechanism of, and the cellular

structure(s) responsible for, this transient impermeability have remained enigmatic.

Experiments performed in this thesis served to confirm, and expand upon the work of other investigators in this area. The data collected lend themselves to the development of a putative, although admittedly incomplete, mechanism explaining *P. aeruginosa* adaptive resistance, to aminoglycoside antimicrobial agents. It appears that the principle reason underlying *P. aeruginosa* adaptive resistance to aminoglycoside antimicrobial agents is significantly reduced cytoplasmic aminoglycoside accumulation in adaptively resistant cells (Perlin and Lerner, 1986; Daikos *et al.*, 1990; confirmed in this thesis). The reduction in cytoplasmic aminoglycoside accumulation in adaptively resistant *P. aeruginosa* suggests that the impediment to cytoplasmic accumulation probably lies either external to, or within the cytoplasmic membrane as bacterial cell death arises coincident with cytoplasmic aminoglycoside appearance and interaction with 30S ribosomal targets (Gilbert, 1995).

Experiments performed in this thesis suggest that cytoplasmic membrane changes may be at least partially responsible for aminoglycoside adaptive resistance in *P. aeruginosa.* One of the cytoplasmic membrane changes was putatively identified as nitrite reductase when quantitative changes in transcription were observed during aminoglycoside adaptive resistance. Nitrite reductase is a periplasmic, cytoplasmic membrane associated protein that serves as the terminal electron donor molecule in the anaerobic respiratory pathway. The term anaerobic respiratory (in anaerobic respiratory pathway) is somewhat misleading, as nitrite reductase has been shown to be constitutively expressed at low levels in aerobically growing *P. aeruginosa* (Silvestrini *et al.*, 1989). High level nitrite reductase expression has been demonstrated in aerobically grown *P. aeruginosa* in the presence of 50 mM KNO_3 (Silvestrini *et al.*, 1989). Increased

transcription of *denA* implies increased cellular nitrite reductase levels, although this was not confirmed. It would be very interesting, with future experiments, to determine if there is dimeric to monomeric nitrite reductase conversion during aminoglycoside adaptive resistance in *P. aeruginosa.* Increased transcription of *denA* also implies reduced aerobic respiratory pathway (where cytochrome c oxidase is the terminal electron donor molecule) function, although this was not confirmed.

Support for the supposition that increased *denA* transcription, and therefore increased anaerobic respiratory pathway use, may at least partially explain *P. aeruginosa* adaptive resistance to aminoglycoside antimicrobial agents can be found in the scientific literature. Aminoglycoside accumulation has previously been demonstrated to be reduced in a variety of aerobic respiratory pathway mutants such as respiratory quinone deficiency mutants (Bryan and van den Elzen, 1976; Acar *et al.*, 1978; Miller *et al.*, 1980) and cytochrome c oxidase deficiency mutants (Bryan *et al.*, 1980; Bryan and Kwan, 1981). One millimolar cyanide treatment of *P. aeruginosa*, although having very limited effect on cellular pmf, for at least 30 minutes, significantly reduced aminoglycoside accumulation during this time (Bryan *et al.*, 1979). Results presented in this thesis have suggested that aminoglycoside adaptive resistance, at least at 1X MIC, was pmf independent as well.

Cyanide is known to bind certain cytochrome redox centres, including cytochrome c oxidase, with high affinity and block their function (Palleroni, 1989). Cyanide production in *P. aeruginosa* has previously been demonstrated to also be subject to *anr* regulation (Ye *et al.*, 1995). Increased *anr* transcription during aminoglycoside adaptive resistance suggests that a coincident increase in cellular cyanide production occurs. This supposition, in turn, suggests reduced cytochrome oxidase activity, and a more significant

role for the anaerobic respiratory pathway (nitrite reductase) in the energetics of the cell. Cytochrome c oxidase is completely inhibited by a cyanide concentration of 1 mM while nitrite reductase is not (Palleroni, 1989). This suggests that cytochrome c oxidase may play an important, although unknown, pmf independent role in aminoglycoside accumulation, as the respiratory pathway molecule or molecules involved in aminoglycoside accumulation are likely unique to the aerobic respiratory pathway. Non-functional, or suboptimally functioning cytochrome c oxidase implies aminoglycoside resistance.

Further, the observation that stationary phase cultures demonstrate significantly reduced aminoglycoside accumulation may also apply to this phenomenon (Gilbert, 1995). Stationary phase cultures produce KCN (ANR regulated) to a concentration of aprroximately 200 to 300 μM (Cunningham and Williams, 1995), suggesting decreased aerobic respiratory pathway function and increased anaerobic respiratory pathway function. These observations suggest that *P. aeruginosa* may regulate the expression of, and or, use of its two principle respiratory pathways in response to aminoglycoside insult. However, the observation, described earlier, that anaerobically maintained *P. aeruginosa* demonstrate significantly more ($P<0.05$) aminoglycoside accumulation than cultures adaptively resistance to gentamicin, at 1X MIC, suggests that additional factors, separate from respiratory pathway regulation, may be involved in aminoglycoside adaptive resistance.

It also appears that nitrite reductase transcription during aminoglycoside adaptive resistance is being positively regulated by ANR, as has been previously described by other investigators (Sawers, 1991; Zimmermann *et al.*, 1991; Ye *et al.*, 1995). ANR has been shown to be involved in anaerobic arginine catabolism, cyanogenesis, as well as

shown to be involved in nitrate respiration. An interesting observation is that adaptively resistant *P. aeruginosa* do not demonstrate an increase in the >100 kDa band (putative 150 kDa α subunit of nitrate reductase) seen in anaerobically grown *P. aeruginosa.* This may suggest that nitrite reductase transcription may be regulated by a cellular factor in addition to ANR or that nitrate reductase production may also be regulated by multiple factors. The regulation of ANR transcription itself remains unknown, as does the method by which cells surviving aminoglycoside exposure transmit the signal(s) for increased *anr* and *denA* transcription.

Consideration of the unstable, yet reproducible nature of adaptive resistance suggests that it does not arise as the result of a mutational event but rather may be a function of subpopulation selection, a regulatory event, or a combination of both. Data supporting this supposition have been previously discussed. In addition, cross resistance to other aminoglycosides, which is characteristic of adaptive resistance, contrasts enzymatic modification and ribosomal mutation which tend to affect specific aminoglycosides (Daikos *et al.,* 1990). Adaptive resistance persists with continuous aminoglycoside exposure and is lost with subculture in aminoglycoside free media (Daikos *et al.,* 1990). Previous work has shown that the degree of adaptive resistance is greater, and the duration longer, with higher initial aminoglycoside exposures (Barclay *et al.,* 1992). Stepwise increases in aminoglycoside concentration allow *P. aeruginosa* to grow in antibiotic concentrations (128 mg/l) that would normally eradicate the parental strain (Gilleland *et al.,* 1989). This observation suggests some form of reversible cellular induction, as these high MIC variants revert to parental susceptibility when grown in aminoglycoside free media (Gilleland *et al.,* 1989; Karlowsky *et al.,* 1994b).

In conclusion, *P. aeruginosa* adaptive resistance to aminoglycoside antimicrobial

agents is a multifactorial process. It is the opinion of the author that several of these factors remain to be addressed. The data collected in this thesis would suggest that, the reduced cytoplasmic accumulation of aminoglycoside, characteristic of *P. aeruginosa* adaptive resistance to aminoglycosides, may be, at least partially, attributable to ANR regulated increases in nitrite reductase transcription.

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