4-QUINOLONE ANTIBACTERIALS AND TEMPERATURE

Thesis submitted by

Aidan Charles Parte

for the degree of

Doctor of Philosophy

in the

University of London

Microbiology Section

Department of Pharmaceutics

The School of Pharmacy

University of London   March 1992

ProQuest Number: 10629216

ProQuest 10629216

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT:

The effects of temperature on the minimum inhibitory concentrations (MICs) of ciprofloxacin, ofloxacin or DR-3355 and on the ability of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* to mutate to resist 5 times the MIC of these 4-quinolones were studied. Reducing the temperature of incubation from 37 to 30°C and then to 25°C only slightly affected MIC values. With *Staph. epidermidis* temperature reduction always reduced its mutation frequency to resist 4-quinolones. The effect of temperature on mutation frequency with the other three species was more variable and in some instances temperature reduction even elevated their mutation rates.

The effect of temperature on the bactericidal activity of ciprofloxacin or DR-3355 against the four bacterial species was also investigated. In contrast to the MIC results it was found that bacterial kill was greatly lessened by temperature reduction. When the values of the apparent activation energies for bacterial death were calculated they were found to be consistent with inhibition of enzymes being the rate-limiting step in the killing of bacteria by these 4-quinolones.

The effect of temperature on the multiplication of the four bacterial species in drug-free media was also studied. It

was found that *Staph. epidermidis* multiplied more rapidly at 30°C than at 25 or at 37°C, and it did not divide at all at 20°C. However, the other three species divided more rapidly as the temperature of incubation was increased.

In agreement with clinical findings it seems that 4-quinolone therapy of infectious sites which are at temperatures less than 37°C may be more prone to failure due to increased mutational resistance or to reduced bactericidal activity or to a combination of both these factors. In addition fomites which are at room temperature and contaminated with bacteria and 4-quinolone residues could act as sources of resistant mutants, particularly in hospitals.

Acknowledgements:

Table of Contents:

Section 1: Introduction:

<u>Table of Contents (continued):</u>   Page

<u>**Table of Contents (continued)**</u>:   Page

Table of Contents (continued): Page

9

Index to Figures (continued):   Page

Index to Figures (continued):   Page

<u>**Index to Figures (continued):**</u>   Page

<u>Index to Figures (continued)</u>:   Page

<u>Abbreviations and symbols:</u>

=	equal to
>	greater than
<	less than
≈	approximately
[y]	concentration of y
A	pre-exponential factor in the Arrhenius equation (4)
Å	Angstrom unit (10^{-8} cm)
α	linking number of a circular DNA molecule
ADP	adenosine-5'-diphosphate
ADPNP	5'-adenylyl ß,γ-imidodiphosphate
ATP	adenosine-5'-triphosphate
bp	base pairs
cal	calories
CIP	ciprofloxacin
CCCP	carbonyl cyanide *m*-chlorophenyl-hydrazone
cfu	colony-forming units
cm	centimetre
conc.	concentration
Da	molecular weight in Daltons
DNA	deoxyribonucleic acid
2,4-DNP	2,4-dinitrophenol
° or °C	degrees centigrade
e	natural base
E_a	apparent activation energy (Calories)
EDTA	ethylene diaminetetraacetic acid
ESS	extracellular slime substance

<u>Abbreviations and symbols (continued)</u>:

G	acceleration due to gravity (9.81 metres second^{-2})
g	gramme
h	hours
IC$_{50}$	concentration required to inhibit supercoiling by 50% in the DNA gyrase supercoiling assay
J	joules
K	degrees Kelvin
k	kilo
l	litre
ln	logarithm to the base e
log$_{10}$	logarithm to the base 10
LPS	lipopolysaccharide
M	molarity (mol l^{-1})
m-AMSA	4'-(acridinylamino)-methanesulphon-*m*-anisidide
µg	microgramme
µm	micrometre
mg	milligramme
ml	millilitre
MIC	minimum inhibitory concentration (µgml^{-1})
min	minutes
mol	moles
MRSA	methicillin-resistant *Staphylococcus aureus*
µ	specific growth rate (h^{-1})
µ$_q$	specific death rate (h^{-1})
N	size of DNA molecule in bp (see equation 3)
N/A	not applicable

Abbreviations and symbols (continued):

N/B	nutrient broth
OFL	ofloxacin
OBC	optimum bactericidal concentration (μgml^{-1})
OBC^{37}	optimum bactericidal concentration at 37°C
omp	outer membrane protein or porin
oz	ounce
P	probability
p	plasmid
%	percent
% w/v	% weight per unit volume (g per 100ml)
PBS	phosphate-buffered saline
pH	$-\log_{10}$ [H^{+}]
R	ideal gas constant (1.9862 calories mol^{-1} K^{-1})
r	correlation coefficient
REP	repetitive extragenic palindromic (DNA sequences)
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecylsulphate
T	Absolute temperature in Kelvins
temp.	temperature
t_d	viable count doubling time in minutes (equation 6)
t_h	viable count halving time in minutes (equation 6)
	helical pitch of DNA
U.V.	ultraviolet
v	velocity of reaction (equation 4)
vs	versus
x	times or multiplied by

SECTION 1:

Introduction:

Topological constraints of the bacterial chromosome:

Bacteria contain two to four copies (depending on their growth rate and stage in the cell division cycle) of a chromosome comprised of double-stranded closed circular DNA, which when relaxed is approximately 1000-times the length of the *Escherichia coli* cell (Krawiec and Riley, 1990). In order for the chromosomes to be accomodated inside the bacterial cell they are condensed by two processes. The first is that 60-70 regions, termed domains, are attached to an RNA core (Worcel, 1974). As each of these domains is about 20µm long a second process known as negative supercoiling occurs whereby the closed circular DNA molecule is coiled in the opposite direction to the Watson-Crick helical structure of the DNA, causing the molecule to compact itself into a smaller volume.

Principles of supercoiling:

When a linear or nicked circular DNA molecule becomes cyclised the two strands become covalently linked. This linkage can be characterised by the linking number, α, which is always an integer. The linking number is the number of times one strand of the DNA passes through the plane of the other strand (Lilley, 1986). In the example described α is the number of duplex turns present in the linear or nicked DNA molecule. By convention α has a positive sign for

right-handed duplex molecules. The DNA molecule is said to be relaxed if there is no torsional strain on the molecule and the linking number of the relaxed DNA is α_0. If duplex turns are removed or added, the DNA molecule becomes under- or over-wound. The linking difference, $\delta\alpha$, between the under- or over-wound DNA molecule and the relaxed DNA molecule is defined as:

$$\delta\alpha = \alpha - \alpha_0 \qquad (1)$$

A negative value for $\delta\alpha$ defines a negatively supercoiled DNA, whilst a positively supercoiled DNA molecule will have a positive value of $\delta\alpha$. The helical periodicity of DNA and hence the value of α_0 is dependent upon environmental conditions such as temperature, pH, ionic concentrations and DNA binding agents (Wang, 1969a, 1969b; Depew and Wang, 1975; Pulleybank et al, 1975). Thus a value of $\delta\alpha$ only applies to a certain DNA molecule under specific conditions. In order to compare the topological properties of different DNA molecules the specific linking difference or the superhelical density, σ, was defined as:

$$\sigma = \delta\alpha / \alpha_0 \qquad (2)$$

All naturally occurring closed circular DNA molecules and chromosomal DNA are negatively supercoiled (Vosberg, 1985), with the exception of the archæobacteria (Kikuchi and Asai, 1984; Bouthier de La Tour et al, 1990). Positive

supercoiling of DNA can be brought about *in vitro* by binding

of dyes (LePecq and Paoletti, 1967; Bauer and Vinograd,

1968; Wang, 1971) and *in vivo* positive supercoiling may

occur transiently through processes such as DNA replication

or transcription (Liu and Wang, 1987; Wu *et al*, 1988).

Supercoiled DNA molecules have higher mobility than relaxed

DNA when electrophoresed in polyacrylimide gel because

supercoiled DNA is more compact than relaxed DNA.

Supercoiled DNA molecules possess a reduced ability to

intercalate dyes such as ethidium bromide, which allows

their superhelical density to be titrated (Keller, 1975).

In vivo supercoiled DNA exists as a population of DNA

molecules with an average superhelical density distributed

about the mean in a Gaussian manner, giving the appearance

of a ladder when electrophoresed and stained (Keller, 1975).

In vivo the bacterial chromosome has on average one negative

supercoil for every 40 turns of the double helix i.e. σ = −

0.025. *In vitro* the DNA is approximately twice as

supercoiled with one negative supercoil in every 20 turns of

the double-helix i.e. σ = −0.05. The reason for the

difference between *in vivo* and *in vitro* levels of

supercoiling is thought to be due to the interaction of the

DNA with histone-like proteins *in vivo*, such as the small

basic protein HU (Drlica and Rouviere-Yaniv, 1987).

Supercoiling of a relaxed closed circular DNA molecule is

associated with an increase in free energy since supercoils

do not occur spontaneously and require energy for their introduction. Pulleybank *et al* (1975) and Depew and Wang (1975) showed that the free energy change (δG) caused by the introduction of supercoils was directly proportional to $\delta\alpha^2$, by the relationship:

$$\delta G = (K \times RT / N) \times \delta\alpha^2 \qquad (3)$$

where R is the ideal gas constant, T is the absolute temperature, N the size of the DNA molecule in base pairs and K is a constant equal to 1050 if N > 2000 base pairs. All processes which lead to relaxation of torsional strain associated with supercoiling are therefore energetically favoured. The decrease in $\delta\alpha$ by 1 in a DNA molecule with σ of -0.05 is accompanied by a free energy change of approximately -6 kcal or -25 kJ (Vosberg, 1985). The potential energy stored in the DNA molecule is available to drive structural changes in the DNA which can be used to facilitate control and maintenance of processes such as DNA transcription, gene expression and DNA replication (Vosberg, 1985).

The torsional stress of a closed circular supercoiled DNA molecule can be relieved (i) by local denaturation of the duplex; (ii) by reducing the helical pitch, for example, by intercalating dyes such as ethidium bromide; (iii) by nicking one or both strands of the duplex; or (iv) by the action of enzymes known as topoisomerases. (i) and (ii) do

not alter linking numbers but (iii) and (iv) do change
linking numbers.

Topoisomerases are so-called because the substrate and
product of the reaction are topological isomers of one
another, identical in all respects except for the physical
state of the DNA. Changes in linking number occur by two
quite separate routes. The first includes linking number
changes in steps of one or multiples of one. Changes in
steps of one are brought about by single-stranded nicks and
alterations of the number of strand crossovers either by
rotation of the DNA about its axis or translocation of one
strand relative to the other (Vosberg, 1985). Type I
topoisomerases catalyse one step linking number changes.
Two step changes of linking number require a transient
double-stranded break through which another segment of the
same DNA molecule passes. Each single passage decreases or
increases the linking number by two, depending on the
direction of passage (Fuller, 1978). Two step changes in
linking number are catalysed by type II topoisomerases.
Type I and type II topoisomerases are found in prokaryotic
organisms. Eukaryotic organisms also possess type I and II
topoisomerases although whether the latter can cause
negative supercoiling is doubtful.

Type I topoisomerases:
Prokaryotic type I topoisomerases selectively relax or
remove negative supercoils from supercoiled DNA in the

absence of ATP. The prototypical enzyme from this class is the *E. coli* omega protein (Wang, 1971) now referred to as *E. coli* topoisomerase I (EC 5.99.1.2). In *E. coli* topoisomerase I is a single subunit protein of molecular weight 105,000 Da coded by the *topA* gene mapping at 28 minutes on the *E. coli* chromosome (Bachmann, 1990). *E. coli* topoisomerase I has four different topoisomerase activities: (i) it relaxes negatively supercoiled DNA (Wang, 1971); it links covalently closed single-stranded DNA rings containing complementary base sequences (Kirkegaard and Wang, 1978); (iii) it forms topological knots in single-stranded DNA rings (Liu *et al*, 1976); (iv) it catenates and decatenates double-stranded circular DNA molecules, providing that one of the DNA molecules has a nick (Tse and Wang, 1980; Low *et al*, 1984). None of these catalytic activities require the hydrolysis of ATP. Another *E. coli* type I topoisomerase quite distinct from the omega protein was discovered by Srivenugopal *et al* (1984) which they called topoisomerase III. It is a 72-76 kDa protein which is unusual in its requirement of K^+ ions in addition to the usual Mg^{2+}. Type I topoisomerases are thought to relax negative supercoils by making a nick in one strand of the DNA so allowing its ends to swivel around the other unbroken strand. The nicked 5' end of the DNA is thought to be covalently bound to the enzyme by a phosphotyrosine link (Tse *et al*, 1980). This was shown by formation of a covalent complex between ^{32}P-labelled single-stranded DNA and topoisomerase I followed by digestion of the DNA with staphylococcal nuclease. The

radiolabel from the DNA was transferred to the enzyme and the amino acid to which the label was bound was found to be tyrosine by paper electrophoresis and by thin layer chromatography (Tse *et al*, 1980). They noted that the covalent complexes that were found may not be the true reaction intermediates since attempts to reform phosphodiester bonds from these intermediates were unsuccessful. The broken strand is then resealed releasing energy from the temporary covalent bond and so there is no net energy requirement. Other prokaryotic type I topoisomerases have been isolated from *Micrococcus luteus* (Kung and Wang, 1977), *Salmonella typhimurium* (Wang and Liu, 1979), *Agrobacterium tumefaciens* (LeBon *et al*, 1978) and *Bacillus megaterium* (Burrington and Morgan, 1978). These enzymes have similar properties to those of the *E. coli* enzyme.

Eukaryotic topoisomerase I enzymes have been isolated from yeast, sea urchin, *Drosophila*, avian erythrocytes, salmon testis, calf thymus, rat liver and *H. sapiens* (Gellert, 1981). Eukaryotic type I topoisomerases are found in chromatin and though they share many properties with prokaryotic type I topoisomerases they differ in some respects (Vosberg, 1985). The two groups of enzymes share the following properties: (i) they both relax and catenate circular DNA; (ii) they both act by making transient single-stranded nicks and covalently bound enzyme-DNA intermediates; (iii) their relaxation activities are

strongly inhibited by single-stranded DNA; and (iv) they do not require ATP. The two groups of enzymes differ in the following respects (Vosberg, 1985): (i) prokaryotic type I topoisomerases attach to the 5′ end of the nick, whereas eukaryotic type I topoisomerases are bound to the 3′ end of the nick; (ii) prokaryotic topoisomerase I does not relax positive supercoils; and (iii) prokaryotic topoisomerase I does not fully relax DNA whereas the eukaryotic enzymes do relax DNA to completion; and (iv) single-stranded chain transfer has only been found with eukaryotic type I topoisomerases.

Reverse gyrase:

An unusual type I topoisomerase with a positive supercoiling activity at temperatures greater than 55°C and which required both ATP and Mg^{2+} was found in the acidothermophilic archæobacterium *Sulfolobus acidocaldarius* (Kikuchi and Asai, 1984). The enzyme, which they called reverse gyrase, was originally classified as a type II enzyme (Kikuchi and Asai, 1984) but is now thought to be a type I topoisomerase (Nadal *et al*, 1988). A survey by Bouthier de La Tour *et al* (1990) found reverse gyrase in all hyperthermophilic bacteria that they studied but not in mesophiles or in moderate thermophiles. The physiological rôle of reverse gyrase is not clear but it may be that the enzyme prevents the DNA from denaturation at the high growth temperatures by tightening the helical pitch of the DNA (Kikuchi and Asai, 1984). An alternative hypothesis is that

positive supercoiling of the DNA may stabilise the DNA in nucleoprotein complexes (Bouthier de La Tour *et al*, 1990).

Type II topoisomerases:

Type II topoisomerases make transient double-stranded nicks in DNA and they require ATP hydrolysis. The prokaryotic type II topoisomerases are known as DNA gyrases and the most studied of these is *E. coli* DNA gyrase (DNA topoisomerase [ATP-hydrolysing], EC 5.99.1.3) (Gellert *et al*, 1976a). Prokaryotic type II topoisomerases have been isolated from *Micrococcus luteus* (Liu and Wang, 1978), *Bacillus subtilis* (Sugino and Bott, 1980), *Ps. aeruginosa* (Miller and Scurlock, 1983), *Staph. aureus* (Takahata and Nishino, 1988), *Citrobacter freundii* (Aoyama *et al*, 1988a). *Micrococcus luteus* gyrase is very similar to the *E. coli* enzyme in its activites, overall structure and has a similar amino acid sequence. DNA gyrase from *Staphylococcus aureus* also has a closely related amino acid sequence to those of *E. coli* and *B. subtilis* DNA gyrases (Hopewell *et al*, 1990). However, the genes for the A and B subunits of *Staph. aureus* gyrase are contiguous rather than being separated on the chromosome as is the case with *E. coli* (see below). Interestingly, the *gyr*B and *gyr*A genes are also contiguous in the halophilic archæobacterium *Haloferax* (Holmes and Dyall-Smith, 1991). An *E. coli* topoisomerase related to gyrase but without negative supercoiling activity was found by Brown *et al* (1979) which they designated topoisomerase II'. It has two subunits, one of which is identical to the DNA gyrase A

subunit, the other subunit being similar to the gyrase B subunit. It is likely that topoisomerase II′ is a proteolytic artefact formed during purification and so this enzyme has not received much further study.

Eukaryotic type II topoisomerases have been isolated from the Drosophila, Xenopus and *Homo sapiens* where it is associated with the chromatin (Berrios *et al*, 1985). Eukaryotic topoisomerase II is thought to consist of two identical subunits of molecular weight 166-175 kDa. Its activities include relaxation of both negatively and positively supercoiled DNA, catenation, decatenation, and knotting and unknotting of circular DNA (Vosberg, 1985). There is no evidence that these enzymes can introduce negative supercoils into DNA (Gellert, 1981). One possible reason why no requirement for a negative supercoiling activity has been found in eukaryotic cells is that in eukaryotic systems DNA is condensed in chromatin by wrapping of the DNA around octameric histone protein complexes to form a left-handed superhelix (Finch *et al*, 1977).

DNA gyrase:

E. coli DNA gyrase consists of four subunits, two identical A subunits of molecular weight 105 kDa and two identical B subunits of molecular weight 95 kDa (Sugino *et al*, 1977; Higgins *et al*, 1978). These subunits are coded for by the *gyr*A gene (at 48 minutes) and the *gyr*B gene (at 83 minutes), respectively, on the chromosome map (Bachmann, 1990).

Proteolytic cleavage of the A subunits of *E. coli* DNA gyrase generates two fragments of 64 and 33 kDa (Reece and Maxwell, 1989). The N-terminal 64 kDa fragment retains the supercoiling and cleaving activity of the A subunits (see below) provided it is combined with intact B subunits (Reece and Maxwell, 1989; 1991). The smaller C-terminal fragment exhibited no enzymatic activity even when combined with intact B subunits. Proteolytic cleavage of the B subunit of *E. coli* DNA gyrase generates two fragments of 43 and 46 kDa, the smaller of which has the ATPase activity and binds coumarin antibacterials (Maxwell, 1990). The 46 kDa domain interacts with the gyrase A subunit and DNA (Maxwell, 1990). The exact number of copies of gyrase per *E. coli* cell is not known but it has been estimated that there are 500 molecules of the A subunit and approximately 50 molecules of the B subunit (Higgins *et al*, 1978).

Negative supercoiling of DNA by DNA gyrase:

The main reaction of DNA gyrase is negative supercoiling, an activity which requires both ATP and Mg^{2+} (Gellert *et al*, 1976a). The coumarin antibacterials coumermycin A_1 and novobiocin inhibit the topoisomerase activities of DNA gyrase by competitive binding to the B subunit ATP binding site (Gellert *et al*, 1976b; Sugino *et al*, 1978). The 4-quinolone antibacterials are thought to inhibit DNA gyrase by interfering with the breakage-reunion activity of gyrase, to form a 4-quinolone-DNA gyrase-DNA complex (see below).

DNA gyrase binds to specific sites on DNA *via* the A subunit
in the absence of ATP to form stable complexes (Fisher *et
al*, 1981; Moore *et al*, 1983; Staudenbauer and Orr, 1981).
DNA gyrase binds to relaxed and linear DNA with a much
higher affinity than to negatively supercoiled DNA (Morrison
et al, 1980). Thus DNA gyrase binds selectively to its
substrate. *E. coli* DNA gyrase binds with up to 10-fold
higher affinity to short sequences of DNA known as
repetitive extragenic palindromic (REP) sequences than it
does to other non-REP DNA sequences (Yang and Ferro-Luzzi
Ames, 1988). REP sequences are highly conserved about a
consensus sequence which exhibits homology to the gyrase
binding sites on plasmid BR322 (pBR322). REP sequences
occupy approximately 0.5% of chromosomal DNA and they are
distributed throughout the chromosome except in structural
genes. Apart from binding of DNA gyrase the function of REP
sequences remain obscure (Yang and Ferro-Luzzi Ames, 1988).

DNA gyrase induces double-stranded cleavage of DNA when the
reaction mixture is incubated with the 4-quinolone oxolinic
acid followed by treatment with sodium dodecylsulphate (SDS)
(Gellert *et al*, 1977; Mizuuchi *et al*, 1980; Snyder and
Drlica, 1979; Fisher *et al*, 1981). Cleavage patterns for
certain sites can be altered by the presence of ATP without
affecting the amount of bound enzyme (Morrison *et al*, 1980;
Fisher *et al*, 1981). Binding of ATP to the B subunit of
gyrase may affect the conformation of the A subunit of
gyrase thereby causing shifting of cleavage site (Sugino *et*

al, 1978; Morrison *et al*, 1980; Rau *et al*, 1987). It is thought that cleavage occurs even in the absence of oxolinic acid albeit at a low frequency (Morrison and Cozzarelli, unpublished data quoted in Morrison *et al*, 1980). Thus, the drug simply enables the cleavage sites to be observed more easily by preventing resealing of the double-stranded breaks.

In studies utilising protection from digestion by Staphylococcal nuclease, exonuclease III and pancreatic DNase I it was shown that DNA gyrase wraps a 120-155 bp length of DNA around its tetrameric structure (Klevan and Wang, 1980; Kirkegaard and Wang, 1981; Morrison and Cozzarelli, 1981; Fisher *et al*, 1981). A region ≈40 bp long is most strongly protected from digestion and this sequence contains the oxolinic acid-promoted cleavage site (Fisher *et al*, 1981). The DNA is wrapped around gyrase in the positive direction (Liu and Wang, 1978) and the enzyme makes a staggered double-stranded break 4 base pairs apart (Kirkegaard and Wang, 1981; Fisher *et al*, 1981) whereby the 5' ends of the DNA strands are believed to be covalently bound to tyrosine residues at position 122 on each of the A subunits of DNA gyrase (Horowitz and Wang, 1987) leaving free the 3'-OH ends of the DNA. The active site tyrosine was mapped to position 122 on the A subunit by formation of the cleaved complex between DNA gyrase and DNA followed by sequencing of the peptide attached to the 5' end of the DNA (Horowitz and Wang, 1987). The formation of phosphotyrosine

links between DNA and protein appears to be common to all
the topoisomerase-DNA interactions that have been mapped
(Tse *et al*, 1980; Champoux, 1981; Rowe *et al*, 1984; Horowitz
and Wang, 1987). In the gyrase A proteins of both *Staph.
aureus* and *B. subtilis* there is a conserved 11 amino acid
sequence which is highly homologous to that surrounding the
catalytic tyrosine-122 of the *E. coli* gyrase A subunit
(Hopewell *et al*, 1990). That this sequence is highly
conserved in the DNA gyrase from *E. coli*, *Staph. aureus* and
B. subtilis implies that it is critical to the topoisomerase
activities of DNA gyrase.

Rau *et al* (1987) utilising transient electric dichroism
studied the structure of the DNA gyrase-DNA complex,
confirming the wrapping of the DNA around the enzyme. They
found that the ends (or "tails") of the wrapped DNA extended
from the complex close to each other at an angle of 120°.
In contrast to findings by Kirkegaard and Wang (1981) that
binding of ATP or its non-hydrolysable analogue ADPNP (5'-
adenylyl ß, ᵟ -imidodiphosphate) had no effect on the
conformation of the enzyme-DNA complex as shown by DNase
protection, Rau *et al* (1987) found that ADPNP *did* induce a
major conformational change in the complex involving folding
of the DNA tails to the enzyme. It was proposed that the
conformational change could bring the DNA segment to be
translocated (probably one of the tails) to the cleavage
site.

More recently, however, Wigley *et al* (1991) investigated the
crystal structure of the 43 kDa N-terminal fragment of the
DNA gyrase B protein complexed with ADPNP. As was described
earlier this 43 kDa fragment possesses the site of ATP
hydrolysis (Maxwell, 1990). It was found to contain a large
hole, approximately 20Å in diameter, running through the
middle. The hole is about the same diameter as a DNA helix
and was proposed to form a gateway through which the DNA may
pass during supercoiling. Wigley *et al* (1991) proposed that
following cleavage of the DNA by the A subunits, binding of
ATP stabilises the interface between the B subunits during
strand-passage, which would involve a major conformational
change being transmitted to the A subunits. Since non-
hydrolysable ADPNP is thought to allow a single supercoiling
cycle, the strand-passage event must occur before ATP
hydrolysis. Wigley *et al* (1991) proposed that the
requirement for hydrolysis of ATP is in the release of the
DNA from within the DNA gyrase-DNA complex, which may
account for the conformational change observed by Rau *et al*
(1987) on addition of ADPNP.

In summary DNA gyrase binds to specific sites on DNA in the
absence of ATP where the A subunits make double-stranded
breaks in which the 5′ ends of the DNA are covalently linked
via a phosphotyrosine bond. Binding of ATP to the B
subunits stabilises the enzyme-DNA complex whereby the
segment of DNA to be translocated is brought to the "gate"
in the DNA. When the DNA has been translocated the double-

stranded breaks in the DNA are resealed to form a circular
DNA molecule with a linking difference of two.

Other activities of prokaryotic type II topoisomerases:
Relaxation of DNA by DNA gyrase occurs approximately 20-40
times more slowly than its supercoiling activity and for
this reason it is thought that relaxation is only a minor
activity of DNA gyrase (Sugino *et al*, 1977; Gellert *et al*,
1977).

Topoisomerases and DNA replication:
DNA gyrase is essential for DNA replication. This was
demonstrated by the fact that gyrase inhibitors also inhibit
in vivo and *in vitro* DNA synthesis. Coumermycin A_1 which
inhibits ATP hydrolysis by the gyrase B subunit inhibits
nucleic acid synthesis *in vivo* (Ryan, 1976) and in
solubilised cells *in vitro* (Ryan and Wells, 1976). The 4-
quinolone nalidixic acid the target site of which is DNA
gyrase inhibits nucleic acid synthesis *in vivo* in both *E.
coli* (Goss *et al*, 1965; Crumplin and Smith, 1976) and *B.
subtilis* (Cook *et al*, 1966). Mutants which are temperature-
sensitive (*ts*) in the *gyr*A gene are unable to replicate
chromosomal DNA and certain bacteriophage DNAs at the non-
permissive temperature (Kreuzer and Cozzarelli, 1979).

DNA gyrase is involved with both the initiation and
elongation steps of DNA replication (Ogasawara *et al*, 1979;
Kreuzer and Cozzarelli, 1979). Plasmids containing the

cloned *E. coli* origin of replication, *oriC*, require DNA
gyrase for replication *in vitro* (Kaguni and Kornberg, 1984).
OriC is also a high-affinity binding site for DNA gyrase
(Lother *et al*, 1984). Negative supercoiling of the origin
is thought to promote efficient binding of replicative
proteins such as the *dna*A protein (Louarn *et al*, 1984).
That the elongation step of DNA replication requires DNA
gyrase was demonstrated by Kreuzer and Cozzarelli (1979)
using pulse-labelling with ^{3}H-thymidine. They found that
DNA synthesis in a *nal*A (now *gyr*A) ts mutant could be shut
off very rapidly by shifting the bacteria from the
permissive to the non-permissive temperature.

DNA gyrase is involved in segregation of daughter
chromosomes by its ability to decatenate circular DNA
molecules i.e. unjoin two or more interlocking rings
(Kreuzer and Cozzarelli, 1980). This activity is believed
to be essential for chromosome segregation following a round
of DNA replication, prior to cell division (Steck and
Drlica, 1984). Another recently discovered type II
topoisomerase from *E. coli*, known as topoisomerase IV, was
also found to be essential for chromosome segregation (Kato
et al, 1990). The new enzyme is coded for by the *parC* and
the *parE* genes which are homologous to the *gyr*A and *gyr*B
genes, respectively. No negative supercoiling activity has
been found for topoisomerase IV but it can relax negatively
supercoiled DNA (Kato *et al*, 1990). In this respect it is
similar to eukaryotic type II topoisomerase.

DNA gyrase may also have structural effects on the chromosome quite separate from its supercoiling activities. Wahle and Kornberg (1988) using protein binding and protection from DNase I and exonuclease III digestion studied partitioning and stability of the plasmid SC101. They found that DNA gyrase bound with up to 40-fold higher affinity to the *par* sequence of the plasmid than to non-specific sequences. The 100 bp *par* sequence is essential for stability of the plasmid but Wahle and Kornberg (1988) found that the function of gyrase was probably not due to its supercoiling or decatenation activities. They concluded that high-affinity binding of DNA gyrase to the *par* sequence could stabilise the tertiary DNA structure, and in this respect gyrase may have a similar rôle to eukaryotic topoisomerase II in the "protein scaffold" of chromatin (Berrios *et al*, 1985).

Topoisomerases, transcription and supercoiling:

As described earlier unwinding of DNA is thermodynamically favoured by negative supercoiling. Negative supercoiling of DNA has been shown to enhance transcription of genes from certain promoters *in vitro* (Tse-Dinh and Beran, 1988) and *in vivo* where inhibition of gyrase by 4-quinolone and coumarin antibacterials was shown to inhibit transcription (Smith *et al*, 1978). Very high levels of negative supercoiling which promote B-Z transitions in DNA also inhibit activation of transcription of genes on pBR322 (Brahms *et al*, 1985). Mutations in the *top*A gene coding for topoisomerase I also

affect transcription of genes. *In vivo* Sternglanz *et al* (1981) demonstrated that a *top* mutant could induce ß-galactosidase and tryptophanase more rapidly and to higher levels compared to its isogenic wild-type strain.

In vivo the average level of DNA supercoiling of the *E. coli* chromosome is thought to be determined by the balance between topoisomerase I and DNA gyrase activities (Pruss *et al*, 1982; Menzel and Gellert, 1983; Wang, 1991). Total deletion mutants in the *top*A gene have been found to be compensated for by secondary mutations in the *gyr*A and *gyr*B genes which code for partially defective proteins, with the result that the cell can survive by achieving an acceptable superhelical density (DiNardo *et al*, 1982; Pruss *et al*, 1982). McEachern and Fisher (1989) showed that one such compensatory mutation was due to an additional alanine-arginine tandem repeat in a region of the B subunit of DNA gyrase where there are normally two alanine-arginine tandem repeats. The mutation could reduce the activity of DNA gyrase by interefering with coupling between the A and B subunits (McEachern and Fisher, 1989). Parenthetically, the recombination event resulting in the additional tandem repeat could have been mediated by DNA gyrase itself (McEachern and Fisher, 1989). Recently, *top*A deletion mutants have been isolated which are dependent on low levels of novobiocin for growth (Hammond *et al*, 1991). In the absence of novobiocin the *top*A deletion mutants, which were previously thought to be non-viable, grow slowly.

Hammond *et al* (1991) proposed that relaxation of DNA by inhibition of gyrase by novobiocin relieves the effect of the *top*A deletion.

In normal wild-type cells Menzel and Gellert (1983) proposed an homeostatic mechanism for the regulation of supercoiling by control of DNA gyrase genes by DNA conformation, based on the following evidence. They found that the rates of synthesis of both the A and B subunits of DNA gyrase *in vivo* could be increased up to 10-fold by treatments that block DNA gyrase activity and so relax DNA. Also, in a cell-free transcription system it was found that relaxed DNA was a good template for DNA gyrase synthesis and that supercoiled DNA was a very poor template (Menzel and Gellert, 1983). In a later *in vivo* study Menzel and Gellert (1987a) using fusions of short *gyr*A and *gyr*B 5′ sequences to the galactokinase gene found that the galactokinase gene could be induced by coumermycin A_1. They also showed that only 20 bp of the 5′ sequence, including a consensus sequence from the *gyr*A and *gyr*B promoters, was required for relaxation-stimulated transcription (Menzel and Gellert, 1987b).

More evidence that supercoiling is controlled homeostatically by balancing enzymatic negative supercoiling and relaxing activities comes from experiments on the control of topoisomerase I gene expression. The *top*A gene coding topoisomerase I, the major relaxing activity in *Escherichia coli*, has four promoters (Tse-Dinh and Beran,

1988). In an *in vitro* transcription system it was found that when all four promoters were negatively supercoiled transcription of topoisomerase I from the promoters was greatest (Tse-Dinh and Beran, 1988).

The 4-quinolones:

The modern fluorinated 4-quinolone antibacterials such as ciprofloxacin and ofloxacin are derivatives of the prototype 4-quinolone nalidixic acid which has been in clinical use since the early 1960s. The chemical structures of several 4-quinolones are shown in Figure 1. The term 4-quinolone for this class of drug was proposed by Smith (1984a) since the full name of the common skeleton from which the drugs are derived is 4-oxo-1,4-dihydroquinoline. Most modern 4-quinolones differ from nalidixic acid in that they possess a fluorine atom at the C6 position and a piperazinyl or a substituted piperazinyl group in place of the methyl group at C7. The structural changes in the modern 4-quinolones tend to make them less hydrophobic than their earlier analogues (Hirai *et al*, 1986^a). The *in vitro* activities of the fluorinated 4-quinolones such as ciprofloxacin (Wise *et al*, 1983; Fass, 1983), ofloxacin (Chin *et al*, 1991), DR-3355 (Tanaka *et al*, 1990), sparfloxacin (Chaudhry *et al*, 1990; Chin *et al*, 1991; Cooper *et al*, 1990) and lomefloxacin (Wise *et al*, 1988) are high enough that they can be considered as broad spectrum antibacterials. Ofloxacin is a raceimic mixture of two optical isomers, DR-3354 (R-[+]-ofloxacin)

Figure 1: structures of some 4-quinolones.
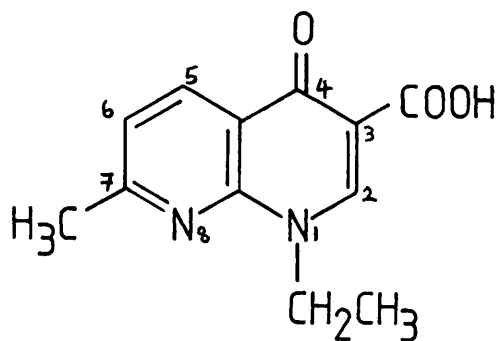
NALIDIXIC ACID
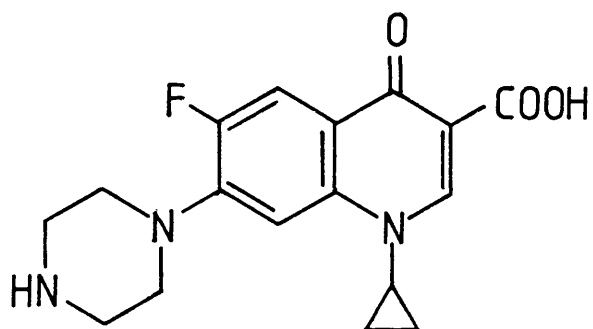
CIPROFLOXACIN
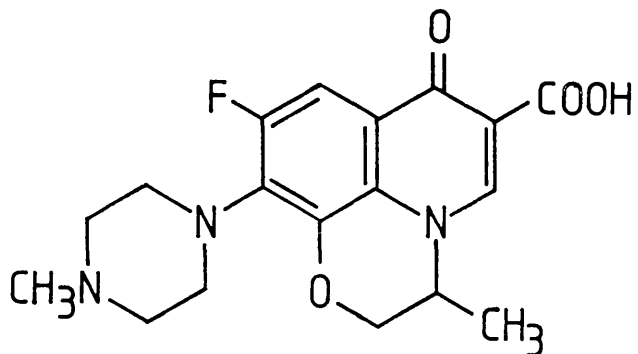
OFLOXACIN
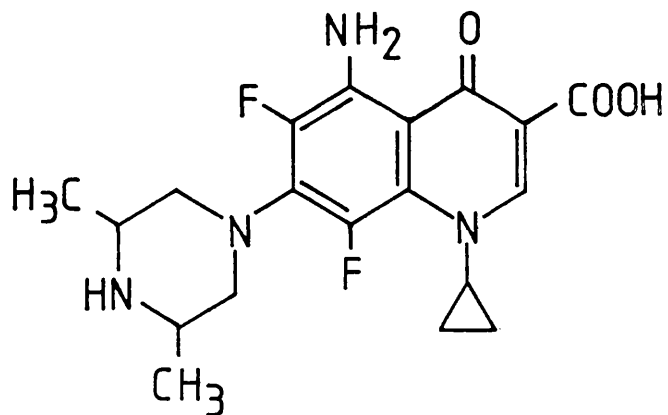
SPARFLOXACIN

and DR-3355 (S-[-]-ofloxacin). The antibacterial potency of DR-3355 is approximately twice that of ofloxacin because DR-3354 has little antibacterial activity (Tanaka *et al*, 1990).

Inhibition of DNA gyrase by 4-quinolones:

The 4-quinolones exert their antibacterial activity by inhibition of DNA gyrase, leading to rapid death of bacteria at concentrations above the minimum inhibitory concentration (MIC) (Crumplin and Smith, 1975). DNA gyrase was identified as the target enzyme of 4-quinolones by Gellert *et al* (1977) who found that *in vitro* the supercoiling activity of gyrase could be inhibited by oxolinic acid, and the supercoiling activity of DNA gyrase from a nalidixic acid-resistant (*nal*A) mutant was resistant to oxolinic acid. However, the concentrations of 4-quinolone required to cause 50% inhibition of the supercoiling activity of DNA gyrase *in vitro*, and 50% inhibition of the breakage-reunion activity *in vitro* are often 10-100 fold higher than the MIC of the 4-quinolone against the bacteria (Liu and Wang, 1978; Sugino and Bott, 1980; Sato *et al*, 1986; Zweerink and Edison, 1986; Miller and Scurlock, 1983; Takahata and Nishino, 1988; Aoyama *et al*, 1988a). A possible reason for the discrepancy could be that the 50% inhibitory concentration (IC_{50}) in the supercoiling assay corresponds to a higher level of inhibition of gyrase than is required for the inhibition of bacterial multiplication which is assayed by MIC determination (Diver *et al*, 1990). Another factor which may be contributory to the discrepancy is the observation that

4-quinolones have been estimated to be concentrated intracellularly by up to 50-fold relative to the extracellular concentration (Kotera et al, 1991).

For some years there has been debate over the fine details of the action of 4-quinolones, largely concerning exactly where the drugs bind i.e. to DNA gyrase itself, to DNA only or to the DNA gyrase-DNA complex formed by the enzyme in its topoisomerase activities. As a result of studies using radio-labelled norfloxacin ligand-binding Shen and Pernet (1985) claimed that at therapeutic concentrations of norfloxacin there was little or no binding of norfloxacin to gyrase. Furthermore, binding of norfloxacin to single-stranded DNA seemed to occur in preference to binding to either linear or relaxed DNA. In addition they claimed that the binding of norfloxacin to relaxed DNA was not altered by the presence of DNA gyrase (Shen and Pernet, 1985). It was also found that thermal denaturation of double-stranded DNA seemed to increase norfloxacin binding to DNA by up to 10-fold. In contrast to the findings of Shen and Pernet (1985) Palu et al (1988) using equilibrium dialysis and fluorescence spectroscopy did not find any evidence for binding of norfloxacin or ofloxacin to DNA. Shen (1989) attributed the negative findings of Palu et al (1988) to the low drug concentration used and poor experimental design.

However, the results of Palu et al (1988) were vindicated by Shen et al (1989a) who later found saturable binding of

norfloxacin to complexes between DNA gyrase and its relaxed DNA substrate, thus retracting their earlier claim that norfloxacin bound to DNA in the absence of gyrase. The binding sites were found to be saturable at the same concentrations of norfloxacin that caused inhibition of DNA supercoiling activity of DNA gyrase *in vitro*. These results suggested that 4-quinolones inhibit DNA gyrase by binding to a site on DNA which is formed by the gyrase-DNA complex. Furthermore, Shen *et al* (1989b) using various DNA species found that binding of norfloxacin to single-stranded DNA was non-specific but that binding to supercoiled DNA in the presence of DNA gyrase was highly cooperative and saturable. Single-stranded sections could form in supercoiled DNA, for example in the cruciform structure (Sinden and Pettijohn, 1984; Dayn et *al*, 1991), or in an easily denaturable region of a supercoil. These results suggested that norfloxacin binds to unpaired bases in supercoil-promoted single-stranded sections of DNA *via* hydrogen bonding and not by intercalation. In fact, Tornaletti and Pedrini (1988) also showed that 4-quinolones do not bind to DNA intercalatively.

Based on the above evidence Shen *et al* (1989c) proposed an amended model for inhibition of DNA gyrase by 4-quinolones. They propose that the bound gyrase molecule creates a single-stranded "bubble" in the DNA during strand passage. The bubble provides a site for four or more 4-quinolone molecules to bind in a cooperative manner. The cooperativity of the interaction is derived from the

structure of the 4-quinolone molecule, which allows stacking
of two 4-quinolone molecules (a phenomenon which has been
observed with nalidixic acid in solution) in the same
orientation on one plane, and hydrophobic bonding between
two other drug molecules in the opposite orientation above
or below the plane of the first two molecules. Hydrogen
bonding occurs between the carboxylic acid group at C3 on
the drug molecule and the hydrogen bond donors of the purine
or pyrimidine bases on the separated DNA strands. The
opposite orientation of the third and fourth molecules would
overcome the problem of electrostatic repulsion between the
carboxylic acid groups of the first two 4-quinolone
molecules and actually account for the cooperativity i.e.
the second, third and fourth drug molecules to bind do so
more strongly than the previous one. The nature of the
gyrase-induced binding site may account in part for the
differences in susceptibility of bacteria to 4-quinolones,
due to the various substituent groups on the quinolone ring.
Thus, binding of the 4-quinolone in the single-stranded
bubble created in the DNA by gyrase, "locks" the gyrase-DNA
complex thereby inhibiting gyrase activity, with all its
knock-on effects on essential cell functions.

However, recent studies on 4-quinolone-resistance mutations
in the *gyr*A and the *gyr*B genes and their effects on the
activities of DNA gyrase have cast doubt on the validity of
the model for 4-quinolone inhibition of DNA gyrase proposed
by Shen *et al* (1989c). It has been shown that 4-quinolone

resistance can be due to changes in the conformation and/or charge on amino acid residues on the A and B subunits of DNA gyrase (Yoshida *et al*, 1988; Yoshida *et al*, 1990a; Yoshida *et al*, 1991a; Hallett and Maxwell, 1991). These findings (described below) suggest that self-association of drug molecules and hydrogen bonding between the drug and DNA as proposed by Shen *et al* (1989c) may not be the principal mode of 4-quinolone inhibition of DNA gyrase.

Spontaneous mutation in the *E. coli* DNA gyrase genes resulting in 4-quinolone-resistance occurs as frequently in the *gyr*A genes as in the *gyr*B genes, but in clinical isolates the *gyr*A mutation is more common (Nakamura *et al*, 1989). Analysis of sequence changes in the *gyr*A gene have shown that mutations result in amino acid substitutions in a relatively hydrophilic region between residues 67 and 106 of the protein (Yoshida *et al*, 1988) close to the catalytic tyrosine-122 (Horowitz and Wang, 1987). Further work by Yoshida *et al* (1990a) showed that spontaneous high-level 4-quinolone resistance was always associated with susbstitution of serine-83 to either leucine or tryptophan. These mutations could lead to local conformational changes in the A subunit. Mutations in the amino acids surrounding serine-83 resulted in lower levels of 4-quinolone resistance. Yoshida *et al* (1990a) called the region between residues 67 and 106 the "4-quinolone-resistance-determining region" of the A subunit.

Hallett and Maxwell (1991) generated two 4-quinolone resistance mutations in the gyrase A subunit by gap misrepair mutagenesis and site-directed mutagenesis. The substitutions were alanine for serine at position 83 and arginine for glutamine at residue 106. Inhibition of the *in vitro* activities of the mutant and wild-type gyrases by ciprofloxacin were assayed. It was found that the supercoiling, relaxation, decatenation and cleavage activities of the mutant enzymes were 10-fold less susceptible to ciprofloxacin than wild-type DNA gyrase. Interestingly, supercoiling and Ca^{2+}-induced cleavage of DNA were virtually unaltered in the mutant enzymes compared to wild-type DNA gyrase. Although the mechanism of Ca^{2+}-induced cleavage is unknown the result suggests that the mutations destabilise the 4-quinolone-DNA gyrase interaction without affecting breakage and reunion (Hallet and Maxwell, 1991).

Spontaneous resistance to 4-quinolones due to mutation in the gyrase B subunit results from amino acid substitutions at residues 426 and 447 (Yoshida *et al*, 1991a). It was shown that substitution of aspartic acid at position 426 for asparagine in the *nal*24 mutant conferred resistance to all 4-quinolones tested. Substitution of lysine for glutamic acid at position 447 in the *nal*31 (*nal*C) mutant conferred resistance to acidic 4-quinolones but rendered bacteria hypersusceptible to the modern amphoteric 4-quinolones such as ciprofloxacin or ofloxacin. The results of Yoshida *et al*

(1991a) with the *nal*31 (*nal*C) mutant support earlier findings by Smith (1984bc). The two classes of mutation were designated type 1 and type 2, respectively (Yoshida *et al*, 1991a). The hydrophobicity profile of the 4-quinolone-resistance-determining region of the B subunit shows that the type 1 and 2 mutations occur in very hydrophilic areas, probably at turns on the surface of the protein (Yoshida *et al*, 1991a). On the basis of these findings they proposed a model for the interaction of the old and new 4-quinolones with the gyrase B subunit which assumed that residues 426 and 447 are physically close to one another. In the wild-type protein the older drugs interact with the two residues *via* their C7 methyl group and the modern drugs interact *via* their C7 piperazinyl ring. Type 1 mutation causes the area to become relatively more positive, reducing the association with all 4-quinolones. Type 2 mutation makes the area more negative, increasing the attraction for the piperazinyl group of the modern 4-quinolones, whilst conferring less attraction for the methyl group of the old 4-quinolones (Yoshida *et al*, 1991a).

The result of the studies of the 4-quinolone-resistance-determining regions of the A and B subunits of gyrase is a proposal that the 4-quinolone-binding site may be a "pocket" bounded by the hydrophilic residues of the 4-quinolone-resistance-determining regions of both subunits of gyrase (Yoshida *et al*, 1991a). It is possible that the conformation and charge of the amino acids in the 4-

quinolone-resistance-determining region affects the nature of the single-stranded DNA bubble in which the 4-quinolones are proposed to bind in the model of Shen *et al* (1989c). Thus the models of Shen *et al* (1989c) and Yoshida *et al* (1991a) may not be mutually exclusive.

Resistance studies have proved invaluable to broader issues of 4-quinolone action. The 4-quinolone-resistant DNA gyrase phenotype is recessive to the sensitive phenotype in *E. coli* (Hane and Wood, 1969). This means that when both the wild-type and 4-quinolone-resistant DNA gyrases co-exist in a partial diploid the bacterium exhibits the sensitive phenotype. The reason for the recessivity of 4-quinolone resistant gyrase could be due to impaired function at the active site of gyrase brought about by the acquisition of 4-quinolone resistance. Sequence changes in the highly conserved gyrase genes, especially affecting the active site, may not be acceptable to the bacterial cell. However, as explained above and contrary to this argument, two *gyr*A mutants with high-level 4-quinolone resistance generated by gap misrepair mutagenesis and site-directed mutagenesis were shown to be unaffected in their supercoiling activities compared to the wild-type (Hallett and Maxwell, 1991).

Another explanation for the recessivity of 4-quinolone resistance is the proposal that 4-quinolones may act as a gyrase "poison" (Kreuzer and Cozzarelli, 1979; Drlica and Franco, 1988) rather in the manner with which eukaryotic

antitumour topoisomerase inhibitors are proposed to act (Nelson *et al*, 1984; D'Arpa and Liu, 1989). This means that even a small number of interactions between 4-quinolones and wild-type gyrase molecules could irreversibly inhibit essential cell functions by locking gyrase to the DNA, regardless of whether or not 4-quinolone-resistant gyrase molecules are present. Evidence that lesion-forming drugs including the 4-quinolones can poison topoisomerases comes from two bacteriophage studies. Firstly, temperature-sensitive DNA gyrase mutants were found to be able to replicate bacteriophage T7 at the non-permissive temperature, whereas treatment of the bacteria with nalidixic acid prevented T7 growth (Kreuzer and Cozzarelli, 1979). Secondly, the intercalative antitumour drug *m*-AMSA reduces the yield of wild-type T4 bacteriophage by 10-fold, even though its target enzyme is the non-essential T4 topoisomerase II (K. N. Kreuzer, unpublished results, quoted in Rowe *et al*, 1984).

A link between the mechanisms of action of *m*-AMSA and 4-quinolones was established by the recent discovery that an *m*-AMSA-resistant mutant topoisomerase from T4 conferred DNA gyrase-like sensitivity to oxolinic acid (Huff and Kreuzer, 1990). The mutation also conferred either cross-resistance or hypersusceptibility to six other antitumour drugs whose target enzyme is mammalian topoisomerase II, suggesting a common mode of action between the drugs. These findings are germane to considerations of toxicity of 4-quinolones

especially as more potent 4-quinolone derivatives are
developed. It is also interesting to note that nucleic acid
synthesis is required for the cytocidal activity of
topoisomerase poisons such as camptothecin, *m*-AMSA and
etoposide (D'Arpa *et al*, 1990) since bacterial cell division
is required for maximum bactercidal activity of 4-quinolones
(Smith, 1984a).

In vitro effects of 4-quinolones:

One unusual and almost ubiquitous feature of the 4-
quinolones is that they do not exhibit the expected dose-
response curve when bacteria are treated with a range of
concentrations of the drug in nutrient broth. As the
concentration of the drug is increased above the MIC,
progressively more death of the bacteria is found, until a
certain concentration (known as the Most Bactericidal
Concentration later termed the Optimum Bactericidal
Concentration or OBC) of the drug is reached (Crumplin and
Smith, 1975). As the concentration of the 4-quinolone is
increased further than the OBC progressively *less* death of
bacteria is found - this phenomenon is known as the
paradoxical or biphasic response, (Crumplin and Smith,
1975). The biphasic response has been observed with all 4-
quinolones tested so far against all organisms except *Ps.
aeruginosa* (Morrissey and Smith, 1990).

Crumplin and Smith (1975) discovered that the biphasic
response is due to secondary inhibition of protein or RNA

synthesis at higher 4-quinolone concentrations. Thus, simultaneous treatment of bacteria with either the protein synthesis inhibitor, chloramphenicol, or the RNA synthesis inhibitor, rifampicin, reduces the bactericidal activity of the 4-quinolones. However, the bactericidal activity of the modern fluorinated 4-quinolones against many organisms cannot be completely abolished by the above treatments, or by suspension of the bacteria in phosphate-buffered-saline (PBS; to prevent bacterial multiplication) leading to the proposition of two bactericidal mechanisms (Smith, 1984a). The first, designated mechanism A (the only mechanism possessed by the older 4-quinolones such as nalidixic or oxolinic acids), is protein- and RNA-synthesis-dependent and requires that the bacteria are capable of division. The second mechanism of action, mechanism B, has none of the prerequisites of mechanism A and has been demonstrated for ciprofloxacin (Smith, 1984a), ofloxacin (Smith, 1984a), Lewin and Amyes, (1989), lomefloxacin (Lewin *et al*, 1989a), pefloxacin and fleroxacin (Lewin and Amyes, 1990) against *E. coli*. However, ciprofloxacin does not exhibit mechanism B against coagulase-negative or coagulase-positive Staphylococci whilst ofloxacin or DR-3355 *do* possess this bactericidal mechanism (Lewin and Smith, 1988; Lewin and Smith, 1989ab; Lewin and Amyes, 1989). A third bactericidal mechanism, designated mechanism C, also operates against non-dividing bacteria but does require protein or RNA synthesis (Ratcliffe and Smith, 1985). Thus 4-quinolones which possess mechanism C can kill bacteria resuspended in

PBS but not when the 4-quinolone is in combination with chloramphenicol or rifampicin. Mechanism C has been found only for enoxacin (Lewin *et al*, 1989a) and norfloxacin (Ratcliffe and Smith, 1985) against *E. coli*, but these drugs do not possess mechanism B against *E. coli*.

The ability of ciprofloxacin to kill *E. coli* in the absence of protein- or RNA-synthesis has recently been shown to involve the A subunit of DNA gyrase (Lewin *et al*, 1991a). As mentioned above ciprofloxacin possesses bactericidal mechanisms A and B against *E. coli* and this 4-quinolone can therefore kill bacteria in the presence of either chloramphenicol or rifampicin (Smith, 1984a). However, it was found that the *nal*A mutation rendered the bacteria insensitive to ciprofloxacin in the presence of either chloramphenicol or rifampicin (Lewin *et al*, 1991a). The *nal*A mutation in *E. coli* confers high-level resistance to 4-quinolones by altering the A subunit of DNA gyrase so it would appear that mechanism B involves this subunit of DNA gyrase. Ciprofloxacin still possessed mechanism B against other 4-quinolone-resistant mutants of *E. coli* which had altered subunit B of DNA gyrase (*nal*C) or altered uptake (*nal*B) or both altered B subunit and uptake (*nal*D).

4-quinolones and the SOS response:

Treatment of bacteria with ultraviolet (U.V.) light or DNA-damaging agents such as 4-quinolones induces at least 17 genes which are part of the SOS response (Little and Mount,

1982). The SOS response is a pleiotropic response to DNA
damage which is under the control of the *rec*A and *lex*A
genes. DNA damage produces an (as yet unidentified)
inducing signal which modifies the RecA protein which then
cleaves the LexA protein which is the repressor for the SOS
system (Little and Mount, 1982). As the SOS response
repairs the damaged DNA the level of the inducing signal
decreases, thereby progressively reducing the magnitude of
the response until normal repression of the SOS genes occurs
(Little and Mount, 1982).

Since 4-quinolones are known to induce the SOS response it
would be expected that the SOS response affects survival of
bacteria treated with the drugs. For several years there
has been debate as to whether or not the SOS response
enhances or reduces the bactericidal activity of 4-
quinolones. Piddock and Wise (1987) found that the RecA-
inducing concentration of several 4-quinolones correlated
well with the antibacterial activity of the drugs in an *E.
coli rec*A::*lac* fusion. They also found that induction of
the SOS response was dependent on protein synthesis and that
at high 4-quinolone concentrations the 61 and 73 kDa heat-
shock proteins were induced. As a result of this study
Piddock and Wise (1987) proposed that DNA damage is the
primary bactericidal mechanism of 4-quinolones and that the
SOS response protects the bacteria from 4-quinolones. Lewin
and Smith (1990) found that DNA breakdown occurred in
bacteria treated with 4-quinolones. DNA breakdown still

occurred in bacteria treated with nalidixic acid and chloramphenicol or rifampicin (to abolish lethality) and so it was proposed that DNA breakdown did not contribute to the bactericidal activity of nalidixic acid. In addition *rec*B and *rec*C mutants which lack exonuclease V do not suffer from DNA breakdown when treated with nalidixic acid even though the bacteria are killed (Lewin and Smith, 1990). Walters *et al* (1989) found that the effect of SOS mutations were highly variable with respect to different 4-quinolones. They found that mutants with a defective SOS response were less susceptible to nalidixic acid and ciprofloxacin, but that SOS induction enhances survival of bacteria treated with enoxacin and fleroxacin.

A study by Lewin *et al* (1989b) suggests that SOS plays no rôle in the cellular response of *E. coli* to nalidixic acid-induced DNA damage. *Rec*A13 and *rec*B21 mutants, which are defective in recombination repair and cannot induce the SOS response when treated with nalidixic acid, were shown to be hypersensitive to nalidixic acid (Lewin *et al*, 1989b). *E. coli* with the *lex*A3 mutation which prevents SOS induction without affecting recombination repair was as sensitive as the wild-type to nalidixic acid. A *rec*A430 mutant which lacks SOS repair but is recombination-proficient was also found to be as susceptible as the wild-type to nalidixic acid. Thus the hypersusceptibility of the *rec*A13 and *rec*B21 mutants to nalidixic acid is due to their defective recombination and the SOS response is irrelevant to survival

of nalidixic acid-treated bacteria (Lewin *et al*, 1989b). In
a continuation of this study, Howard and Smith (1991) found
that *rec*A and *lex*A mutants were hypersusceptible to
ciprofloxacin and ofloxacin and that the *rec*A mutant was
more hypersensitive than the *lex*A mutant. Since the *rec*A
mutant is hypersusceptible to nalidixic acid, ciprofloxacin
and ofloxacin it was suggested that recombination repair
repairs DNA damage caused by bactericidal mechanism A which
is common to all three drugs (Smith, 1984a). These findings
also suggest that the SOS response repairs DNA damage caused
by mechanism B of ciprofloxacin or ofloxacin (Howard and
Smith, 1991).

One of the effects of SOS induction is filamentation of the
bacterial cell thought to be due to the expression of the
*sul*A (*sfi*A) gene product which inhibits cell division
(Little and Mount, 1982; Diver and Wise, 1986; Phillips *et
al*, 1987). At concentrations of ofloxacin or pefloxacin
equal to three-times the MIC value, Vincent *et al* (1991)
found that filamentation of *E. coli* W7 (*dap*A *lys*A) was
followed by a lytic event which required active protein
synthesis and could therefore be abolished by
chloramphenicol. Shortening of glycan chain-length may have
been caused by increased transglycosylase activity, and
other alterations in the cell wall peptidoglycan were seen.
Quite different results were found when the bacteria were
treated with high concentrations of 4-quinolones. When the
bacteria were treated with 128-times the MIC value of

ofloxacin or pefloxacin, filamentation and lysis was absent,
and in agreement with findings by Crumplin and Smith (1975),
protein synthesis was completely inhibited, and the death
rate was reduced. Vincent *et al* (1991) proposed that the
bactericidal activity of 4-quinolones may be related to
lysis and changes in peptidoglycan integrity. In a more
recent study Georgopapadakou and Bertasso (1991) also found
filamentation of *E. coli* treated with low concentrations of
fleroxacin. In addition they observed formation of large
nucleoids in midcell (which was reversible upon removal of
fleroxacin) which suggests inhibition of nucleoid
segregation. As the concentration of fleroxacin was
increased the size of the nucleoid decreased which suggests
that fleroxacin inhibited DNA replication. Furthermore, the
MICs of several fluorinated 4-quinolones were found to be
close to the concentration required to inhibit replicative
DNA synthesis by 50%. Inhibition of nucleoid segregation
could have been part of the SOS response (as described
above) but it may also be caused by inhibition of either DNA
gyrase or DNA topoisomerase IV, both of which are thought to
be essential for partition of daughter chromosomes after
replication (Kreuzer and Cozzarelli, 1980; Steck and Drlica,
1984; Kato *et al*, 1990).

Uptake of 4-quinolones into the bacterial cell:
Before a molecule of a 4-quinolone reaches its site of
action, the DNA gyrase-DNA complex, it must be taken up from
the surrounding medium into the cytoplasm. Uptake

mechanisms have been most extensively studied in Gram negative bacteria, such as *E. coli* or *Ps. aeruginosa*. These organisms have outer membranes which pose penetration problems to a broad range of antibacterial agents (Nikaido, 1988, 1989; Dechène *et al*, 1990; Bryan and Bedard, 1991). Bedard *et al* (1987) found that in *E. coli* 4-quinolone uptake was non-active, because uptake was non-saturable and was not reduced by metabolic uncouplers such as 2,4-dinitrophenol (2,4-DNP) or carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP). As a result they proposed that 4-quinolones entered bacteria utilising diffusion through the outer membrane proteins and the lipid bilayer. A more recent study (Diver *et al*, 1990) on the contrary would seem to suggest that there may be an active transport mechanism for the uptake of 4-quinolones, since treatment with 2,4-DNP or the electron transport inhibitors potassium cyanide or sodium azide did seem to reduce drug accumulation. The differences between this study and that of Bedard *et al* (1987) were attributed to the use of different 4-quinolone concentrations and incubation temperatures. Diver *et al* (1990) also found that CCCP stimulated 4-quinolone uptake but this could have been due to structural perturbations at the both the outer and inner membranes (Helgerson and Cramer, 1976, cited by Diver *et al*, [1990]) and not due to interference with the proton-motive force. In agreement with Diver *et al* (1990) Kotera *et al* (1991) found that EDTA reduced uptake of 4-quinolones and that norfloxacin could be concentrated intracellularly by 50-fold compared to the medium even in the presence of

EDTA. The intracellular concentration of norfloxacin was attributed to an interaction with phospholipids but may have involved another process.

4-quinolones were shown to penetrate the outer membrane through the outer membrane porin ompF, in a study of porin-deficient mutants that were found to be partially resistant to 4-quinolones (Hirai et al, 1986a). These findings were supported by the isolation of spontaneous 4-quinolone-resistant mutants that were found to lack the same porin (Hirai et al, 1986b; Hooper et al, 1986). However, MIC values caused by mutation of outer membrane porin genes rarely exceed wild-type MIC values by greater than four-fold. On the other hand, hydrophilic cephalosporins, which enter bacteria solely via porins, exhibited MIC values in porin-deficient mutants as high as 64-fold greater than wild-type MIC values. The importance of OMPs in resistance development is dependent on whether or not the drug has alternative means of uptake.

Hirai et al (1986a) also found in lipopolysaccharide-deficient ("rough") mutants of Salmonella typhimurium that the MIC of 4-quinolones was decreased compared to the wild-type, and that the size of the MIC decrease correlated with the hydrophobicity of the 4-quinolone. These findings would seem to suggest that 4-quinolones can cross the outer membrane through non-porin pathways. Chapman and Georgopapadakou (1988) in studies using fleroxacin proposed

that the drug diffused through the lipid bilayer having
first disorganised it by chelation of divalent cations i.e.
uptake was "self-promoted". Their proposals are based on
the following evidence. Fleroxacin had four main effects on
the outer membrane of *E. coli*: (i) it caused release of
lipopolysaccharide from the outer membrane; (ii) it
increased cell-surface hydrophobicity; (iii) it increased
permeability to ß-lactams and (iv) it sensitised bacteria to
lysis by detergents such as SDS. All of these effects were
antagonised by magnesium ions and fleroxacin uptake was
reduced and its MIC was increased by the presence of added
magnesium. Chapman (unpublished results, quoted in Chapman
and Georgopapadakou, [1988]) found that all fluorinated 4-
quinolones can permeabilize the outer membrane equally well.
He concluded that use of the porin pathway is dependent on
the hydrophobicity of the 4-quinolone i.e. the less
hydrophobic the more it uses the outer membrane porins for
access into bacteria.

Although there is still some debate as to whether or not 4-
quinolone uptake is an active process, an active efflux
mechanism for norfloxacin has been identified in 4-
quinolone-susceptible *E. coli* (Cohen *et al*, 1988). They
found that in agreement with a previous study with enoxacin
(Bedard *et al*, 1987) norfloxacin uptake was rapid in energy-
depleted cells, but that in energised cells norfloxacin
accumulation was reduced. Efflux was found to be mediated
by carriers (therefore saturable) at the inner membrane, but

mutants lacking ompF exhibited greater energy-dependent decrease in norfloxacin accumulation. The reasons for this phenomenon are not clear since loss of ompF should affect both uptake and exit rates to the same extent with no effect on steady state intracellular norfloxacin levels. It is likely that the equilibrium concentration of the 4-quinolone in *E. coli* is dependent on the rates of passive diffusion through porins and through the lipid bilayer, together with the rate of efflux through the active efflux system.

In *Pseudomonas aeruginosa* the principal porin, F, produces much larger channels than those in *E. coli* but they still confer intrinsically lower permeability to hydrophilic antimicrobials (Nikaido *et al*, 1991). This paradox may be due to *Ps. aeruginosa* porin F being of uniform diameter through the whole width of the membrane, whereas in *E. coli* the porin may have a short constriction. Uniform pores seem to offer more "drag resistance" to solute diffusion (Nikaido *et al*, 1991). Ciprofloxacin accumulation in *Ps. aeruginosa* is extremely rapid, reaching steady-state levels within one minute of exposure. Uptake is non-saturable suggesting that accumulation is by diffusion (Celesk and Robillard, 1989). The uncoupler CCCP increased ciprofloxacin uptake by up to six-fold, but a ciprofloxacin-resistant mutation, *cfx*B, caused four-fold less accumulation than the wild-type, but only in the presence of CCCP or 2,4-DNP. The *cfx*B mutant has an additional 51 kDa OMP which may reduce outer membrane permeability, reducing ciprofloxacin

concentration in the cytoplasm. The effect of the mutation is ameliorated when the active efflux system is operating (Celesk and Robillard, 1989).

A study by Chamberland et al (1989) found that in vitro selection of mutants resistant to norfloxacin resulted in reduction of the 25.5 kDa outer membrane protein, G, and loss of a 40 kDa protein. These changes caused significantly reduced norfloxacin uptake and were associated with cross-resistance to ß-lactams, tetracycline or chloramphenicol. Similar outer membrane alterations were found in ciprofloxacin-resistant mutants of Ps. aeruginosa isolated during ciprofloxacin therapy of patients suffering from cystic fibrosis (H. R. Rabin and L. E. Bryan, unpublished results, quoted in Chamberland et al, [1989]). Chemical mutagenesis of Ps. aeruginosa followed by selection of latamoxef-resistant mutants was found to result in decreased permeability to latamoxef and reduction in outer membrane proteins C, D, E1 and E2 (Yamano et al, 1990). The mutation also conferred 16- and 32-fold increases in the MICs of ciprofloxacin and ofloxacin, respectively, suggesting that at least one of the proteins C, D, E1 or E2 functions as a porin channel. Since the levels of four outer membrane proteins were only reduced it is possible that the mutation was in a regulatory gene controlling expression of the four proteins (Yamano et al, 1990).

Uptake of 4-quinolones into Gram-positive bacteria has not been as extensively studied as that in Gram-negative bacteria. In clinical isolates of *Staphylococcus aureus* sparfloxacin and norfloxacin uptake was found to be non-saturable and an energy-dependent reduction in accumulation was observed (Yoshida *et al*, 1991b). Sparfloxacin uptake was up to 6-fold higher than that of norfloxacin in the presence or the absence of CCCP, and the improved permeation is probably due to the moderate lipophilicity of the sparfloxacin molecule. The differences in uptake of 4-quinolones between drug-resistant and -susceptible isolates of *Staph. aureus* were abolished by CCCP. These findings imply that energy-dependent reduction of 4-quinolone accumulation may contribute to clinical resistance to 4-quinolones (Yoshida et *al*, 1991b).

Clinical uses of 4-quinolones:

The fluorinated 4-quinolones are used for the treatment of a wide range of infections caused by both Gram-negative and Gram-positive bacteria. One of the main advantages of modern 4-quinolones is that high serum and tissue concentrations can be achieved after oral administration (Wise *et al*, 1986; Wise and Donovan, 1987). Since its introduction ciprofloxacin has proved invaluable in several areas of antimicrobial chemotherapy including elimination of meningococci from carriers (Renkonnen *et al*, 1987), treatment of respiratory tract infections (Raoof *et al*, 1986) especially Pseudomonas infection in cystic fibrosis

patients (LeBel *et al*, 1986 ; Pedersen *et al*, 1987; Jensen
et al, 1987; Steen *et al*, 1989), skin and soft-tissue
infections (Fass, 1986; Wood and Logan, 1986; Valainis *et
al*, 1987; Eron, 1987; Self *et al*, 1987), gastrointestinal
                                              ; fass, 1987
and urinary tract infections (Gasser *et al*, 1987; Tolkoff-
Rubin and Rubin, 1987), sexually transmitted diseases
(Oriel, 1986; Fong *et al*, 1987), osteomyelitis (Lesse *et al*,
1987) and prophylaxis in neutropenic patients (Smith *et al*,
1986). Although ofloxacin is generally less potent than
ciprofloxacin, it may be more effective in the treatment of
Staphylococcal infection since it possesses bactericidal
activity against non-dividing Staphylococci (Lewin and
Smith, 1988; Lewin and Smith, 1989b). Ofloxacin is largely
renally cleared and ciprofloxacin is cleared by both renal
and non-renal pathways (Lode *et al*, 1990). Thus
ciprofloxacin should be used in preference to ofloxacin in
patients with partial renal impairment (Lode *et al*, 1990),
whereas patients with total renal failure should receive
ofloxacin because of the convenience of less frequent dosing
(Wolfson and Hooper, 1991).

Resistance to the 4-quinolones:

One of the greatest problems in antimicrobial chemotherapy
is the development of resistance to a particular therapeutic
agent. This often means that an infection cannot be treated
effectively by the drug of first choice because a
sufficiently high concentration of the antibacterial can no
longer be attained in order to inhibit the causative

organism. Resistance effectively reduces the useful lifespan of a particular drug leading to a constant and ever-increasing search for new drugs and more potent derivatives of previously effective drugs. Resistance can be carried on plasmid-encoded genes or it can occur through mutation of chromosomal genes (Bryan, 1988). Plasmid-mediated resistance has been discovered for virtually all classes of antibacterial drugs and is especially serious because the resistance can spread vertically to other species and even other genera.

The 4-quinolones, nitrofurantoin and polymyxin have not been proven to suffer from plasmid-mediated resistance (Courvalin et al, 1990; Courvalin, 1990). However, one case of what was thought to be plasmid-mediated 4-quinolone resistance (Munshi et al, 1987) has subsequently been shown to be due to carriage of a mutator plasmid which increases the rate of spontaneous mutation of chromosomal genes (Ambler et al, 1991). Bacteria can only become resistant to 4-quinolones by mutation of chromosomal genes, coding for altered DNA gyrase subunits, or affecting the accumulation mechanism(s) of the drugs or both (Lewin et al, 1990).

There are several explanations for the lack of plasmid-mediated-resistance to 4-quinolones. As mentioned earlier the 4-quinolone-resistant DNA gyrase phenotype is recessive to the sensitive phenotype (Hane and Wood, 1969), a phenomenon which can be explained by the "poison" hypothesis

(Kreuzer and Cozzarelli, 1979; Drlica and Franco, 1988).
Drug inactivation as a mechanism of resistance is unlikely
because the 4-quinolones are entirely synthetic agents and
bacteria have not been exposed to them before in nature. It
is thus improbable that bacteria could develop enzymatic
means to destroy the 4-quinolone structure. A more "active"
reason why 4-quinolones have not been shown to suffer from
plasmid-mediated resistance is that 4-quinolones inhibit
conjugation of R plasmids, probably due to inhibition of
donor DNA synthesis (Weisser and Wiedemann, 1987; Hooper et
al, 1989). Curing of plasmids by 4-quinolones has been
observed in vivo with Serratia marcescens and E. coli
(Mehtar et al, 1987; Lewin et al, 1989d). Antagonism of DNA
gyrase by coumermycin A_1 also inhibits conjugation of
plasmids and Hfr mating (Wolfson et al, 1982; Hooper et al,
1989). It is possible however, that plasmids could carry
genes coding for altered uptake mechanisms resulting in
reduced uptake or enhanced efflux of 4-quinolones, but so
far this mechanism has not been reported (Courvalin, 1990).

The frequency with which bacteria become resistant to the 4-
quinolones in vitro is low, and varies for each drug and
bacterium. The mutation rates are generally between 10^{-6}
and 10^{-11} in Gram-negative and Gram-positive bacteria (Smith,
1986a; 1990ab; Hooper et al, 1986; Robillard and Scarpa,
1988; Felmingham et al, 1988; Legakis et al, 1989; Watanabe
et al, 1989; Yoshida et al, 1990b; Gootz and Martin, 1991).
It is difficult to isolate 4-quinolone-resistant mutants of

E. coli KL16 at 37°C (Smith, 1986a; Felmingham *et al*, 1988),
but Smith (1986a) showed that lowering the selection
temperature increased the mutation frequency to
ciprofloxacin and norfloxacin resistance. Odell and
Crumplin (1990) also found that *E. coli* CSH50, *K. aerogenes*,
K. pneumoniae, *Proteus mirabilis* and *S. marcescens* exhibited
temperature optima (32°C) for the isolation of 4-quinolone-
resistant mutants.

Mechanisms of resistance to 4-quinolones:

As described above, resistance to 4-quinolones can occur by
mutation of the genes coding for DNA gyrase and/or of genes
affecting permeability to the drugs. Mutations in DNA
gyrase arising *in vitro* and in clinical isolates leading to
4-quinolone resistance have been described in many bacteria.
These bacteria include *E. coli* (Gellert *et al*, 1977; Higgins
et al, 1978; Hooper *et al*, 1986; Yoshida *et al*, 1988; 1990a;
1991a; Nakamura *et al*, 1989; Hallett and Maxwell, 1991), *Ps.
aeruginosa* (Miller and Scurlock, 1983; Yoshida *et al*,
1990b), *Citrobacter freundii* (Aoyama *et al*, 1988b),
Enterobacter cloace (Lucain *et al*, 1989), *Neisseria
gonorrhoeae* (Stein *et al*, 1991), *Campylobacter jejuni* (Gootz
and Martin, 1991), *Serratia marcescens* (Masecar and
Robillard, 1991), *Staph. aureus* (Takahata and Nishino,
1988), *B. subtilis* (Sugino and Bott, 1980) and *Enterococcus
faecalis* (Nakanishi *et al*, 1991a).

Mapping of resistance mutations to specific amino acid

changes in DNA gyrase has only been done in *E. coli*, *Staph. aureus* and *Staph. epidermidis*. In *E. coli* 4-quinolone-resistance mutations in the *gyr*A and *gyr*B genes are clustered in both genes, leading to the proposition of "4-quinolone-resistance-determining regions" in both the A and the B subunits of DNA gyrase (Yoshida *et al*, 1990a; 1991a). The details of both 4-quinolone-resistance-determining regions were described earlier in the section entitled "Inhibition of DNA gyrase by 4-quinolones". To summarise, spontaneous mutation in the *gyr*A gene causing high-level resistance to 4-quinolones was always associated with the substitution of the serine residue at postion 83 for either leucine or trytophan in the gyrase A subunits (Cullen *et al*, 1989; Yoshida *et al*, 1988; 1990a). Mutations in residues adjacent to serine-83 resulted in lower levels of resistance to 4-quinolones. Analagous mutations have been mapped in the DNA gyrase A subunit from three clinical isolates of *Staph. aureus* which emerged during ciprofloxacin therapy. The amino acid changes were substitution of serine for leucine at position 84 and/or serine for proline at residue 85 (Sreedharan *et al*, 1990). The similarity of these changes in *Staph. aureus* to those seen in *E. coli* is perhaps not surprising when one considers that the DNA gyrase from both organisms exhibit strong homology (Hopewell *et al*, 1990). Furthermore, in a ciprofloxacin-resistant clinical isolate of *Staph. epidermidis* a novel substitution at serine-84 for phenylalanine has been identified (Sreedharan *et al*, 1991). This change is analagous to the substitution

of serine for leucine at position 84 seen in *Staph. aureus.*

Spontaneous mutation in the *E. coli* gyrase B subunit can result in two phenotypic classes of resistance to 4-quinolones (Yoshida *et al*, 1991a). Type 1 mutation resulted from substitution of aspartic acid for asparagine at residue 426 and conferred resistance to all 4-quinolones tested. Type 2 mutation resulted from substitution of lysine for glutamic acid at residue 447 and conferred resistance to the earlier acidic 4-quinolones but hypersusceptibility to modern amphoteric 4-quinolones. Yoshida *et al* (1991a) proposed that resistance to 4-quinolones due to mutations in DNA gyrase occurred in a "pocket" bounded by hydrophilic residues from both the A and B subunits.

Bacteria that have become resistant to 4-quinolones due to mutations affecting permeability mechanisms have been identified in mutants isolated *in vitro* and in isolates from clinical specimens. In *E. coli* impermeability mutations normally affect the outer membrane porin F, coded for by the *omp*F gene (Hirai *et al*, 1986ab; Bedard *et al*, 1989). Mutants lacking ompF exhibit small MIC increases of between two- and four-fold relative to the wild-type MIC for old and modern 4-quinolones (Hirai *et al*, 1986a). Mutations in ompF often cause pleiotropic resistance i.e. resistance to unrelated classes of antibiotics, since many antibacterials permeate the bacterial cell *via* this porin. One such mutation is the *nor*B mutation which confers resistance to

norfloxacin, chloramphenicol, cefoxitin or tetracycline
(Hirai *et al*, 1986b).

Changes in the lipopolysaccharide (LPS) layer also affect
susceptibilty of bacteria, especially to hydrophobic 4-
quinolones. "Rough" mutants of *S. typhimurium*, deficient in
LPS, are less susceptible to hydrophobic 4-quinolones such
as nalidixic acid or flumequine compared to the wild-type,
but remain as susceptible to hydrophilic 4-quinolones such
as ciprofloxacin and norfloxacin (Hirai *et al*, 1986a). A
low-level norfloxacin-resistant mutant, *nor*C, of *E. coli* has
been shown to have altered ompF along with changes in the
LPS structure. This mutant has the unusual phenotype of
being resistant to norfloxacin and ciprofloxacin but
hypersusceptible to nalidixic acid, hydrophobic antibiotics
and detergents (Hirai *et al*, 1986b).

Since an active-efflux mechanism has been demonstrated for
norfloxacin in *E. coli* that were susceptible to the drug
(Cohen *et al*, 1988) it is conceivable that resistance could
arise through enhanced active efflux of 4-quinolones.
Indeed, such a resistance mechanism has been identified in
clinical isolates of *Staph. aureus* whereby the uncoupler
CCCP reduced susceptibility of 4-quinolone-resistant mutants
to wild-type levels (Yoshida *et al*, 1991b). Another efflux-
mediated resistance mutation in *Staph. aureus* which has been
well characterised is *nor*A (Ubukata *et al*, 1989; Yoshida *et
al*, 1990c). The *nor*A gene codes for a 42 kDa membrane

protein which confers 16- to 64-fold increases in MICs of
hydrophilic 4-quinolones compared to the wild-type MIC, but
little or no resistance to hydrophobic 4-quinolones. CCCP
only abolishes resistance to hydrophilic 4-quinolones
suggesting that the *nor*A gene codes for an active-efflux
system which is specific to hydrophilic 4-quinolones
(Yoshida *et al*, 1990c).

In *Ps. aeruginosa* resistance to 4-quinolones due to
impermeability is extremely heterogeneous and often
pleiotropic. In an experimental peritonitis model in mice,
three pefloxacin-resistant mutants of *Ps. aeruginosa* were
isolated (Michea-Hamzehpour *et al*, 1991a). In two strains
there was reduced expression of a 47 kDa protein, probably
D2, and increased MICs of imipenem. Both of these strains
had altered LPS profiles and higher calcium levels in the
outer membrane. One of these strains also had an altered
gyrase, and the third strain only had an altered DNA gyrase.
In a more recent study by Michea-Hamzehpour *et al* (1991b)
mutants of *Ps. aeruginosa* selected on pefloxacin had
increased levels of the LPS 3-deoxy-D-mannooctulosnic acid.
More hydrophilic 4-quinolones such as ciprofloxacin or
norfloxacin had 8 to 16-fold increases in MIC. In strains
which produced protein D2 but not in D2-deficient strains
uptake of sparfloxacin was strongly inhibited by L-lysine or
imipenem. L-lysine and imipenem are both taken up *via* D2 by
facilitated diffusion so it was proposed that sparfloxacin
may also be taken up by this mechanism (Michea-Hamzehpour,

1991b).

The *nfx*B mutation results in the appearance of a new 54 kDa
outer membrane protein and a 16-fold increase in the MIC of
norfloxacin relative to the wild-type MIC (Hirai *et al*,
1987). The *nfx*B mutant also confers hypersusceptibility to
ß-lactams and aminoglycosides. The *nfx*C mutation results in
decreased expression of a 46 kDa outer membrane protein and
increased production of a 50 kDa protein (Fukuda *et al*,
1990). The *nfx*C mutation confers 8- to 32-fold increases in
the MICs of fluoroquinolones compared to the wild-type in
addition to cross-resistance to imepenem and chloramphenicol
(Fukuda *et al*, 1990). As is found with the *nfx*B mutation,
*nfx*C confers hypersusceptibility to ß-lactams and
aminoglycosides. Yet another mutant has been identified
which has reduced levels of protein G along with the loss of
a 40 kDa outer membrane protein, resulting in reduced
norfloxacin uptake (Chamberland *et al*, 1989). This mutant
also exhibited cross-resistance to ß-lactams, tetracycline
chloramphenicol.

Legakis *et al* (1989) found that changes in the LPS layer of
Ps. aeruginosa were associated with ciprofloxacin resistance
in three spontaneous mutants. Two of the three mutants
exhibited cross-resistance to ß-lactams and aminoglycosides.
One of these mutants had increased levels of a 54 kDa outer
membrane protein and the other mutant had decreased levels

of a 12 kDa outer membrane protein (Legakis *et al*, 1989).

LPS changes were also observed in clinical isolates of *Ps.*

aeruginosa resistant to ciprofloxacin (Masecar *et al*, 1990).

The LPS changes were accompanied by decreased sensitivity of

the DNA gyrase supercoiling assay to inhibition by

ciprofloxacin but Masecar *et al* (1990) attributed only a

minor rôle to the LPS changes in the resistance phenotype.

Clinical resistance to the 4-quinolones:

Clinical mutational resistance to the 4-quinolones has been

observed to increase in Europe from the early- to mid-

1980's, albeit from low levels (less than 1%) (Kresken and

Weidemann, 1988) for three organisms in particular: *Ps.*

aeruginosa, Staph. aureus and *Klebsiella pneumoniae*. In a

study covering the ex-Federal Republic of Germany, Austria

and Switzerland, resistance of most bacteria to 4-quinolones

remained unchanged at less than 4% between 1983 and 1986

(Kresken and Weidemann, 1988). However, for *Ps. aeruginosa*

during the same period the frequency of resistance to

ofloxacin and ciprofloxacin increased from over 3% to more

than 10%. Smaller increases in prevalence of clinical

resistance were reported for *Staph. aureus* (Kresken and

Weidemann, 1988; Blumberg *et al*, 1989). Very high rates of

ciprofloxacin-resistance have been reported in methicillin-

resistant *Staph. aureus* (MRSA) (Harnett *et al*, 1991) and

frequently isolated from patients that had not even received

ciprofloxacin therapy (Smith *et al*, 1990). Although the

percentage of 4-quinolone resistance in clinical isolates is

not nearly as high as for other classes of antimicrobial
agent, resistance is becoming a problem in 4-quinolone
therapy of infections caused by both *Ps. aeruginosa* and
Staph. aureus.

It is emerging from a number of recent studies that
resistance development occurs in certain types of patient
and more readily at certain sites of infection.
Ciprofloxacin is being increasingly used for treatment of
broncho-pulmonary infections by *Ps. aeruginosa* in patients
with cystic fibrosis (CF) (Bosso *et al*, 1987; Jensen *et al*,
1987; Steen *et al*, 1989). Resistance to ciprofloxacin has
often been reported to develop during therapy, possibly due
to altered pharmacokinetics in CF patients (LeBel *et al*,
1986; Jensen *et al*, 1987; Steen *et al*, 1989). The
resistance mechanism has been reported to be a persistence
phenomenon involving alteration of DNA gyrase and
impermeability to the drug (Diver *et al*, 1991).

Skin and soft-tissues are a major site for resistance
development by *Staph. aureus* (Fass, 1986; Righter, 1987;
Valainis *et al*, 1987; Parry *et al*, 1989) and *Ps. aeruginosa*
(Licitra *et al*, 1987; Neu *et al*, 1989). Osteomyelitis
infection caused by *Ps. aeruginosa* is also prone to
resistance development to 4-quinolones (Lesse *et al*, 1987;
Gilbert *et al*, 1987; Trexler Hessen *et al*, 1987; Neu *et al*,
1989). In osteomyelitis resistance may be prone to develop
since in osteomyelitis conditions are anaerobic (Lesse *et*

al, 1987) a factor which has been shown to abolish the bactericidal activity of 4-quinolones *in vitro* (Morrissey *et al*, 1990; Lewin *et al*, 1989c; Lewin *et al*, 1991b). These workers also found that when very high numbers of bacteria are present conditions became sufficiently anaerobic to abolish the bactericidal activity of 4-quinolones *in vitro*. Possible reasons for clinical or bacteriological failure at the above sites of infection may be reduced tissue penetration of the drug, poor drainage of the wound, the presence of necrotic tissue and, as mentioned above, high numbers of organisms (Parry *et al*, 1989; Neu *et al*, 1989; Ball, 1990). The coagulase-negative Staphylococci, especially *Staph. epidermidis*, are emerging as important nosocomial pathogens, especially in infections of foreign-body implants, the use of which is increasing (Peters, 1988; Archer, 1988). Development of 4-quinolone resistance in *Staph. epidermidis* and *Ps. aeruginosa* leading to clinical failure is likely to occur in such patients (Parry *et al*, 1989).

SECTION 2:

Materials and Methods

Bacterial strains:

Escherichia coli KL16 — Prototrophic K12 derivative (Hfr, *thi*-1, *rel*A, *spo*T1) (Hane and Wood, 1969).

Pseudomonas aeruginosa C17LN22 — Clinical isolate (Smith, 1990).

Staphylococcus aureus E3T — Coagulase positive. Derivative of strain E3 lacking penicillinase plasmid (Lewin and Smith, 1988).

Staphylococcus epidermidis SK360 — Coagulase negative, clinical isolate (Tennent *et al*, 1988).

These bacteria were subcultured fortnightly on nutrient agar, incubated at 37°C overnight and refrigerated until use.

Antibacterial agents:

Ciprofloxacin from Bayer, Newbury, U. K.

Ofloxacin from Hoechst, Hounslow, U.K.

DR-3355 from Daiichi Seiyaku, Tokyo, Japan.

Ciprofloxacin and DR-3355 were dissolved in sterile distilled water. Ofloxacin was dissolved in 0.02ml of 0.5M sodium hydroxide per mg and immediately diluted with sterile distilled water.

All other chemical reagents were Analar grade from BDH (Poole).

Media:

Media and glassware was sterilised by autoclaving for 20 minutes at 10 pounds per square inch. The same single batch of Oxoid Nutrient Broth No.2, was used for preparing all broths for growth medium and for diluents throughout this thesis. This was to ensure that the divalent cation concentration remained constant as divalent cations are known to antagonise some 4-quinolones (. Smith and Ratcliffe 1986). To make nutrient broth, 25g of Nutrient Broth powder was dissolved per litre of distilled water and dispensed in suitable volumes for autoclaving.

Nutrient agar plates for subculturing and performing viable counts were made as per nutrient broth except that Lab M agar as gelling agent was added to give a final strength of 1.5% w/v prior to autoclaving. After the molten agar had cooled to 55°C in a water bath plates were poured in sterile plastic Petri dishes (Sterilin). After the plates had set they were inverted and overdried for 45 minutes at 45°C. Plates were refrigerated for up to one week before use.

Phosphate Buffered Saline (PBS) consisted of 0.9% w/v sodium chloride in M/40 phosphate buffer at pH 4. 0.5M phosphate buffer was made by mixing 80ml of 0.5M di-sodium hydrogen orthophosphate.12H$_2$0 with 20ml sodium di-hydrogen orthophosphate in distilled water. The pH was adjusted to 7.4 with 1M NaOH and PBS was dispensed into suitable volumes for autoclaving.

Subcultivation:

Bacterial strains and mutants were subcultured on solid media by the "wire-out" technique. This involved taking a small amount of a bacterial colony on a flamed and cooled platinum-iridium wire loop and streaking it across the surface of one edge of the plate from left to right. The loop was flamed again and allowed to cool, and the right hand side of the first streak on the plate was further streaked out to the right. This was repeated once from the right hand side of the second streak and the final streak was made by a zig-zag movement of the sterile loop from the right hand side of the third streak. The plate was incubated overnight at 37°C and refrigerated until use.

Viable counting:

This was performed by making an appropriate dilution of the sample in nutrient broth and spreading 0.1ml on a nutrient agar plate. After the inocula had soaked in the plates were inverted and incubated at 37°C for two days when the colonies were counted.

Minimum Inhibitory Concentration Tests:

The minimum inhibitory concentration (MIC) was defined as the minimum concentration of a 4-quinolone that completely inhibited visible colony formation. It was determined by inoculating nutrient agar plates containing increasing concentrations of the 4-quinolone with the undiluted overnight cultures and with 10^{-2} and 10^{-4} dilutions of these cultures prepared in sterile nutrient broth, using a Denley multipoint inoculator, which delivered approximately 1µl.

Consequently the mean inoculum size tested was approximately 6×10^5 colony forming units (cfu), 6×10^3 cfu and 60 cfu, respectively. The concentration range used was a geometrically-based progression increasing successively by 25-50% at each incremental step. The ratios used were 1, 1.5, 2, 3, 4, 5 and 7.5. To make each plate the appropriate drug solution and sterile distilled water were pipetted into sterile 1oz bottles to a final volume of 3ml. Then hot sterile nutrient agar was added to a total volume of 20ml mixed by inversion and poured into a sterile Petri dish. These were overdried and used immediately. Plates were inoculated, allowed to dry and incubated at the appropriate temperature for up to three days. The results were read each day, using a drug free nutrient agar plate as the reference control plate.

Determination of mutation frequency:

The mutation frequency is the rate at which bacteria can become phenotypically different (in this case resistant to a 4-quinolone) by mutation. It was determined by inoculating 4-quinolone-containing nutrient agar plates with concentrated bacterial cultures, incubating them and counting resistant colonies. If the initial inoculum size is known, the frequency with which the bacteria become resistant can be calculated.

In this thesis the ability of up to approximately 4×10^{10} bacteria to mutate to resist 5 times the MIC (5x the MIC) obtained using approximately 6×10^5 cfu at 25, 30 and 37°C, was studied. The 4-quinolone-containing plates were prepared as for the MIC determinations. *E. coli*, *Ps. aeruginosa*, *Staph. aureus* and *Staph. epidermidis* were first grown statically at 37°C overnight in 200ml of nutrient broth in a 2 litre flask. These were then shaken at 150 cycles per minute for a further 5 hours at 37°C on an orbital incubator (Gallenkamp). For *E. coli*, *Staph. aureus* and *Staph. epidermidis* the cultures were centrifuged (MSE) at 6,000 x G for 20 minutes and resuspended in 10ml of sterile nutrient broth, resulting in an approximate 20-fold increase in cell concentration. The viable counts of the concentrated cultures were estimated on nutrient agar using 0.1ml volumes of 10^{-7} and 10^{-8} dilutions made in sterile nutrient broth. The viable counts of the concentrated cultures were approximately 6×10^{10} cfu ml^{-1} for *E. coli* and

Staph. aureus. 0.1ml aliquots of the cultures were spread on each of five plates containing 4-quinolones for each of the three temperatures studied. Thus $\approx 6 \times 10^9$ cfu were spread on each plate and the total number of bacteria tested at each temperature was approximately 3×10^{10} cfu. Plates were allowed to dry, inverted and then incubated at the appropriate temperature for up to 7 days. Colonies were counted each day.

The viable counts of the concentrated cultures of *Staph. epidermidis* were approximately $1-2 \times 10^{10}$ cfu ml^{-1}. For experiments with ciprofloxacin at 37 and 30°C, and with ofloxacin and DR-3355 at 37, 30 and 25°C, 0.1ml aliquots of the concentrated culture were spread on each of five-ten 4-quinolone-containing plates at each temperature. Thus, approximately $7.5 \times 10^9 - 1.5 \times 10^{10}$ cfu were tested in each of the above experiments. Preliminary experiments with ciprofloxacin at 25°C found that the mutation frequency of *Staph. epidermidis* to ciprofloxacin resistance was very low. To increase the number of bacteria studied, 0.1ml aliquots were spread on each of 25 ciprofloxacin-containing plates. In this experiment approximately 4.14×10^{10} cfu were tested. Plates were allowed to dry, inverted and then incubated at the appropriate temperature for up to 7 days. Colonies were counted each day.

Preliminary experiments with *Ps. aeruginosa* found high mutation frequencies to ciprofloxacin resistance.

Therefore, in subsequent experiments with ciprofloxacin,
ofloxacin and DR-3355 the cultures were not concentrated.
Instead the viable count of the shaken culture was
determined on nutrient agar using 0.1ml volumes of 10^{-6} and
10^{-7} dilutions made in sterile nutrient broth. The viable
count of the shaken cultures was approximately 3×10^9 cfu ml^{-1}
and 0.2ml volumes were spread on each of five 4-quinolone-
containing plates at each temperature. Thus $\approx 3 \times 10^9$ cfu were
tested at each temperature. Plates were allowed to dry,
inverted and then incubated at the appropriate temperature
for up to 7 days. Colonies were counted each day.

Randomly selected mutants from all temperatures were
subcultivated on the same concentration of drug from which
they were isolated and re-incubated at the the same
temperature. Mutants that grew after subcultivation at 25
or 30°C were further subcultured onto nutrient agar plates
containing 5x the MIC at 37°C of the 4-quinolone and
incubated at 37°C. This was also done using drug-free
nutrient agar plates to check for drug dependence. If a
mutant did not grow after its initial isolation this failure
rate was taken into account when the proportion of stable
mutants was calculated.   The mutation frequency was
calculated after considering the stability of the mutants
and applying Chauvenet's criterion. (Geigy, 1955) Chauvenet's criterion
is a statistical method used for eliminating values which
may differ markedly from the others in a series of
determinations. If one or more value is eliminated by

Chauvenet's criterion the mean and variance are recalculated. The statistical significance of differences between the mutation frequencies observed at each temperature was determined by the two-tailed student t-test. If P was <0.050 then the difference was regarded as being significant.

In order to characterise mutants a variety of identification tests were performed on selected mutants. For *E. coli*, the tests were: (i) indole production from lactic acid; (ii) the methyl red test and (iii) the Voges-Proskauer test. These standard tests give +, +, and − results, respectively for wild type *E. coli*.

For *Pseudomonas aeruginosa*, the tests were: (i) Liquefaction of gelatin; (ii) the Hugh and Liefson test for glucose oxidation and (iii) aerobic utilisation of glucose to produce acid. These 3 tests give characteristic positive results for wild-type *Pseudomonas aeruginosa*.

For *Staph. aureus*, a slide coagulase test was performed because this test is characteristic of wild-type *Staph. aureus* and an important pathogenicity determinant for this organism. For *Staph. epidermidis*, the API Staph (Bio Merieux S.A., Marcy-l'Etoile, France) rapid diagnostic strip test was performed; the resulting coded identification number was 6706113 for the wild-type organism.

With all of the above tests with the exception of the coagulase test for *Staph. aureus*, the expected results were found. With *Staph. aureus* there was loss of coagulase activity with a very small number of mutants isolated on 5x the MIC of DR-3355: this phenomenon has been reported previously for mutants resistant to ofloxacin (Smith, 1990a).

Determination of the bactericidal activity of 4-quinolones:
The bactericidal activity of ciprofloxacin and DR-3355 was determined for *E. coli*, *Ps. aeruginosa*, *Staph. aureus* and *Staph. epidermidis* at 20, 25, 30 and 37°C by inoculating temperature-equilibrated 4.9ml volumes of nutrient broth containing a range of ciprofloxacin or DR-3355 concentrations with 0.1ml of an overnight nutrient broth culture. For *E. coli, Staph. aureus* or *Staph. epidermidis* experiments were also carried out in PBS to inhibit bactericidal mechanism A. Treatment of bacteria in PBS does not inhibit mechanisms B or C: in order to resolve these two mechanisms the activity of the 4-quinolone must be determined in nutrient broth with chloramphenicol since bactericidal mechanism C does not kill bacteria in the presence of chloramphenicol. However, rifampicin is bactericidal against the Staphylococci, and preliminary experiments with *Staph. epidermidis* showed that the bacteriostatic activity of chloramphenicol against this organism was highly variable. Thus, for *E. coli, Staph. aureus* or *Staph. epidermidis* experiments were carried out in

nutrient broth and in PBS. The inoculum for these experiments was an overnight nutrient broth culture which had been centrifuged at 4000rpm (MSE) for 20 minutes and resuspended in PBS. This was repeated once. The reaction mixtures were incubated for 3 hours and the viable count determined on nutrient broth by plating either the undiluted reaction mixture or appropriate dilutions made in sterile nutrient broth. The % survival at each concentration was calculated by comparing the viable count after 3 hours with that of a drug-free control at time zero. The concentration range used was $0.03-9\mu gml^{-1}$ for *E. coli* and $0.3-90\mu gml^{-1}$ for *Staph. aureus* and *Staph. epidermidis*.

For *Ps. aeruginosa* the bactericidal activity of ciprofloxacin or DR-3355 was not determined in PBS because the 4-quinolones do not exhibit a biphasic response against this organism although they do possess mechanism A at lower concentrations (Morrissey and Smith, 1990). Also, the very rapid death rate of this organism treated with ciprofloxacin or DR-3355 meant that the bactericidal activity was determined after 30 minutes incubation with ciprofloxacin or DR-3355 in the concentration range $0.03-9\mu gml^{-1}$. The viable counts were made as above and the % survival was calculated as before.

Determination of the specific growth rate:

The growth rates of the bacteria in this thesis were determined at 20, 25, 30 and 37°C in nutrient broth simultaneously for up to 5 hours. For each temperature studied, 35ml of double-strength nutrient broth was sterilised in 150ml glass bottles and 33.6ml of sterile distilled water was added. When the broth was equilibrated to the required temperature, 1.4ml of an overnight culture (this represents a 1 in 50 dilution of the culture) was added to the broth (total volume= 70ml) and thoroughly mixed. A sample was taken immediately to determine the viable count at time zero, and 5ml aliquots of the inoculated broth were distributed into sterile 1oz bottles. This was done to ensure that the inoculum was identical for each time point. These were incubated in a water bath and every 1/2 hour one bottle was removed (and not replaced) and the viable count determined as described previously. With *Staph. epidermidis* at 20°C the viable count was determined every hour. The reason that the bottles were discarded after one viable count was to ensure that the reaction volume and hence the level of aeration of the broth remained constant throughout the experiment.

The specific growth rate, μ, is the slope of the straight line portion of a plot of $\log_{10}$ viable count against time. It means that in that part of the growth curve the viable count doubles in a constant time interval known as the doubling time or t_d (Pirt, 1975). The slope of the line was

calculated using the "lreg" (simple linear regression)
computer program on Unix.

Determination of the specific death rate:

The death rates of the bacteria treated with ciprofloxacin
or DR-3355 were also determined at 20, 25, 30 and 37°C over
4 hours. The concentration of ciprofloxacin or DR-3355 used
was the optimum bactericidal concentration (OBC) as
determined at 37°C. The method for determining the death
rates at each temperature was essentially the same as for
determining the growth rate except that an appropriate
volume of a 4-quinolone solution was added to the double-
strength nutrient broth and the volume was made up with
sterile distilled water.

Preliminary experiments treating *Ps. aeruginosa* with $3\mu gml^{-1}$
ciprofloxacin in nutrient broth over 4 hours showed that
this organism was killed extremely rapidly at all
temperatures studied. Thus the specific death rate of this
organism when treated with either ciprofloxacin or DR-3355
was determined by sampling every 5 minutes for 30 minutes.
Also, because the sampling interval was small the death
curves for each temperature were determined individually on
the same day.

The specific death rate for the bacteria treated with 4-
quinolones, μ_q, is the slope of the straight portion of a
plot of log_{10} viable count against time and it means that

the viable count halves in a constant time interval, which

will be called t_h. The slope of the line was calculated

using the "lreg" (simple linear regression) program on Unix.

SECTION 3:

Effects of temperature on the mutation frequency to 4-quinolone resistance:

The effect of selection temperature on the frequency of mutation to 4-quinolone resistance was determined with *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* at 25, 30 and 37°C. To ensure that the bacteria were exposed to the same relative 4-quinolone selective pressure at each temperature, in terms of multiples of the minimum inhibitory concentration (MIC), the effect of temperature on the MICs of ciprofloxacin, ofloxacin and DR-3355 was first determined for each of the bacterial species.

Tables 1, 2 and 3 show the MICs for ciprofloxacin, ofloxacin and DR-3355, respectively, against three different inocula of of *E. coli*, *Ps. aeruginosa*, *Staph. aureus* and *Staph. epidermidis*. The mean inoculum sizes were approximately 6×10^5 colony forming units (cfu), 6×10^3 cfu and 60 cfu.

For ciprofloxacin (Table 1), the susceptibility of *E. coli* was much greater than that of *Ps. aeruginosa* or the two Gram positive species, *Staph. aureus* or *Staph. epidermidis*. At 37°C the MICs for the lowest inoculum were 0.0075, 0.075, 0.15 and $0.2 \mu gml^{-1}$, respectively.

With *E. coli*, lowering the temperature of incubation reduced

Table 1: Effects of temperature on the minimum inhibitory concentrations of ciprofloxacin.

BACTERIA	INOCULUM SIZE	MINIMUM INHIBITORY CONCENTRATION (µgml⁻¹)		
		25°C	30°C	37°C
E. coli	6×10^5 cfu 6×10^3 cfu 60 cfu	0.005 0.004 0.004	0.0075 0.0075 0.0075	0.02 0.01 0.0075
Pseudo. aeruginosa	6×10^5 cfu 6×10^3 cfu 60 cfu	0.15 0.15 0.1	0.15 0.1 0.1	0.2 0.075 0.075
St. aureus	6×10^5 cfu 6×10^3 cfu 60 cfu	0.1 0.1 0.1	0.15 0.1 0.1	0.2 0.15 0.15
St. epidermidis	6×10^5 cfu 6×10^3 cfu 60 cfu	0.4 0.4 0.3	0.3 0.3 0.3	0.2 0.2 0.2

cfu= colony forming units

the MIC for all three inoculum sizes tested. The effect was most pronounced with the highest inoculum size, the MIC decreasing 4-fold from 0.02µgml⁻¹ at 37°C to 0.005µgml⁻¹ at 25°C; with the intermediate inoculum size the MIC at 37°C was 0.01µgml⁻¹ decreasing to 0.004µgml⁻¹ at 25°C. With the lowest inoculum size the MIC at both 37°C and 30°C was 0.0075µgml⁻¹ which decreased to 0.004µgml⁻¹ at 25°C.

With *Ps. aeruginosa*, the MIC response to temperature for the highest inoculum size was the same as that observed with all inoculum sizes of *E. coli*, but was the opposite of that observed with the lower inoculum sizes of *Ps. aeruginosa*. For the highest inoculum size, the MIC decreased from

$0.2\mu gml^{-1}$ at 37°C to $0.15\mu gml^{-1}$ at either 30 or 25°C.
However, with the intermediate inoculum size the MIC
progressively increased 2-fold from 0.075 to $0.15\mu gml^{-1}$
between 37 and 25°C, and with the lowest inoculum size the
MIC also increased from $0.075\mu gml^{-1}$ at 25°C to $0.1\mu gml^{-1}$ at
either 30 or 25°C.

With *Staph. aureus* as with *E. coli*, lowering the incubation
temperature reduced the MIC for all inoculum sizes tested.
With the highest inoculum size, the MIC progressively
decreased from 0.2 to $0.1\mu gml^{-1}$ between 37 and 25°C, and for
the two lower inocula the MICs decreased from $0.15\mu gml^{-1}$ at
37°C to $0.1\mu gml^{-1}$ at either 30 or 25°C.

With *Staph. epidermidis*, the temperature trend was quite
different from that of any of the other organisms tested
because the MIC values for the higher two inoculum sizes
increased from $0.2\mu gml^{-1}$ at 37°C to $0.3\mu gml^{-1}$ at 30°C and to
$0.4\mu gml^{-1}$ at 25°C. The trend for the 10^{-4} inoculum was the
same except that at 25°C the MIC was identical to that at
30°C.

Ofloxacin (Table 2) was found to be less potent than
ciprofloxacin (Table 1), and in addition the overall ranking
of susceptibility of the four bacteria was different with
the two drugs. As before, *E. coli* was the most susceptible
of the bacteria tested, but *Staph. aureus* and *Staph.*

epidermidis were more susceptible than *Ps. aeruginosa*. The MICs at 37°C for the lowest inoculum were 0.03, 0.15, 0.2 and 0.4µgml^{-1}, respectively.

Table 2: Effects of temperature on the minimum inhibitory concentration of ofloxacin:

BACTERIA	INOCULUM SIZE	MINIMUM INHIBITORY CONCENTRATION (µgml^{-1})		
		25°C	30°C	37°C
E. coli	6x10^5 cfu 6x10^3 cfu 60 cfu	0.02 0.015 0.015	0.03 0.02 0.02	0.03 0.03 0.03
Pseudo. aeruginosa	6x10^5 cfu 6x10^3 cfu 60 cfu	1.0 0.75 0.4	0.75 0.75 0.5	0.75 0.5 0.4
St. aureus	6x10^5 cfu 6x10^3 cfu 60 cfu	0.075 0.075 0.075	0.15 0.15 0.15	0.2 0.15 0.15
St. epidermidis	6x10^5 cfu 6x10^3 cfu 60 cfu	0.3 0.3 0.2	0.3 0.3 0.3	0.2 0.2 0.2

cfu= colony forming units

With *E. coli* at the highest inoculum size the MIC was 0.03µgml^{-1} at 37 or 30°C and decreased to 0.02µgml^{-1} at 25°C. With both the other inoculum sizes the MIC values decreased from 0.03 to 0.02 to 0.015µgml^{-1} at 37, 30 and 25°C, respectively.

With *Ps. aeruginosa* the temperature of incubation had variable effects on the MIC of ofloxacin. With the highest inoculum size at 37 and 30°C the MIC was 0.75µgml^{-1}, increasing to 1.0µgml^{-1} at 25°C. With the intermediate

inoculum size the MIC value at 37°C was 0.5µgml^{-1} increasing

to 0.75µgml^{-1} at either 30 or 25°C. With the lowest inoculum

size the MIC values at 25 and 37°C were identical at

0.4µgml^{-1} with the MIC value at the 30°C being 0.5µgml^{-1}.

With *Staph. aureus* at the two lower inoculum sizes the MIC

value at 37 or 30°C was 0.15µgml^{-1}, decreasing to 0.075µgml^{-1}

at 25°C. The results were similar for the highest inoculum

except that at 37°C the MIC was 0.2µgml^{-1}.

With *Staph. epidermidis* the MICs for all the inoculum sizes

at 37 and 30°C were the same, at 0.2 and 0.3µgml^{-1},

respectively. At 25°C the MIC for the higher two inoculum

sizes was 0.3µgml^{-1} but with the lowest inoculum size the MIC

value was 0.2µgml^{-1}.

Table 3 shows the results for DR-3355. Overall, this drug

was approximately twice as active as ofloxacin (Table 2),

and only slightly less active than ciprofloxacin (Table 1)

as determined by its MIC values for the bacteria studied.

The ranking of susceptibility of the bacteria was the same

as that for ofloxacin. The MICs for the lowest inoculum

size at 37°C were 0.015, 0.075, 0.1 and 0.2µgml^{-1} for *E.*

coli, Staph. aureus, Staph. epidermidis and *Ps. aeruginosa*,

respectively.

With *E. coli* the MIC obtained with the largest inoculum size

decreased from 0.02 to 0.0075µgml^{-1} between 37 and 25°C, and

with the other two inoculum sizes the MIC values at 37 or

30°C were 0.015µgml^{-1} decreasing to 0.0075µgml^{-1} at 25°C.

With *Ps. aeruginosa* at the highest inoculum size the MIC

value at 37°C was 0.4µgml^{-1} increasing to 0.5µgml^{-1} at 30 or

25°C. For the intermediate inoculum size the MIC value at

37°C was 0.2µgml^{-1} increasing to 0.4µgml^{-1} at either 30 or

25°C. The trend with the lowest inoculum size was similar

except that at 25°C the MIC value was 0.3µgml^{-1}.

Table 3: Effects of temperature on the minimum inhibitory concentration of DR-3355.

BACTERIA	INOCULUM SIZE	MINIMUM INHIBITORY CONCENTRATION (µgml^{-1})		
		25°C	30°C	37°C
E. coli	6x10^5 cfu 6x10^3 cfu 60 cfu	0.0075 0.0075 0.0075	0.015 0.015 0.015	0.02 0.015 0.015
Pseudo. aeruginosa	6x10^5 cfu 6x10^3 cfu 60 cfu	0.5 0.4 0.3	0.5 0.4 0.4	0.4 0.2 0.2
St. aureus	6x10^5 cfu 6x10^3 cfu 60 cfu	0.04 0.04 0.04	0.075 0.075 0.075	0.075 0.075 0.075
St. epidermidis	6x10^5 cfu 6x10^3 cfu 60 cfu	0.1 0.1 0.075	0.15 0.1 0.1	0.1 0.1 0.1

cfu= colony forming units

With *Staph. aureus* the temperature trend was identical for

all three inoculum sizes tested. The MIC values at 37 or

30°C were 0.075µgml⁻¹ and decreased to 0.04µgml⁻¹ at 25°C.

With *Staph. epidermidis* the temperature of incubation had no
effect on the MIC (0.1µgml⁻¹) of the intermediate inoculum
size. With the highest inoculum size the MIC at 37 or 25°C
was 0.1µgml⁻¹ but increased to 0.15µgml⁻¹ at 30°C. With the
lowest inoculum size the MIC values at 37 or 30°C were
0.1µgml⁻¹ and decreased to 0.075µgml⁻¹ at 25°C.

From these results it can be seen that the temperature of
incubation marginally affected the susceptibility of the
bacteria to ciprofloxacin, ofloxacin or DR-3355 as
determined by MIC tests. The general trend was that
reducing the temperature from 37 to 30°C, or from 30 to
25°C, either had no effect on, or increased the
susceptibility of the bacteria to the drugs. The most
obvious exception to this was with *Staph. epidermidis* and
ciprofloxacin, where the MIC increased as the incubation
temperature was decreased.

Thus, in order for the selective pressure to be standardised
for the isolation of 4-quinolone-resistant mutants, the
bacteria were exposed to five times the MIC value obtained
using the highest inoculum size (5x the MIC) at each
temperature. Nutrient agar plates containing 5x the MIC
values of ciprofloxacin, ofloxacin or DR-3355 were
inoculated with concentrated cultures of bacteria as

described in the "Materials and Methods" section and
incubated for up to 7 days at 25, 30 or 37°C. Mutant
colonies were counted and selected mutants from each
temperature were subcultivated and characterised as
described in the "Materials and Methods" section. The
number of mutants that failed subcultivation were taken into
account in subsequent calculations. After application of
Chauvenet's criterion to the counts the number of mutants
was compared to the viable count on drug-free nutrient agar
to calculate the frequency with which the bacteria mutated
to 4-quinolone resistance. The mutation frequencies for
each bacteria-4-quinolone combination at 25, 30 and 37°C are
shown in Table 4.

**Table 4: Effect of temperature on the mutation frequency to
resist 5x the MIC of 4-quinolones.**

		MUTATION FREQUENCY TO 5x THE MIC		
BACTERIA	DRUG	25°C	30°C	37°C
E. coli	CIP	1.75×10^{-8}	2.88×10^{-8}	6.24×10^{-9}
	OFL	2.63×10^{-8}	3.13×10^{-8}	5.32×10^{-8}
	DR-3355	2.87×10^{-8}	1.55×10^{-8}	1.26×10^{-8}
Pseudo. aeruginosa	CIP	1.42×10^{-7}	1.38×10^{-7}	1.07×10^{-7}
	OFL	3.10×10^{-8}	8.18×10^{-8}	8.39×10^{-8}
	DR-3355	1.49×10^{-8}	1.91×10^{-8}	5.40×10^{-8}
St. aureus	CIP	2.96×10^{-8}	4.80×10^{-8}	1.57×10^{-8}
	OFL	9.65×10^{-8}	3.34×10^{-8}	1.53×10^{-8}
	DR-3355	8.04×10^{-8}	8.48×10^{-9}	1.13×10^{-7}
St. epidermidis	CIP	4.05×10^{-10}	2.48×10^{-9}	2.70×10^{-8}
	OFL	–	2.06×10^{-8}	1.42×10^{-7}
	DR-3355	–	1.09×10^{-8}	8.43×10^{-8}

MIC=Minimum Inhibitory Concentration; CIP=Ciprofloxacin;
OFL=Ofloxacin; – =Mutants were unstable.

For each bacterial species tested the significance of the
differences between the mutation frequencies at 37 and 30°C,

30 and 25°C, and 37 and 25°C was determined by the two-tailed student t-test. Tables 5, 6 and 7 show the probability (P) that the mutation rates for ciprofloxacin, ofloxacin and DR-3355, respectively, were significantly different. Differences between the mutation rates were regarded as being significant if P= <0.050.

Figure 2 shows the mutation frequency to resist 5x the MIC of ciprofloxacin as a function of selection temperature for $E.$ $coli$, $Ps.$ $aeruginosa$, $Staph.$ $aureus$ and $Staph.$ $epidermidis$. Ciprofloxacin-resistant mutants were found for all of the bacteria tested at all of the temperatures

Figure 2: Mutation frequency to resist 5x the MIC[37] of ciprofloxacin.
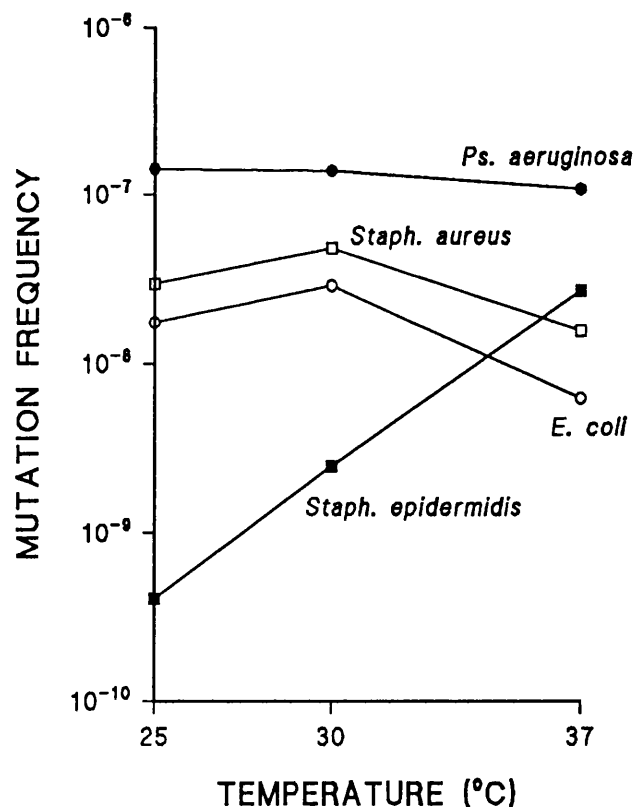
studied, although the frequency at which they were isolated varied for each of the species and was affected differently by the incubation temperature. For *E. coli*, the mutation rate at 37°C was 6.24×10^{-9} increasing to 2.88×10^{-8} at 30°C but when the incubation temperature was reduced to 25°C the mutation frequency decreased to 1.75×10^{-8}. Thus there was a temperature optimum for isolation of mutants resistant to ciprofloxacin, which was 30°C. This phenomenon has been previously reported by Smith (1986a) who was unable to isolate any ciprofloxacin-resistant mutants at 37°C in 10^{12} bacteria studied. A possible reason for this discrepancy is that Smith (1986a) used a different isolation method to that used here. In this thesis the inocula were spread onto 4-quinolone-containing plates (see the "Materials and Methods" section) but Smith (1986a) used overlays containing higher numbers of bacteria to inoculate the drug plates. The differences between the mutation rates at 37, 30 and 25°C were all found to be significant by the student *t*-test (Table 5).

With *Staph. aureus* a similar temperature profile to that observed with *E. coli* was found except that the mutation rates for *Staph. aureus* were slightly higher at all three temperatures. At 37°C the mutation rate was 1.57×10^{-8}, peaking to 4.80×10^{-8} at 30°C and decreasing to 2.96×10^{-8} at 25°C. The difference between the mutation frequencies at 25 and 30°C was found to be not significant ($P = >0.050$) by the student *t*-test (Table 5), but the differences between the

mutation frequencies at either 25 and 37°C, or 30 and 37°C

Table 5: *P* values for the significance of the difference between the mutation frequencies to ciprofloxacin resistance at 25, 30 and 37°C as determined by the two-tailed student *t*-test.

BACTERIA	25 *vs* 30°C	30 *vs* 37°C	25 *vs* 37°C
E.coli	0.010	0.001	0.001
Ps. aeruginosa	>0.050	0.050	0.010
Staph. aureus	>0.050	0.050	0.050
Staph. epidermidis	0.001	0.001	0.001

were significant. In addition, it was found that the acquisition of ciprofloxacin resistance had no effect on coagulase activity because when 12 mutants were tested by the slide coagulase test all were found to be coagulase-positive.

With *Ps. aeruginosa* the mutation frequencies were very high at all of the temperatures studied, being in the 10^{-7} region. As was found with *E. coli* and with *Staph. aureus* a temperature optimum for selection of ciprofloxacin-resistant mutants occurred, but with *Ps. aeruginosa* it was at 25°C. The mutation frequency increased from 1.07×10^{-7} at 37°C to 1.38×10^{-7} at 30°C and to 1.42×10^{-7} at 25°C. There was no significant difference (*P*= >0.050) as determined by the student *t*-test between the mutation rates at 25 or 30°C, but

the mutation frequency at 37°C was significantly lower than that observed at either of the two lower temperatures (Table 5).

In marked contrast to the results found with the other three bacterial species, *Staph. epidermidis* exhibited a progressive *decrease* in the mutation frequency to ciprofloxacin resistance as the temperature was reduced from 37 to 30°C and from 30 to 25°C. There was as much as an order of magnitude difference with each temperature increment, which not surprisingly proved to be significant ($P= 0.001$, Table 5). Only one quarter of mutants isolated at 25°C could be subcultivated on the same concentration of ciprofloxacin from which they were isolated. However, the mutation frequency at 37°C (2.70×10^{-8}) was higher than that found for either *E. coli* or for *Staph. aureus* at 37°C, but the mutation frequency of *Ps. aeruginosa* to ciprofloxacin resistance was highest of all.

Figure 3 shows the effect of selection temperature on mutation frequency of the four bacteria to resist 5x the MIC of ofloxacin. As before, mutants were found for all of the bacteria tested at all three temperatures, but the temperature profiles were quite different from those observed with ciprofloxacin (Figure 2). With *E. coli* reducing the selection temperature from 37 to 30 to 25°C decreased the mutation frequency progressively from 5.32×10^{-8} to 3.13×10^{-8} and then to 2.63×10^{-8}, respectively.

Figure 3: Mutation frequency to resist 5x the MIC[37] of ofloxacin.
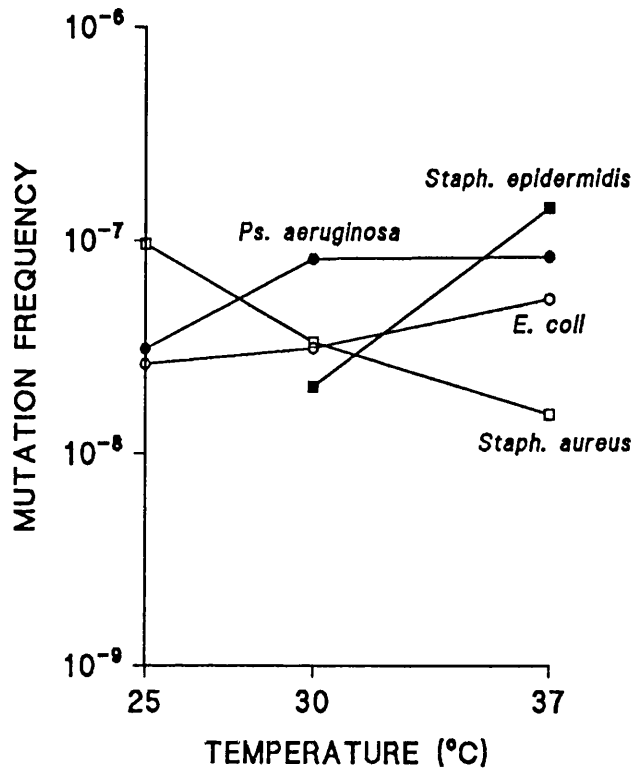
The mutation frequencies at either 25 or 30°C were significantly lower than that at 37°C, but the difference between the mutation rates at 25 and 30°C was not significant ($P= >0.050$) as determined by the student t-test (Table 6). These mutation rates were individually higher than those to ciprofloxacin resistance at either 25, 30 or 37°C despite no temperature optimum for ofloxacin resistance being observed. These findings are in contrast to the results of Smith (1986a) who was unable to isolate ofloxacin-resistant mutants at 37, 30 or 25°C in the 10^{12} bacteria tested at each temperature. A possible methodological reason for the differences between these findings and those of Smith (1986a) was mentioned earlier.

With *Ps. aeruginosa* the mutation frequencies at 37 and 30°C were similar ($P=$ >0.050, Table 6) at 8.39×10^{-8} and 8.18×10^{-8}, respectively, decreasing sharply to 3.10×10^{-8} at 25°C. The mutation rate at 25°C differed significantly from those obtained at the two higher temperatures (Table 6). These frequencies were lower than those to ciprofloxacin resistance (Figure 2), with the greatest difference being at 25°C.

TABLE 6: *P* **values for the significance of the difference between the mutation frequencies to ofloxacin resistance at 25, 30 and 37°C as determined by the two-tailed student *t*-test.**

BACTERIA	25 *vs* 30°C	30 *vs* 37°C	25 *vs* 37°C
E. coli	>0.050	0.010	0.010
Ps. aeruginosa	0.010	>0.050	0.010
Staph. aureus	0.001	0.010	0.001
Staph. epidermidis	–	0.001	–

With *Staph. aureus* reducing the selection temperature progressively increased the mutation rate from 1.53×10^{-8} at 37°C to 3.34×10^{-8} at 30°C and to 9.65×10^{-8} at 25°C. Table 6 shows that all these mutation frequencies differed significantly from each other. Acquisition of ofloxacin resistance did not cause any loss of coagulase activity in any of the 54 mutants tested. These findings are in contrast to those of Smith (1986b) who found that coagulase

activity was lost in 71% of mutants of *Staph. aureus* which
became resistant to 5x the MIC of ofloxacin at 37°C.
However, in agreement with Smith (1986b) no loss of
coagulase was found with the acquisition of ciprofloxacin-
resistance.

With *Staph. epidermidis* a similar trend to that found with
ciprofloxacin was observed in that the mutation rate
decreased from the high value of 1.42×10^{-7} at 37°C to
2.06×10^{-8} at 30°C and this difference was significant (Table
6). Mutants were isolated at 25°C at a frequency of 3.00×10^{-9}
but none of the 10 mutants that were tested could be
subcultured on the same concentration of ofloxacin at 25°C.
As stated in the "Materials and Methods" section the
proportion of mutants that failed subcultivation was taken
into account when calculating the mutation frequency. Since
the mutants isolated at 25°C were unstable they were omitted
from Figure 3. The temperature profile for *Staph.
epidermidis* was the opposite to that of *Staph. aureus*,
exhibiting either the highest or the lowest mutation rates
out of the four bacteria tested at 37 or 25°C.

Figure 4 shows the effect of selection temperature on the
mutation frequency of the four bacteria to resist 5x the MIC
of DR-3355. Even though DR-3355 is the active antibacterial
isomer of the racemic mixture ofloxacin, the mutation
frequency-temperature profile was quite different to that of
ofloxacin (Figure 3).

Figure 4: Mutation frequency to resist 5x the MIC[37] of DR-3355.
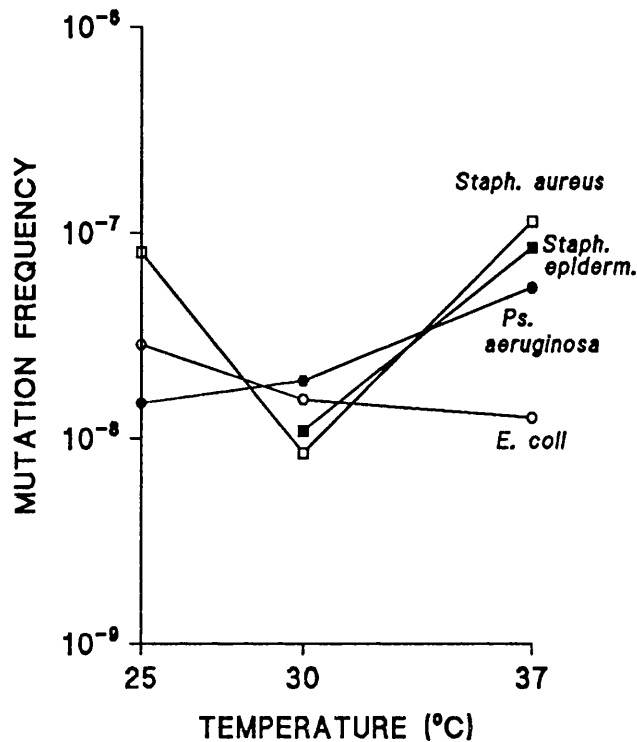
With *E. coli* the mutation rate to resist 5x the MIC of DR-3355 at 37°C was 1.26×10^{-8}, increasing slightly to 1.55×10^{-8} at 30°C and increasing again to 2.87×10^{-8} at 25°C. The difference between the mutation rates at 37 and 30°C was found to be not significant by the student *t*-test (*P*= >0.050), but the mutation frequency at 25°C was significantly higher than that at either 30 or 37°C (Table 7). The mutation rates to resist DR-3355 were lower than those for resistance to ofloxacin at 37 and 30°C, and virtually the same at 25°C (Figure 5). The mutation rates for resistance to ciprofloxacin were lower than those for the other two drugs at either 37 or 25°C, but similar to that for ofloxacin at 30°C.

Figure 5: Mutation frequency of *E. coli* to resist 5x the MIC[37] of 4-quinolones.
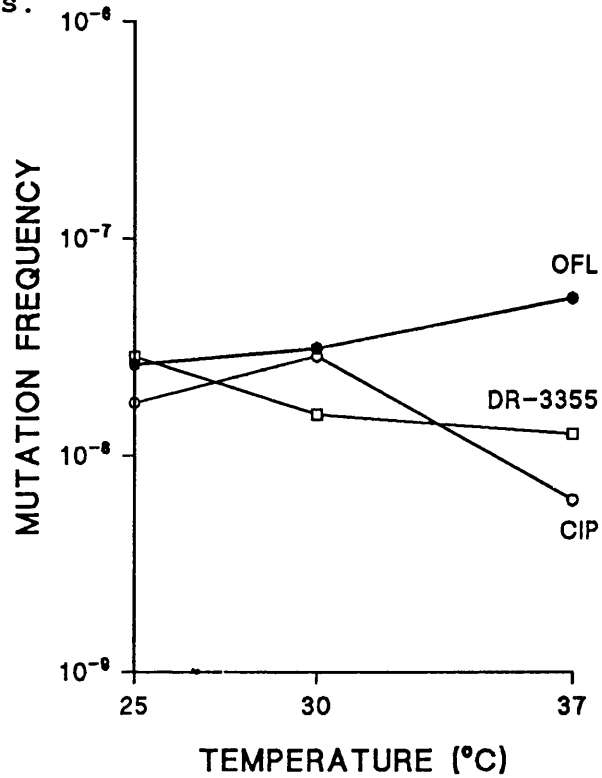
TABLE 7: *P* values for the significance of the difference between the mutation frequencies to DR-3355 resistance at 25, 30 and 37°C as determined by the two-tailed student *t*-test.

BACTERIA	25 *vs* 30°C	30 *vs* 37°C	25 *vs* 37°C
E. coli	0.001	>0.050	0.010
Ps. aeruginosa	>0.050	0.001	0.001
Staph. aureus	0.050	0.001	>0.050
Staph. epidermidis	–	0.001	–

With *Ps. aeruginosa* lowering the incubation temperature from 37 to 30 to 25°C progressively decreased the mutation frequency from 5.40×10^{-8} to 1.91×10^{-8} and then to 1.49×10^{-8}, respectively (Figure 4). The mutation rates at 25 and 30°C were not significantly different from each other ($P=$ >0.050), but they were significantly lower than that at 37°C (Table 7). These mutation rates were lower than those to resist either ciprofloxacin or ofloxacin at all three temperatures studied (Figure 6). Overall, decreasing the incubation temperature increased the mutation rate to resist ciprofloxacin but decreased the mutation rate to resist either ofloxacin or DR-3355.

Figure 6: Mutation frequency of *Ps. aeruginosa* to resist 5x the MIC[37] of 4-quinolones.
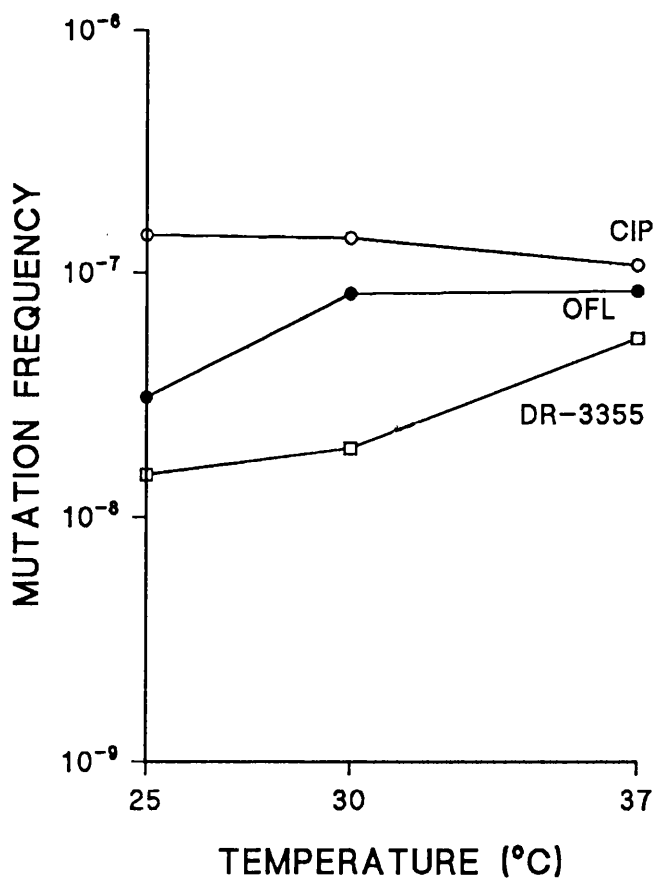
With *Staph. aureus* the mutation frequency-temperature

profile was very unusual in that the mutation rate for

resistance to 5x the MIC of DR-3355 at 30°C (8.48×10^{-9}) was

significantly lower than the very high rates found at either

37 (1.13×10^{-7}) or at 25°C (8.04×10^{-8}) (Table 7). Indeed, the

mutation rates at 37 and 25°C were not significantly

different as determined by the student *t*-test ($P=$ >0.050,

Table 7). The results were so unusual that the experiment

was repeated several times and the data presented is from a

single experiment, the results of which were typical of the

other experiments. The temperature profile for the

frequency of isolation of mutants resistant to 5x the MIC of

DR-3355 was very different to those found either for

Figure 7: Mutation frequency of *Staph. aureus* to resist 5x

the MIC[37] of 4-quinolones.
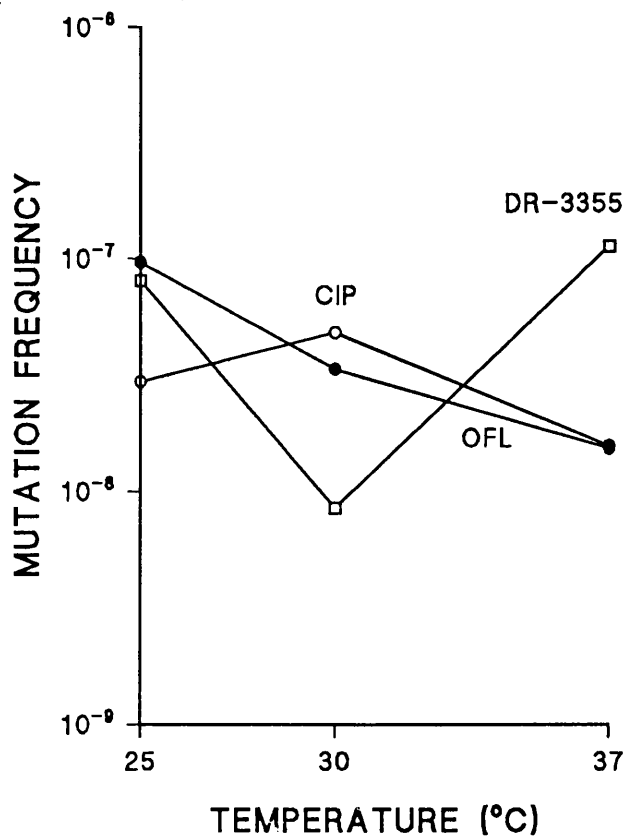
ciprofloxacin or for ofloxacin resistance (Figure 7).
Unlike the findings with either ciprofloxacin or ofloxacin
the acquisition of DR-3355-resistance affected coagulase
activity, because 3 (≈6%) of the 48 mutants tested had
become coagulase-negative.

With *Staph. epidermidis* (Figure 4) the temperature profile
for DR-3355 resistance was similar to that of ofloxacin
although the mutation rates were slightly lower at 8.43×10^{-8}
and 1.09×10^{-8} at 37 and 30°C, respectively, but higher than
those found with ciprofloxacin (Figure 8). The mutation
frequencies at 30 and 37°C were significantly different
(Table 7).

**Figure 8: Mutation frequency of *Staph. epidermidis* to resist
5x the MIC[37] of 4-quinolones.**
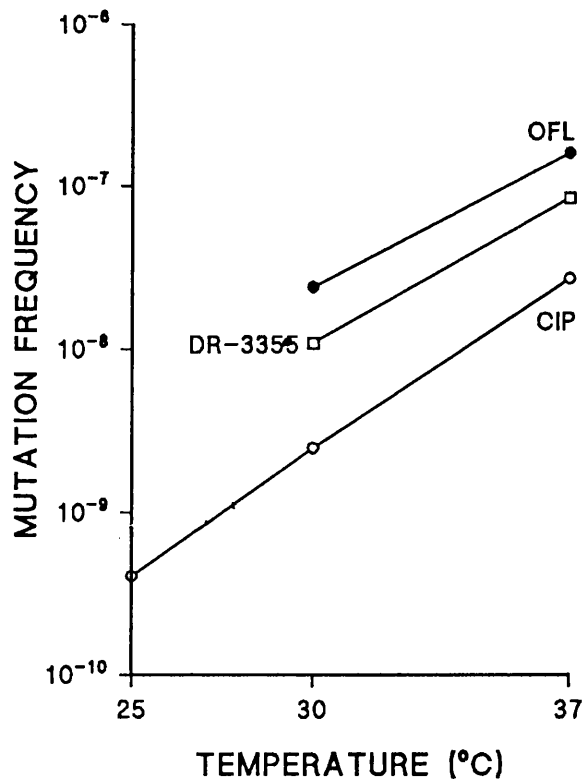
At 25°C mutants were initially isolated at a frequency of 8.75×10^{-9}. However, as was found with ofloxacin none of 10 mutants isolated at 25°C that were tested was able to withstand subcultivation on 5x the MIC of DR-3355 at 25°C and since these mutants were unstable they were omitted from Figure 4.

All mutants isolated which resisted any one of the three drugs tested could be subcultivated at 37°C on 5x the MIC value at 37°C, so none was a temperature-sensitive mutant.

In conclusion, mutants that resisted 5x the MIC of ciprofloxacin, ofloxacin or DR-3355 were isolated at 25, 30 or 37°C for *E. coli*, *Ps. aeruginosa*, *Staph. aureus* or *Staph. epidermidis*. The temperature of selection *did* affect the frequency at which the mutants could be isolated but no temperature trend was found for all drug-bacteria combinations except for *Staph. epidermidis*, where reducing the temperature of incubation always decreased the mutation frequency. Interestingly, with *Staph. epidermidis* three quarters of mutants isolated on ciprofloxacin at 25°C and all mutants that were isolated at 25°C on ofloxacin or on DR-3355 were unstable. The most unusual temperature profile was that seen with *Staph. aureus* and DR-3355 (Figure 4), whereby its mutation rates at 25 and 37°C were much higher than that observed at 30°C.

The generally high mutation rates to 4-quinolone resistance found here agree well with the emergence of clinical resistance over the last decade, especially with *Ps. aeruginosa* and *Staph. aureus* (Kresken and Weidemann, 1988; Blumberg *et al*, 1988). Resistance development also seems to be more likely to occur at certain sites of infection, such as decubitus ulcers or in abscesses, where conditions may be anaerobic and there are large numbers of organisms (Ball, 1990). Such sites of infection are at lower-than-body temperature so it seems possible that a contributory factor in the development of resistance to ciprofloxacin by *Ps. aeruginosa* or *Staph. aureus* is the greater mutation rate at lower temperatures. On the other hand, resistance development by *Staph. epidermidis* seems less likely to occur at sites which are at lower-than-body temperature, and mutants that were resistant at lower temperatures were found to be less stable than those isolated at 37 or 30°C.

SECTION 4:

Effect of temperature on the bactericidal activities of 4-quinolones:

The effects of temperature on the bactericidal activities of ciprofloxacin and DR-3355 were investigated against *E. coli*, *Ps. aeruginosa*, *Staph. aureus* and *Staph. epidermidis* by exposing the bacteria to a range of concentrations of either of the two drugs, as described in the methods section, at 20, 25, 30 or 37°C. As described in the "Introduction" section some 4-quinolones possess more than one bactericidal mechanism against certain bacterial species (Smith, 1984a). Mechanism A requires that the bacteria be capable of protein- and RNA-synthesis and cell division. Thus, mechanism A is inhibited when bacteria are treated in nutrient broth simultaneously with chloramphenicol or rifampicin, or when the bacteria are suspended in phosphate-buffered saline (PBS), respectively. Mechanism B has none of the prerequisites of mechanism A. Bactericidal mechanism C kills non-dividing bacteria but requires that the bacteria are capable of protein- and RNA-synthesis (Ratcliffe and Smith, 1985). Thus, 4-quinolones that possess mechanism C are bactericidal in PBS but not in nutrient broth plus either chloramphenicol or rifampicin. Rifampicin is bactericidal against Staphylococci and preliminary experiments with chloramphenicol showed that its activity against *Staph. epidermidis* was highly variable. Thus with *E. coli*, *Staph. aureus* and *Staph. epidermidis* the activity

of either ciprofloxacin or DR-3355 was determined in nutrient broth and in PBS to inhibit bactericidal mechanism A after 3 hours. The bactericidal activities against *Ps. aeruginosa* were determined after 30 minutes in nutrient broth only because of the hypersusceptibility of this organism to 4-quinolones (Morrissey and Smith, 1990). The activity of the 4-quinolones against this species was not determined in PBS because the 4-quinolones do not exhibit a biphasic reponse against *Ps. aeruginosa* (Morrissey and Smith, 1990). By plotting the log of percentage survival (compared to a drug-free control) against the log of 4-quinolone concentration the bactericidal "profile" of the drug can be seen. For *E. coli* and *Ps. aeruginosa* the concentration range used for both drugs was 0.03-9µgml^{-1}, and for the Staphylococci the concentration range used was 0.3-90µgml^{-1}.

Bactericidal activity of ciprofloxacin against *E. coli*:
Figure 9 shows the bactercidal profiles of ciprofloxacin against *E. coli* in nutrient broth and in PBS at 37°C. In nutrient broth ciprofloxacin exhibited a biphasic response, first demonstrated with nalidixic acid by Crumplin and Smith (1975) i.e. progressively more bactericidal activity was observed as the concentration of ciprofloxacin was increased from 0.03µgml^{-1} up to a concentration known as the most bactericidal concentration or the optimum bactericidal concentration (OBC), in this case 0.15µgml^{-1} ciprofloxacin (Table 8). At the OBC survival was 0.08% and as the

Table 8: Effect of temperature on bactericidal activity of ciprofloxacin against _E. coli_.

Medium	Nutrient Broth		PBS
Temp. (°C)	OBC (μgml^{-1})	% Survival	% Survival
20	0.3	5.45	25.30
25	0.15	1.00	21.56
30	0.3	0.56	20.00
37	0.15	0.08	8.58

concentration was increased beyond 0.15μgml^{-1} the bactericidal activity was progressively reduced until at 9μgml^{-1} ciprofloxacin survival had increased to approximately 3%. It can also be seen from Figure 9 that when the experiment was conducted in PBS at 37°C the biphasic response was abolished and at the OBC survival in PBS was 8.58% representing approximately 107-fold less activity compared to that in nutrient broth.

Figure 10 shows the bactericidal profiles of ciprofloxacin against _E. coli_ in nutrient broth and in PBS at 30°C. A biphasic response was observed in nutrient broth and it was abolished in PBS. The OBC was 0.3μgml^{-1} which was slightly higher than that observed at 37°C (Figure 9) and the survival at the OBC in nutrient broth at 30°C was 0.56% which was also higher than that at 37°C (Table 8). As the concentration of ciprofloxacin was increased beyond the OBC the survival increased progressively to 10.82% at 9μgml^{-1}. When the experiment was performed in PBS, as the concentration of ciprofloxacin was increased from 0.03 to

Figure 9: Bactericidal activity of ciprofloxacin against *E. coli* at 37°C.
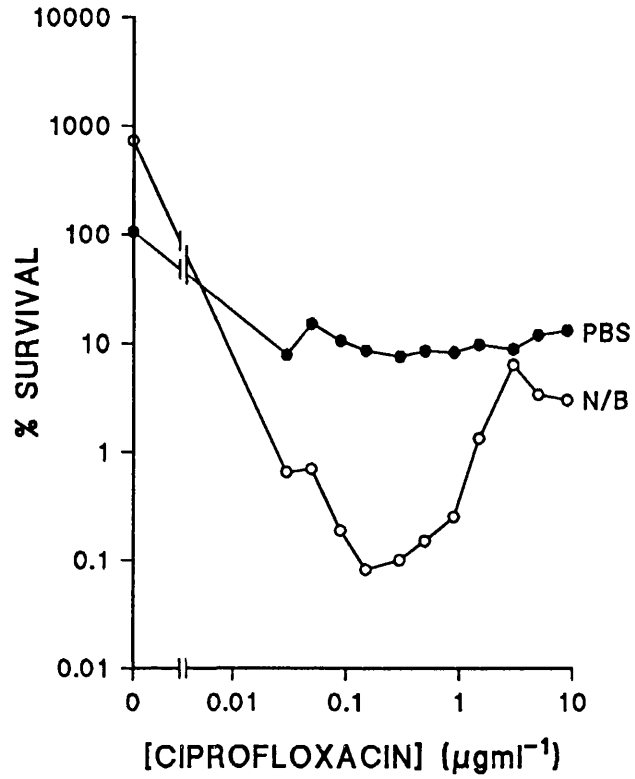
Figure 10: Bactericidal activity of ciprofloxacin against *E. coli* at 30°C.
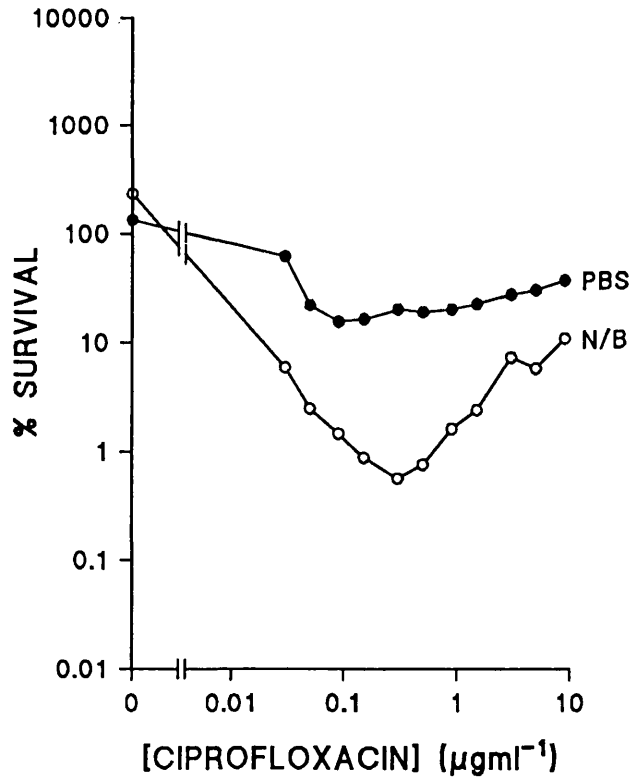
$0.09\mu gml^{-1}$ survival dropped from 61.54 to 15.85%. As the concentration was increased from $0.09\mu gml^{-1}$ there was progressively less death until at $9\mu gml^{-1}$ survival was approximately 37%. However, at the OBC, survival in PBS was 20.00% which was about 36-fold higher than that observed in nutrient broth.

Figure 11 shows the bactericidal profiles of ciprofloxacin against *E. coli* in nutrient broth and in PBS at 25°C. As before, a biphasic response was exhibited in nutrient broth and it was abolished in PBS. The OBC was $0.15\mu gml^{-1}$ which was the same as that found at 37°C (Table 8), but at the OBC the survival at 25°C was 1.00%, compared to survivals of 0.08 and 0.56% at the OBCs obtained at 37 and 30°C, respectively. As the concentration of ciprofloxacin was increased beyond the OBC progressively less death was seen and at $9\mu gml^{-1}$ survival was 12.67%. In PBS there was little activity at any of the concentrations tested: survival at the OBC was 21.56%, which was about 22-fold higher than that in nutrient broth.

Figure 12 shows the bactericidal profiles of ciprofloxacin against *E. coli* in nutrient broth and in PBS at 20°C. A biphasic response was exhibited in nutrient broth and the OBC ($0.3\mu gml^{-1}$) was the same as that at 30°C but at 20°C the survival at the OBC was 5.45%. Above the OBC the survival increased progressively to 25.56% at $9\mu gml^{-1}$ ciprofloxacin. In PBS there was more survival at all concentrations of

Figure 11: Bactericidal activity of ciprofloxacin against *E. coli* at 25°C.
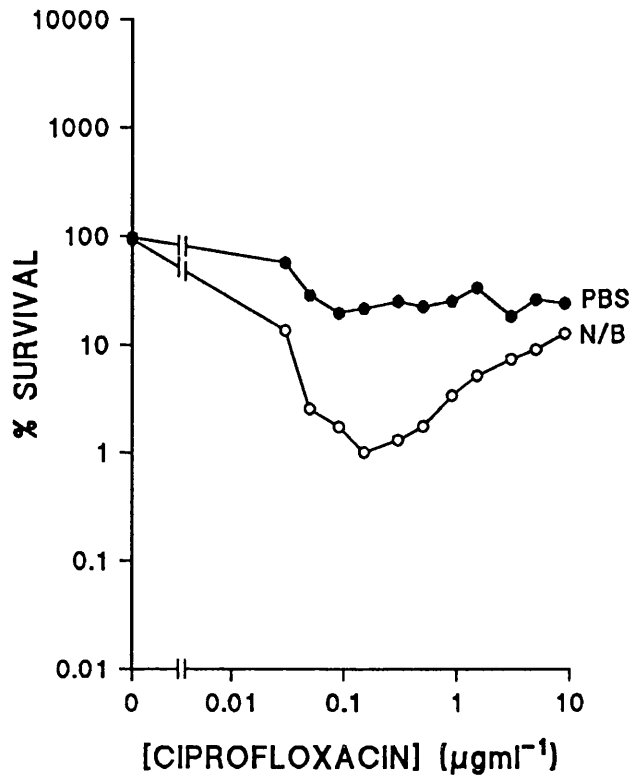
Figure 12: Bactericidal activity of ciprofloxacin against *E. coli* at 20°C.
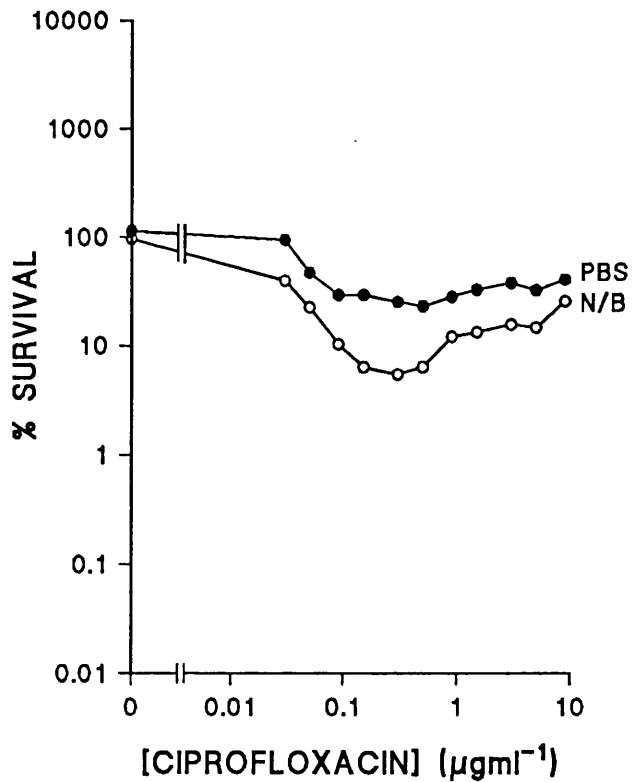
ciprofloxacin compared to that found in nutrient broth with the survival never being below 23%. The relative increase in survival at the OBC in PBS (25.30%) compared to that in nutrient broth was approximately 5-fold.

To compare separately the effects of temperature on the activities of ciprofloxacin in nutrient broth and in PBS, composite profiles for all four temperatures studied were plotted (Figures 13 and 14, respectively). Overall, lowering the temperature had a more pronounced effect on the activity of ciprofloxacin in nutrient broth alone, as can be seen from the greater separation of the profiles in nutrient broth at each temperature. At 20°C there was 68-fold less activity compared to that observed at 37°C in nutrient broth, and as the temperature of incubation was reduced from 37 to 30 to 25 and then to 20°C the OBCs were 0.15, 0.3, 0.15 and 0.3μgml^{-1} ciprofloxacin, respectively. At the OBCs in PBS the relative reduction in activity was 3-fold between 37 and 20°C (Figure 14, Table 8). It is also notable from Figure 14 that PBS reduced the bactericidal activity of ciprofloxacin to about the same level at each temperature across the concentration range tested. Experiments in PBS measure the activity of bactericidal mechanisms B and C while experiments in nutrient broth measure the activities of bactericidal mechanisms A, B and C. Thus, it would seem that bactericidal mechanism A is much more affected by temperature than the other bactericidal mechanisms of ciprofloxacin. This can be seen most clearly in Table 8.

Figure 13: Bactericidal activity of ciprofloxacin in nutrient broth against *E. coli* 20-37°C.
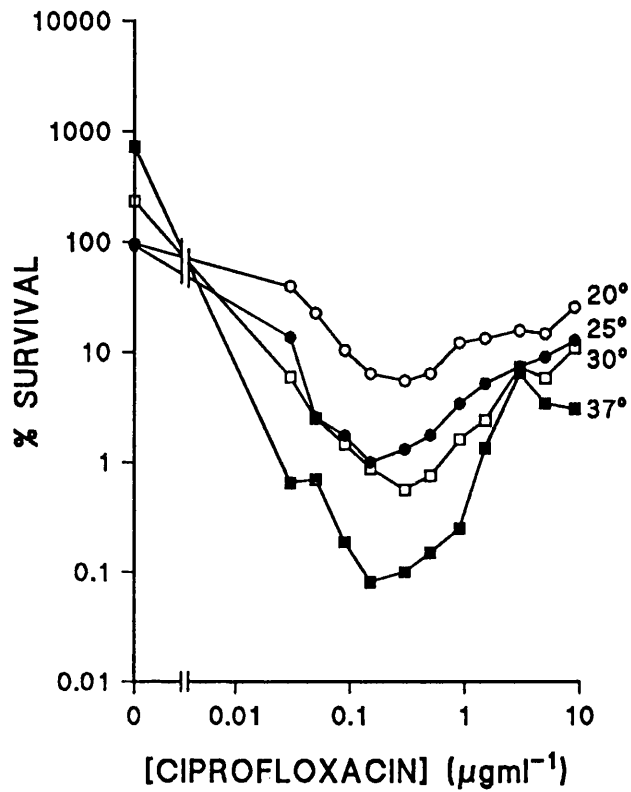
Figure 14: Bactericidal activity of ciprofloxacin in PBS against *E. coli* at 20-37°C after 3 hours.
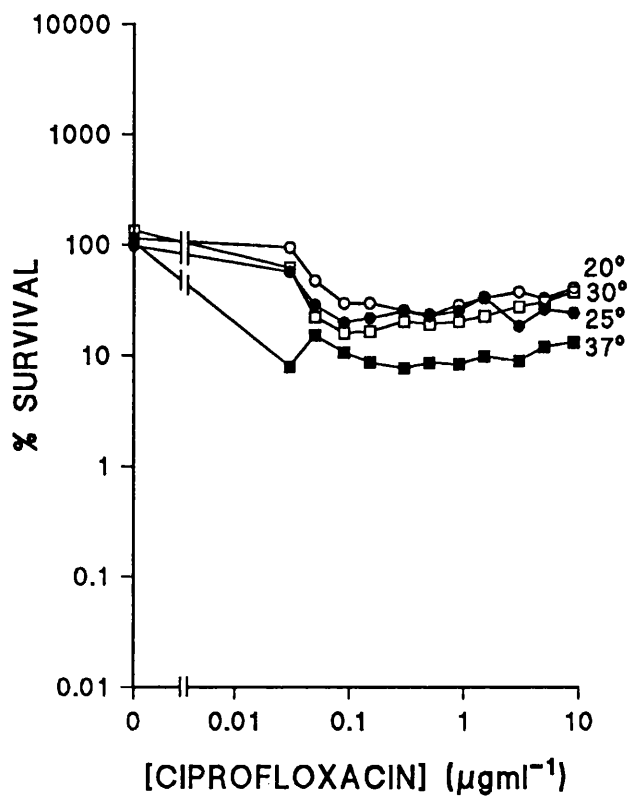
Bactericidal activity of DR-3355 against *E. coli*:

Figure 15 shows the bactericidal profiles of DR-3355 against

E. coli in nutrient broth and in PBS at 37°C. In nutrient

broth a biphasic response was exhibited and the OBC was

slightly higher than that for ciprofloxacin at $0.3\mu gml^{-1}$

(Table 9). However, the survival at the OBC of DR-3355 was

0.05% which was lower than found with ciprofloxacin, and as

the concentration of DR-3355 was increased beyond the OBC

survival increased to 1.73% at $9\mu gml^{-1}$. In PBS the biphasic

response was abolished and survival was less than 10% across

the whole range of DR-3355 concentrations tested. At the

OBC survival was 5.38% which was approximately 108-fold

greater than that in nutrient broth.

**Table 9: Effect of temperature on bactericidal activity of
DR-3355 against *E. coli*.**

Medium	Nutrient Broth		PBS
Temp. (°C)	OBC (μgml^{-1})	% Survival	% Survival
20	0.3	2.80	9.65
25	0.3	0.58	7.39
30	0.3	0.38	7.14
37	0.3	0.05	5.38

Figure 16 shows the bactericidal profiles of DR-3355 against

E. coli in nutrient broth and in PBS at 30°C. DR-3355

exhibited a familiar biphasic response and the OBC was the

same as that found at 37°C but the survival was increased

approximately 8-fold to 0.38% at the OBC at 30°C (Table 9).

As the concentration of DR-3355 was increased from $0.3\mu gml^{-1}$

Figure 15: Bactericidal activity of DR-3355 against *E. coli* at 37°C after 3 hours.
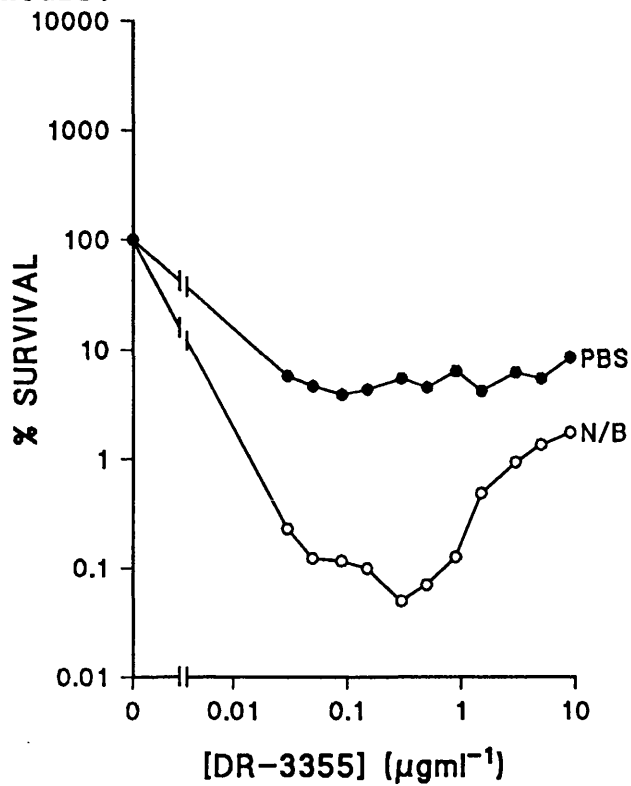
Figure 16: Bactericidal activity of DR-3355 against *E. coli* at 30°C after 3 hours.
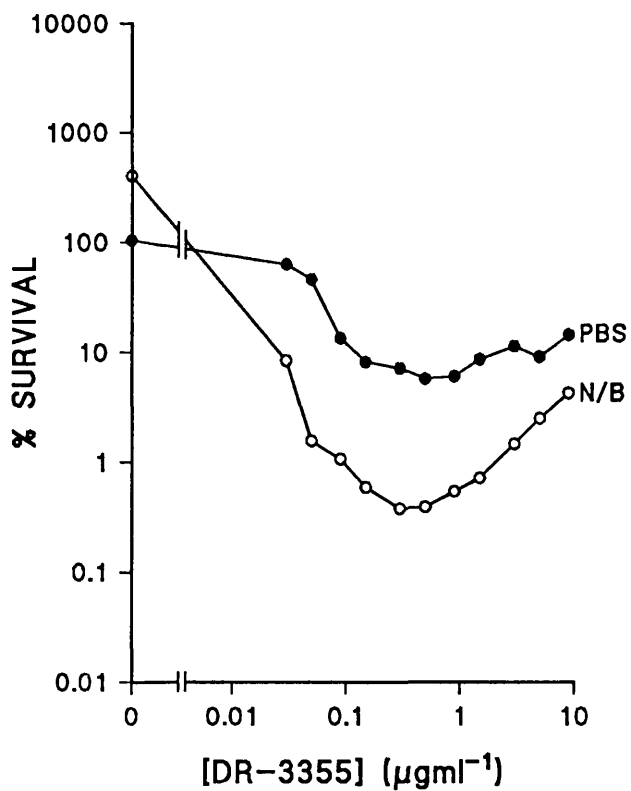
to 9µgml^{-1} survival increased progressively to 4.25%. In PBS

at 30°C the survival at the OBC was 7.14%. In PBS a

moderate biphasic response was seen, whereby at

concentrations of DR-3355 greater than 0.5µgml^{-1} the survival

increased from 5.71 to 14.29% at 9µgml^{-1}.

Figure 17 show the bactericidal profiles of DR-3355 against

E. coli in nutrient broth and in PBS at 25°C. A biphasic

response was exhibited in nutrient broth and the OBC was the

same as that found at 37 or at 30°C (0.3µgml^{-1}). The

survival at the OBC in nutrient broth was 0.58% which was

approximately 12-fold lower than that found at the OBC at

37°C (Table 9). As the concentration of DR-3355 was

increased above the OBC survival increased progressively to

7.40% at 9µgml^{-1} DR-3355. In PBS there was a minor biphasic

response and at the OBC survival was approximately 13-fold

higher (7.39%).

Figure 18 shows the bactericidal profiles of DR-3355 against

E. coli in nutrient broth and in PBS at 20°C. A weak

biphasic response was exhibited in nutrient broth and the

OBC was the same (0.3µgml^{-1}) as that found at the higher

temperatures. The biphasic response was not very pronounced

since at the lowest concentration tested (0.03µgml^{-1})

survival was 4.63% decreasing slightly to 2.80% at the OBC

and then increasing to 13.54% at 9µgml^{-1}. Survival at the

OBC was 56-fold higher than that found at 37°C (Table 9).

In PBS a weak biphasic response was also seen and the

Figure 17: Bactericidal activity of DR-3355 against *E. coli* at 25°C after 3 hours.
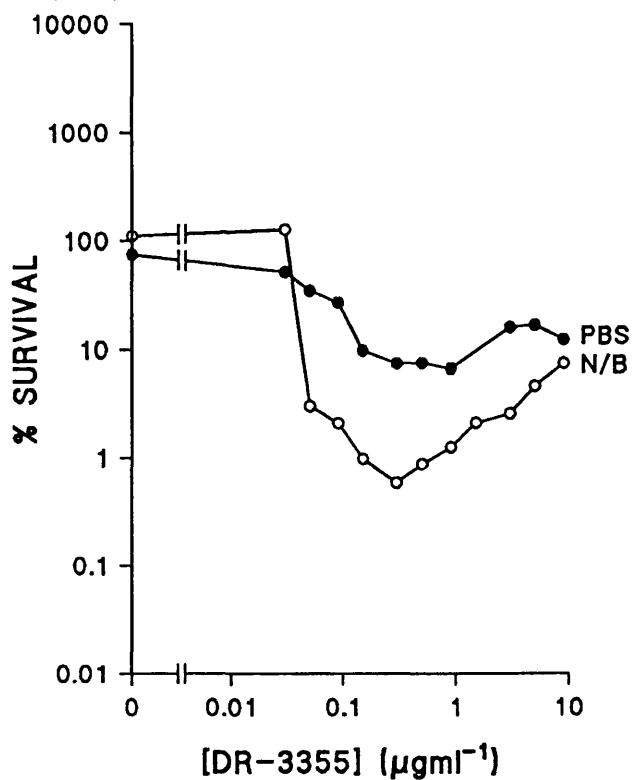
Figure 18: Bactericidal activity of DR-3355 against *E. coli* at 20°C after 3 hours.
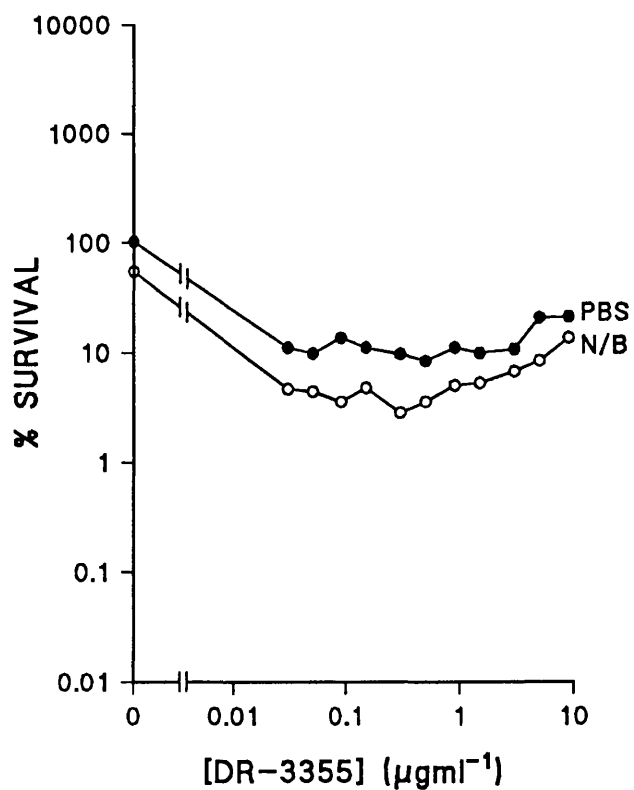
relative difference between the survival at the OBC in PBS
(9.65%) and in nutrient broth was about 3-fold.

As was done with the results for ciprofloxacin, composite
profiles for DR-3355 against *E. coli* at 20, 25, 30 and 37°C
in nutrient broth and in PBS were plotted (Figures 19 and
20, respectively). As was found with ciprofloxacin lowering
the temperature of incubation had a much more pronounced
effect on the activity of DR-3355 in nutrient broth (Figure
19) compared to that in PBS (Figure 20, Table 9). The
increase in survival at the OBC as the temperature was
reduced from 37 to 20°C was approximately 56-fold and 2-
fold, respectively. Once again this would suggest that
bactericidal mechanism A is more affected by temperature
than mechanisms B or C.

Both ciprofloxacin and DR-3355 exhibited bactericidal
mechanisms A and either B or C against *E. coli* at 20, 25, 30
and 37°C, since suspension of the bacteria in PBS abolished
the biphasic response but did not completely inhibit all
bactericidal activity. Overall, the temperature of
incubation had little or no effect on the OBC of either of
the 4-quinolones tested. The OBCs of ciprofloxacin were
slightly lower than those of DR-3355 but the bactericidal
activity of ciprofloxacin was slightly less than that of DR-
3355.

Figure 19: Bactericidal activity of DR-3355 in nutrient broth against *E. coli* at 20-37°C after 3 hours.
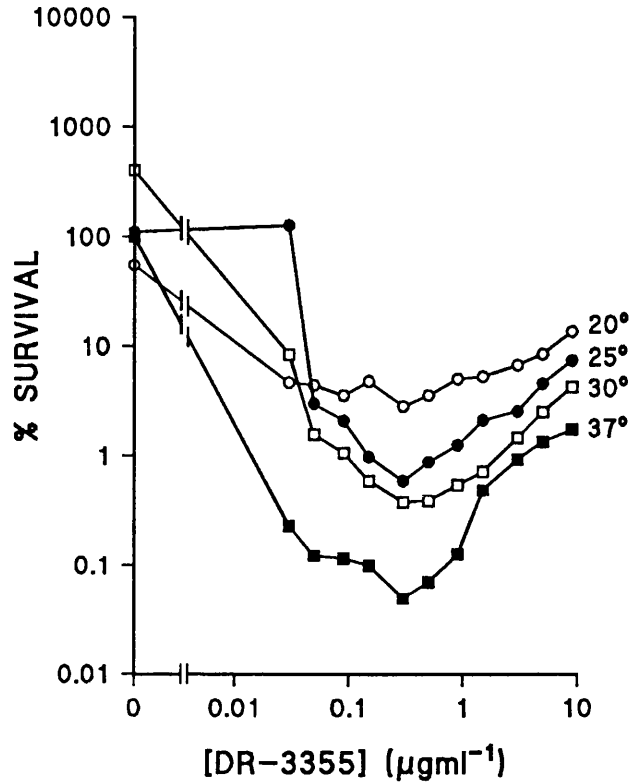
Figure 20: Bactericidal activity of DR-3355 in PBS against *E. coli* at 20-37°C after 3 hours.
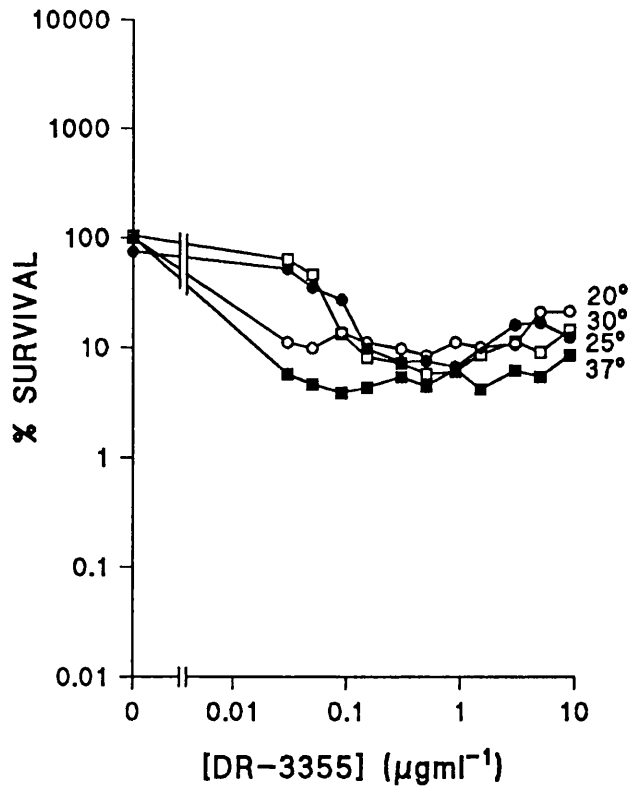
Bactericidal activity of ciprofloxacin against *Ps.*

***aeruginosa*:**

In agreement with results found previously by Morrissey and

Smith (1990) ciprofloxacin did not exhibit a biphasic

response against *Ps. aeruginosa* in nutrient broth at 37°C or

at the three lower temperatures studied (Figure 21).

Therefore at the highest concentration tested ($9\mu gml^{-1}$) the

survival was lowest. At all four temperatures studied the

bactericidal activity of ciprofloxacin was extremely high.

Survival was lower after 30 minutes treatment with $9\mu gml^{-1}$ of

ciprofloxacin than after 3 hours treatment with the OBC of

ciprofloxacin ($0.15\mu gml^{-1}$) against *E. coli*.

Figure 21: Bactericidal activity of ciprofloxacin against

***Ps. aeruginosa* at 20-37°C after 30 minutes.**
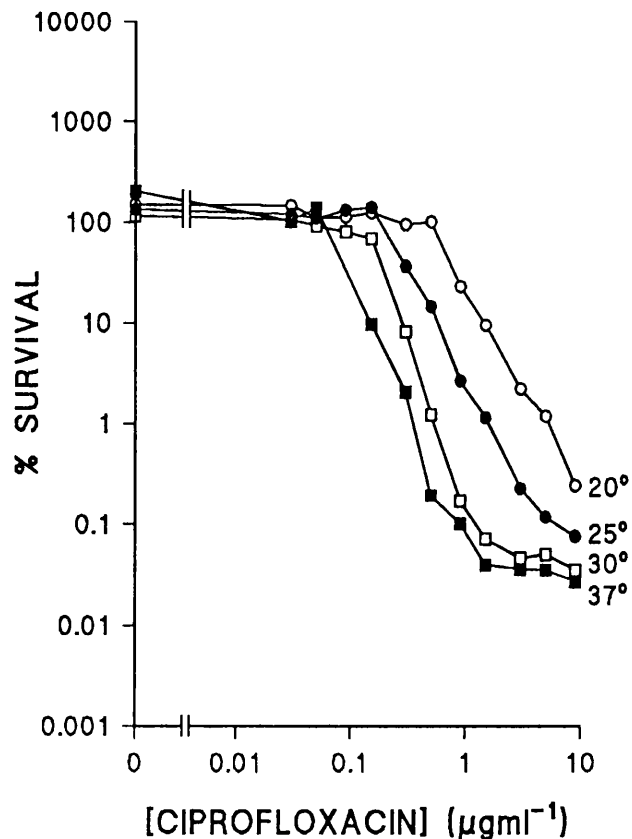
At 9µgml^{-1} ciprofloxacin the survival of *Ps. aeruginosa* after 30 minutes was 0.03, 0.04, 0.08 or 0.24% at 37, 30, 25 or 20°C, respectively. The temperature of incubation also affected the minimum concentration of ciprofloxacin required for bactericidal activity. The concentrations required to kill 90% of the bacteria at 37, 30, 25 or 20°C were 0.15, 0.3, 0.5, or 1.5µgml^{-1}, respectively. As the concentration of ciprofloxacin was increased beyond these values approximately the same decrease in survival was observed at each temperature. Hence the bactericidal profiles were virtually parallel except at 30 and 37°C where above 1.5µgml^{-1} each incremental increase in concentration had little effect on survival. To achieve approximately 1% survival at 37, 30, 25 or 20°C required 0.3, 0.5, 1.5 or 5µgml^{-1} ciprofloxacin, respectively (Figure 21).

Bactericidal activity of DR-3355 against *Ps. aeruginosa*:
The bactericidal profiles of DR-3355 against *Ps. aeruginosa* in nutrient broth at 20, 25, 30 and 37°C (Figure 22) were generally similar to those found with ciprofloxacin (Figure 21) in that once again there was no biphasic response and the bactericidal activity curves were parallel at all four temperatures even at the highest concentrations tested. Furthermore, the concentrations required to cause 90% death were slightly higher for DR-3355 than for ciprofloxacin which at 37, 30, 25, or 20°C were 0.5, 0.9, 0.9 or 1.5µgml^{-1} DR-3355, respectively. At the highest concentration of DR-3355 tested (9µgml^{-1}) there was lower survival at 37

Figure 22: Bactericidal activity of DR-3355 against *Ps.*

***aeruginosa* at 20-37°C after 30 minutes.**
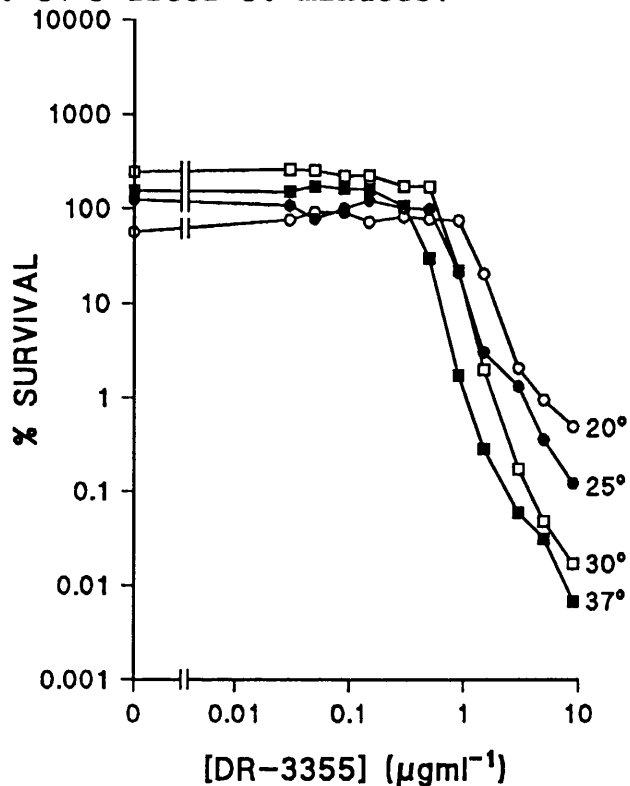
(0.007%) or 30°C (0.02%) than there was with 9μgml⁻¹

ciprofloxacin at either 37 or 30°C. However, at 25 and 20°C

the survival at 9μgml⁻¹ DR-3355 was 0.12 and 0.49%,

respectively, which was slightly higher than that found with

ciprofloxacin. To achieve approximately 1% survival at 37,

30, 25 or 20°C required 0.9, 1.5, 3 or 5μgml⁻¹ DR-3355,

respectively, again a little higher than the concentrations

required with ciprofloxacin.

Bactericidal activity of ciprofloxacin against *Staph.*

***aureus*:**

Figure 23 shows the bactericidal profiles of ciprofloxacin

against *Staph. aureus* in nutrient broth and in PBS at 37°C.

In nutrient broth a biphasic response was exhibited and the

Figure 23: Bactericidal activity of ciprofloxacin against
Staph. aureus **at 37°C after 3 hours.**
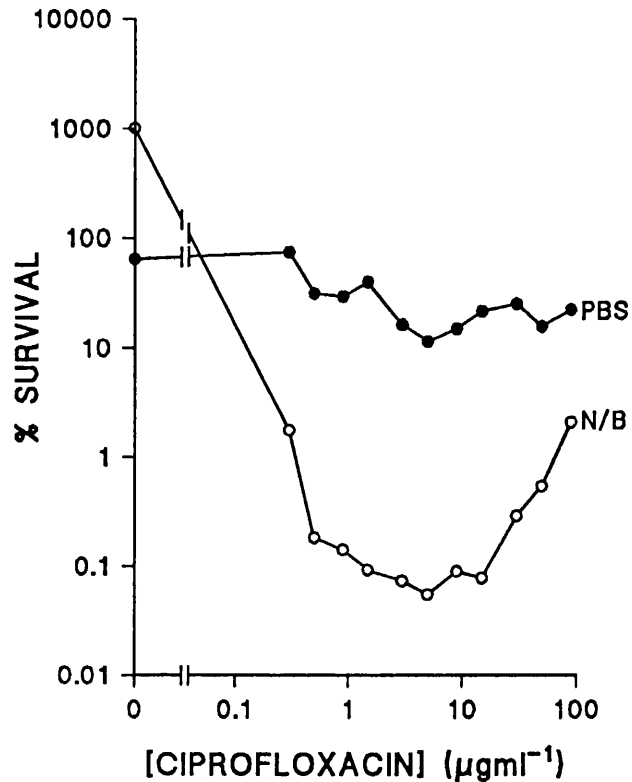
OBC was 5μgml^{-1} (Table 10). Thus, in terms of the OBC
ciprofloxacin was 33-times more active against *E. coli* (OBC=
0.15μgml^{-1}) than against *Staph. aureus* but survival of both
organisms was similar at the OBCs, being 0.08 and 0.06%,
respectively. With *Staph. aureus* in nutrient broth as the
concentration of ciprofloxacin was increased above the OBC
there was progressively less activity and survival at the
highest concentration tested (90μgml^{-1}) was 2.08%. In PBS
the biphasic response was abolished and survival at the OBC
was 11.39% which was approximately 190-fold higher than that
in nutrient broth. The low activity of ciprofloxacin in PBS
was probably because ciprofloxacin does not possess
bactericidal mechanisms B or C and only possesses

bactericidal mechanism A against *Staph. aureus* (Lewin and Smith, 1989a).

Table 10: Effect of temperature on bactericidal activity of ciprofloxacin against *Staph. aureus*.

Medium	Nutrient Broth		PBS
Temp. (°C)	OBC (μgml^{-1})	% Survival	% Survival
20	15	5.16	45.10
25	15	0.58	11.71
30	9	0.05	10.53
37	5	0.06	11.39

Figure 24 shows the bactericidal profiles of ciprofloxacin against *Staph. aureus* in nutrient broth and in PBS at 30°C. As was found at 37°C there was a biphasic response in nutrient broth and the OBC was 9μgml^{-1} ciprofloxacin and survival at the OBC was 0.05% which was slightly lower than that observed at 37°C (Table 10). Apart from these differences the profiles in nutrient broth at 37 or 30°C were similar especially at or above concentrations of 5μgml^{-1}. In PBS the activity was low at all concentrations of ciprofloxacin tested and survival was increased by about 211-fold (to 10.53%) compared to that in nutrient broth, a greater reduction in activity compared to that found at 37°C (Figure 23). As was found at 37°C ciprofloxacin only exhibited bactericidal mechanism A against *Staph. aureus* at 30°C.

Figure 24: Bactericidal activity of ciprofloxacin against
Staph. aureus at 30°C after 3 hours.
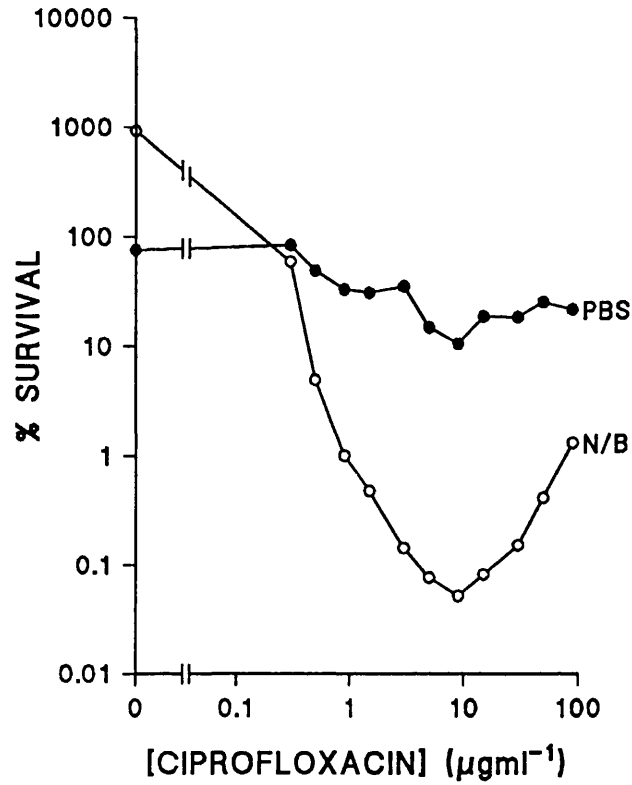
Figure 25: Bactericidal activity of ciprofloxacin against
Staph. aureus at 25°C after 3 hours.

Figure 25 shows the bactericidal profiles of ciprofloxacin against *Staph. aureus* in nutrient broth and in PBS at 25°C. A biphasic response was exhibited in nutrient broth and the OBC was 15µgml^{-1} which was higher than that at either 30°C (9µgml^{-1}) or 37°C (5µgml^{-1}). Survival at the OBC at 25°C was 0.58%, which was about 10-fold higher than the survival at the OBCs at 37 or 30°C (Table 10). In PBS there was a minor biphasic response and at the OBC (in nutrient broth) survival was 11.71%. The relative reduction in the activity of ciprofloxacin in nutrient broth compared to in PBS was only about 20-fold at 25°C, compared to around 200-fold at either 37 or 30°C.

Figure 26 shows the bactericidal profiles of ciprofloxacin against *Staph. aureus* in nutrient broth and in PBS at 20°C. Although there was only a minor biphasic response in nutrient broth the OBC was 15µgml^{-1} and the survival at the OBC was 5.16% which was 86-times higher than that found at the OBC at 37°C (Table 10). As the concentration of ciprofloxacin was increased beyond the OBC survival increased slightly to 9.34% at the highest concentration tested. In PBS there was little activity at any concentration of ciprofloxacin tested with survival at the OBC being 45.10%, which was approximately 9-fold higher than in nutrient broth.

Figure 26: Bactericidal activity of ciprofloxacin against
Staph. aureus at 20°C after 3 hours.
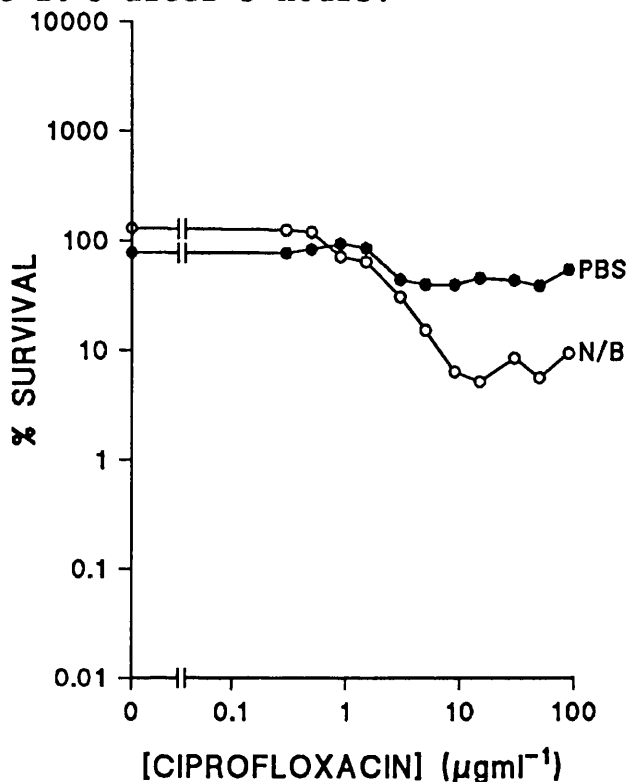
As can be seen from the composite profiles in nutrient broth
at 20, 25, 30 and 37°C (Figure 27) taken from Figures 26,
25, 24 and 23, respectively, the activity of ciprofloxacin
was reduced (by 86-fold) progressively as the temperature of
incubation was decreased from 37 to 20°C. Figure 27 also
clearly shows the similarity of the profiles at 30 and 37°C
at concentrations of ciprofloxacin at or above $5\mu gml^{-1}$. It
also shows the large differences between the activities of
ciprofloxacin at the lower concentrations studied at all
four temperatures. The composite profiles in PBS at 20, 25,
30 and 37°C are shown in Figure 28. As was found with
ciprofloxacin or DR-3355 against *E. coli*, reducing the
temperature of incubation from 37 to 20°C had only a minor
effect (a 4-fold reduction) on the activity of ciprofloxacin

Figure 27: Bactericidal activity of ciprofloxacin in nutrient broth against *Staph. aureus* at 20–37°C after 3 hours.
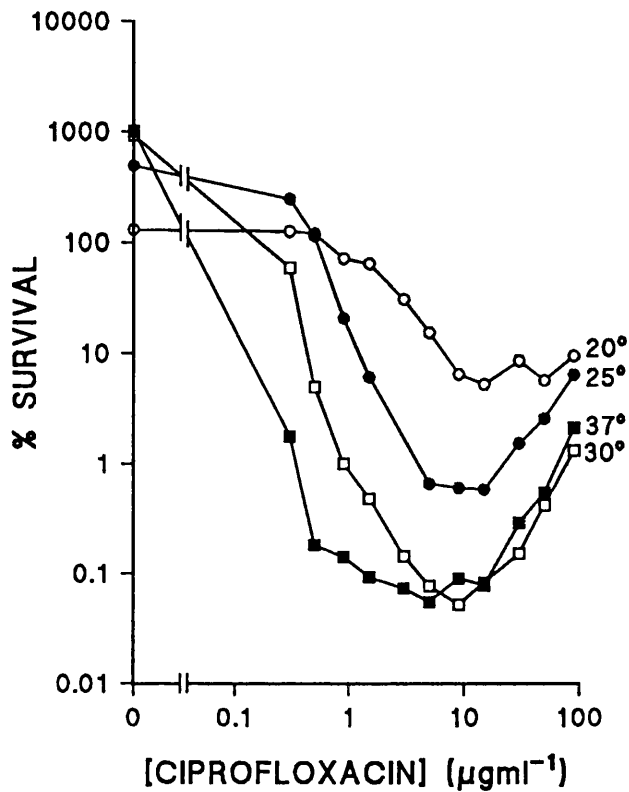
Figure 28: Bactericidal activity of ciprofloxacin in PBS against *Staph. aureus* at 20–37°C after 3 hours.
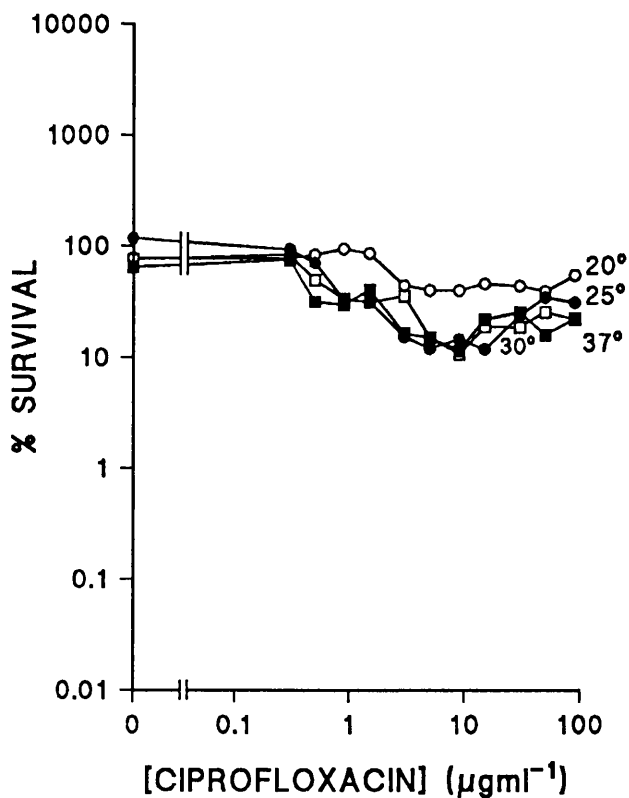
against *Staph. aureus* in PBS. In agreement with previous

findings at 37°C by Lewin and Smith (1989)[a] it was found that

the activity of ciprofloxacin against *Staph. aureus* in PBS

was drastically reduced compared to that in nutrient broth

especially at 37 or 30°C. Thus, it was found that

ciprofloxacin only possessed bactericidal mechanism A

against this organism and rather like mechanism A against *E.*

coli temperature had a dramatic effect on its activity.

Bactericidal activity of DR-3355 against *Staph. aureus*:
Figure 29 shows the bactericidal profiles of DR-3355 against

Staph. aureus in nutrient broth and in PBS at 37°C. In

nutrient broth there was a biphasic response with an OBC of

$5\mu gml^{-1}$ DR-3355 and survival at the OBC was 0.008% (Table

11). Above the OBC survival increased progressively to

0.08% at $90\mu gml^{-1}$ DR-3355. In PBS the biphasic response was

abolished and survival of the bacteria was higher at all DR-

3355 concentrations tested. In PBS increasing the

concentration of DR-3355 from 0.5 to $3\mu gml^{-1}$ decreased

survival from 52.38 to 4.24%, respectively, and further

increases in the concentration of DR-3355 marginally

increased the activity in PBS. At the OBC in PBS survival

was 3.57% which was 457-times higher than the survival in

nutrient broth. Although suspension of the bacteria in PBS

reduced the activity of DR-3355 at 37°C the activity of DR-

3355 was still much better than that of ciprofloxacin in

PBS. Thus, in addition to bactericidal mechanism A, DR-3355

exhibited either mechanism B or C against *Staph. aureus*. As

Figure 29: Bactericidal activity of DR-3355 against *Staph. aureus* at 37°C after 3 hours.
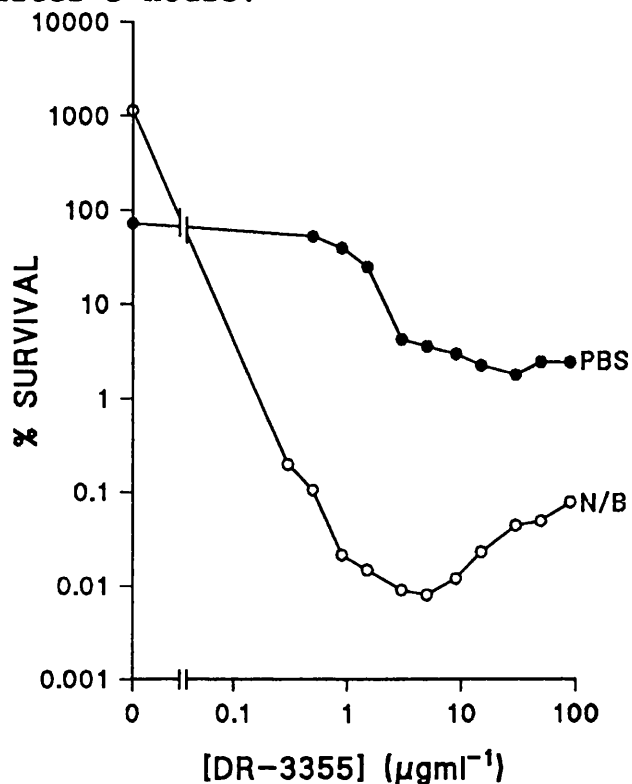
Table 11: Effect of temperature on bactericidal activity of DR-3355 against *Staph. aureus*.

Medium	Nutrient Broth		PBS
Temp. (°C)	OBC (μgml^{-1})	% Survival	% Survival
20	5	.37	2.60
25	3	.52	7.27
30	3	.02	2.32
37	5	.008	3.57

was explained above the bactericidal activity of DR-3355 was not determined in the presence of chloramphenicol so bactericidal mechanisms B and C could not be resolved. However, Lewin and Amyes (1989) found that DR-3355 exhibited bactericidal mechanisms A and B against *Staph. aureus* at 37°C.
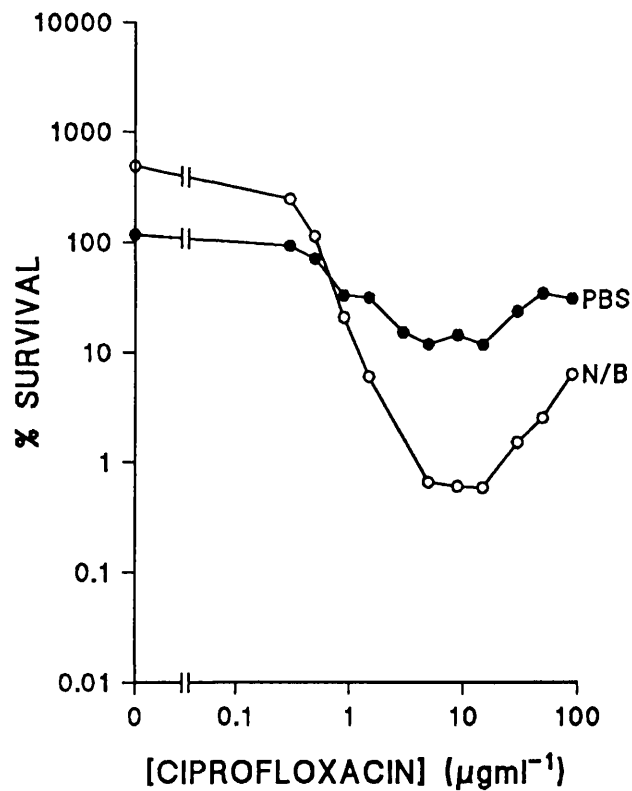
Figure 30 shows the bactericidal profiles of DR-3355 against
Staph. aureus in nutrient broth and in PBS at 30°C. There
was a biphasic response in nutrient broth and the OBC was
$3\mu gml^{-1}$ DR-3355, which was lower than the OBC found at 37°C
(Figure 29, Table 11). Survival in nutrient broth at the
OBC was 0.02% which was 4-times higher than that found at
the OBC at 37°C. As was found at 37°C (Figure 29) in PBS
increasing the concentration of DR-3355 from 0.3 to $3\mu gml^{-1}$
had a large effect on activity, reducing survival from
115.26 to 2.32%, respectively. Further increases in the
concentration of DR-3355 had little effect on the activity
in PBS. In PBS survival at the OBC was 2.32% which was
approximately 116-fold higher than in nutrient broth at
30°C.

Figure 30: **Bactericidal activity of DR-3355 against *Staph.***
***aureus* at 30°C after 3 hours.**
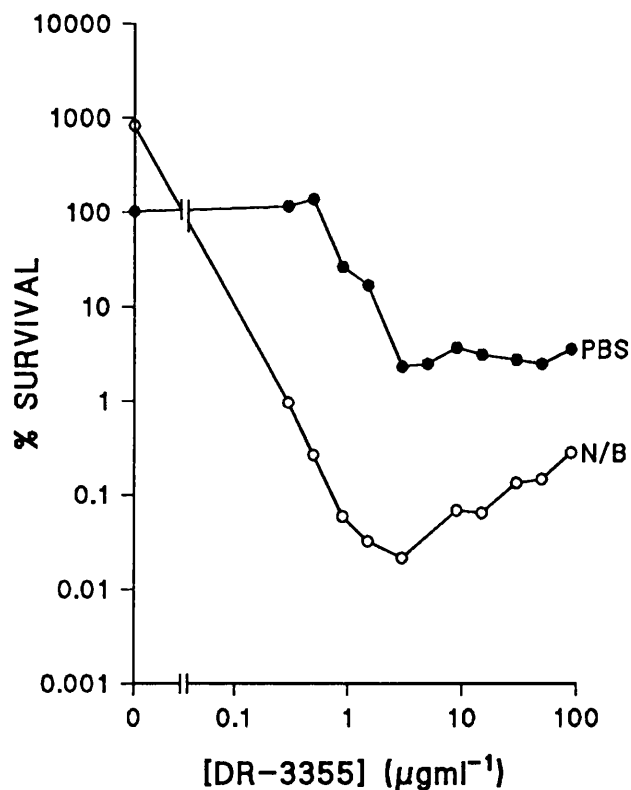
Figure 31 shows the bactericidal profiles of DR-3355 against
Staph. aureus in nutrient broth and in PBS at 25°C.   There
was a biphasic response in nutrient broth which was
abolished in PBS.   In nutrient broth survival decreased
progressively from 3.88% at 0.5µgml⁻¹ DR-3355 to 0.52% at the
OBC which was 3µgml⁻¹: survival was 65-times higher than at
the OBC at 37°C (Table 11).   As the concentration of DR-3355
was increased above the OBC survival increased progressively
to 2.88% at 90µgml⁻¹ DR-3355.   In PBS at 25°C the survival at
the OBC was 7.27%, which was about 14-times higher than that
found in nutrient broth and higher than the survival found
in PBS at 30 or 37°C.

**Figure 31: Bactericidal activity of DR-3355 against *Staph.
aureus* at 25°C after 3 hours.**
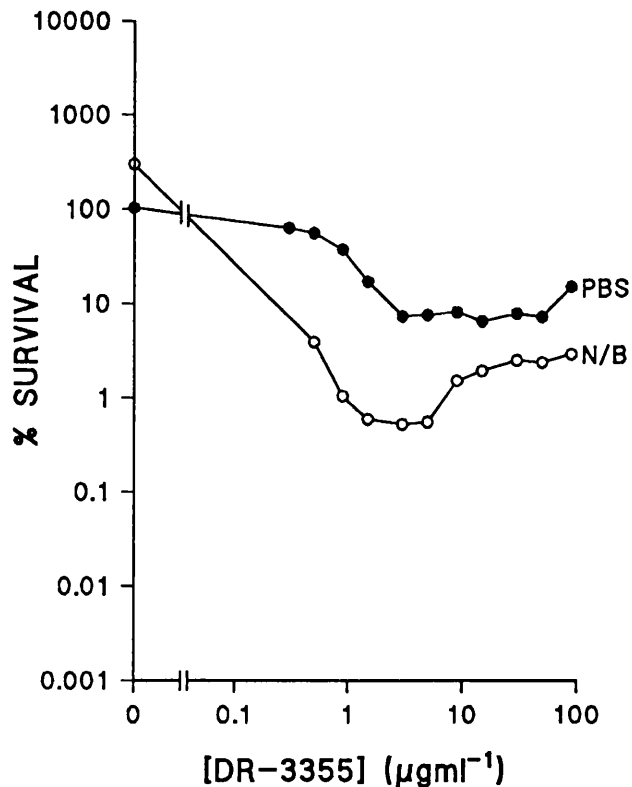
Figure 32 shows the bactericidal profiles of DR-3355 against *Staph. aureus* in nutrient broth and in PBS at 20°C. There was a biphasic response in nutrient broth with an OBC of $5\mu gml^{-1}$ and the survival at the OBC was 0.37%. There was little difference in survival between 3 and $15\mu gml^{-1}$ DR-3355 and at concentrations of DR-3355 above the OBC survival increased to 1.58% at $90\mu gml^{-1}$. The OBC at 20°C was the same as that found at 37°C but higher than the OBCs at either 30 or 25°C, and the activity was approximately 46-times lower at 20°C compared to 37°C (Table 11). In PBS there was a minor biphasic response (OBC= $9\mu gml^{-1}$) and at $5\mu gml^{-1}$ DR-3355 (the OBC in nutrient broth) survival was 2.60% which was 7-fold higher than that in nutrient broth.

Figure 32: Bactericidal activity of DR-3355 against *Staph. aureus* at 20°C after 3 hours.
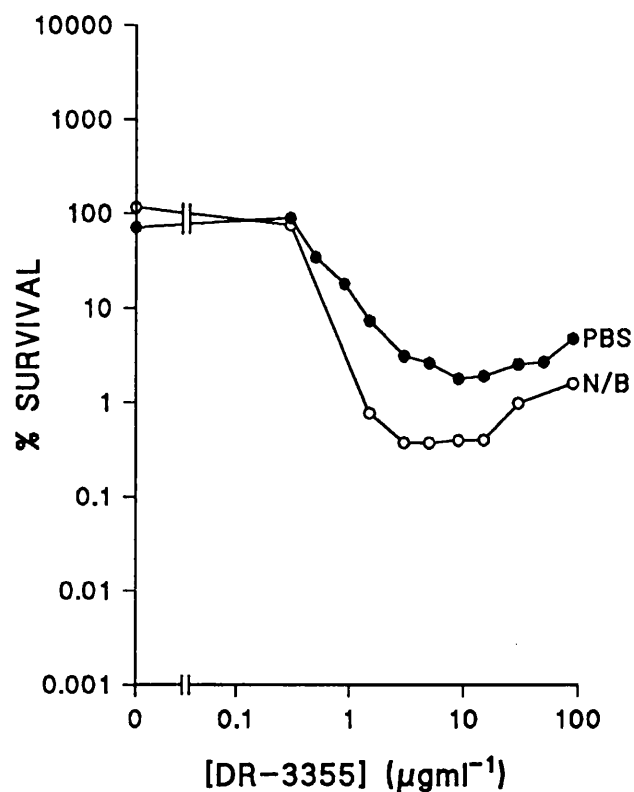
Figure 33 shows the composite profiles in nutrient broth at 20, 25, 30 and 37°C taken from Figures 32, 31, 30 and 29, respectively. The shape of the profiles at 30 or 37°C were similar and survival was lower at 37°C compared to 30°C at all concentrations tested. Also, with the profiles at 20 and 25°C at concentrations of DR-3355 above $1.5\mu gml^{-1}$ the survival at 20°C was lower than at 25°C, and the overall relative decrease in the activity as the incubation temperature was reduced from 37 to 20°C was approximately 46-fold. This relative reduction in the activity was roughly half that seen with ciprofloxacin (86-fold): could this be due to the possession of an additional mechanism of action by DR-3355 (Lewin and Amyes, 1989)?

Figure 33: Bactericidal activity of DR-3355 in nutrient broth against *Staph. aureus* at 20-37°C after 3 hours.
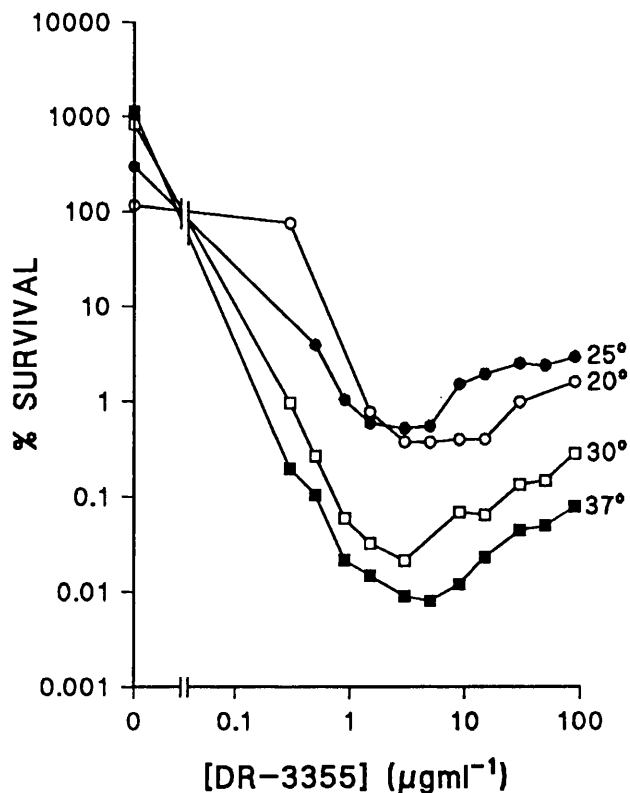
Figure 34 shows the profiles of DR-3355 in PBS at 20, 25, 30 and 37°C taken from Figures 32, 31, 30 and 29, respectively. Figure 34 shows that the temperature of incubation had little effect on the activity of DR-3355 in PBS, with the activity at 20, 30 or 37°C being very similar. At concentrations of DR-3355 at or above $3\mu gml^{-1}$, the activity of DR-3355 in PBS at 25°C was lower than that found at the other three temperatures. Lowering the temperature of incubation from 37 to 20°C had a much larger effect on the activity of DR-3355 in nutrient broth compared to that in PBS. Once again this would seem to suggest that bactericidal mechanism A of DR-3355 against *Staph. aureus* was very much more affected by temperature than its

Figure 34: Bactericidal activity of DR-3355 in PBS against *Staph. aureus* at 20-37°C after 3 hours.
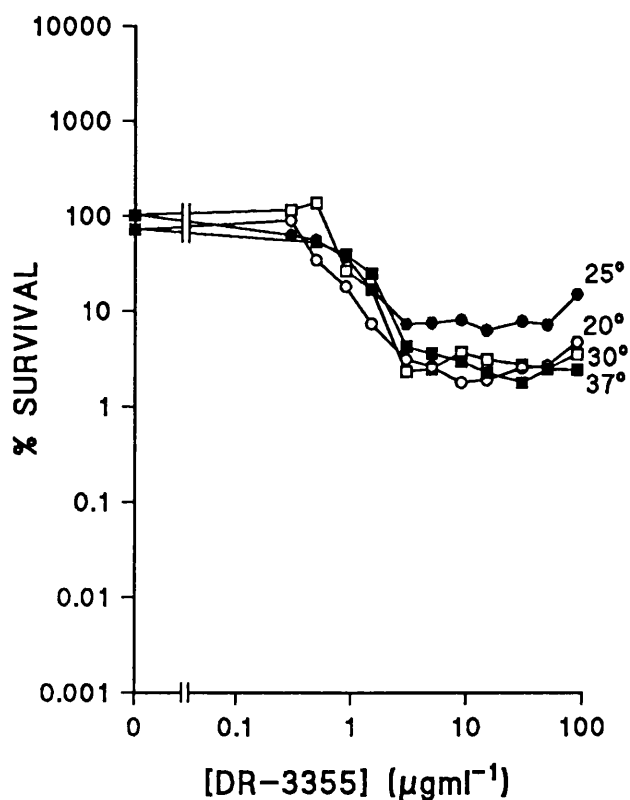
bactericidal mechanisms B or C. DR-3355 was more active in terms of having lower OBCs and lower survival at the OBCs than was ciprofloxacin against *Staph. aureus* (Tables 11 and 10, respectively). Although the OBCs of DR-3355 against *Staph. aureus* were higher than the OBCs of either ciprofloxacin or DR-3355 against *E. coli*, survival at the OBCs was lower with DR-3355 against *Staph. aureus* than with the Gram-negative organism treated with either ciprofloxacin or DR-3355 (compare Tables 8, 9 and 11).

Bactericidal activity of ciprofloxacin against *Staph. epidermidis*:

Figure 35 shows the bactericidal profiles of ciprofloxacin against *Staph. epidermidis* in nutrient broth and in PBS at 37°C. Ciprofloxacin exhibited a biphasic response in nutrient broth with an OBC of $5\mu gml^{-1}$. Survival decreased from 2.35% at $0.3\mu gml^{-1}$ to 0.07% at the OBC, which was a similar level of survival to that found with either *E. coli* or *Staph. aureus* treated with their OBCs of ciprofloxacin at 37°C (Figures 8 and 10, respectively). At concentrations of ciprofloxacin above the OBC the survival of the bacteria increased progressively to 3.03% at the highest concentration tested ($90\mu gml^{-1}$). When the bacteria were treated in PBS the biphasic response was greatly diminished and the activity of ciprofloxacin was drastically reduced compared to that in nutrient broth: at the OBC in PBS the survival was reduced by approximately 548-fold to 38.37% (Table 12). Thus, ciprofloxacin did not seem to exhibit

Figure 35: **Bactericidal activity of ciprofloxacin against** *Staph. epidermidis* at 37°C after 3 hours.
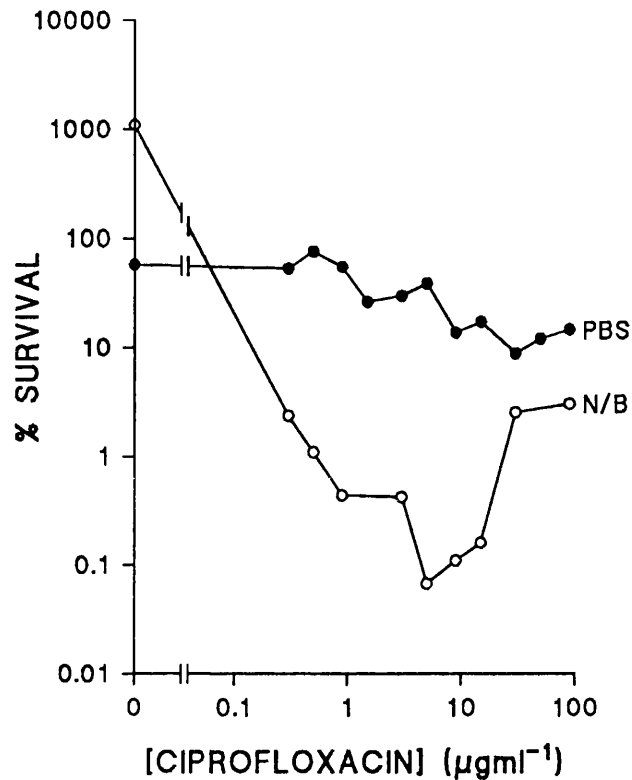
either bactericidal mechanism B or C against *Staph. epidermidis* at 37°C, but it clearly exhibited mechanism A.

Table 12: **Effect of temperature on bactericidal activity of ciprofloxacin against** *Staph. epidermidis*.

Medium	Nutrient Broth		PBS
Temp. (°C)	OBC (μgml^{-1})	% Survival	% Survival
20	9	5.75	35.51
25	3	0.65	42.50
30	5	1.13	117.86
37	5	0.07	38.37

Figure 36 shows the bactericidal profiles of ciprofloxacin against *Staph. epidermidis* in nutrient broth and in PBS at 30°C. As was found at 37°C ciprofloxacin exhibited a

biphasic response in nutrient broth but it was less pronounced at 30°C than at the higher temperature. The OBC at 30°C was 5µgml^{-1} and survival (1.13%) at the OBC was approximately 16-fold higher than that obtained at 37°C. Indeed, survival at 30°C was about 10 to 20-times higher than at 37°C at all concentrations tested except at 30-90µgml^{-1} where survival was similar. When the bacteria were treated in PBS the bactericidal activity of ciprofloxacin was completely abolished at all concentrations tested: survival ranged from 148.21% at 0.3µgml^{-1} to 57.14% at 50µgml^{-1}, and at the OBC survival was 117.86% which was about 104-fold higher than that in nutrient broth. Hence, ciprofloxacin seemed to only exhibit mechanism A against *Staph. epidermidis* at 30°C.

Figure 37 shows the bactericidal profiles of ciprofloxacin against *Staph. epidermidis* in nutrient broth and in PBS at 25°C. There was a biphasic response in nutrient broth and the OBC was 3µgml^{-1} which was lower than that found at either 30 or 37°C (5µgml^{-1}). Survival at the OBC was 0.65% which was about half of the survival at 30°C but about 10-times higher than that at 37°C (Table 12). As was found at 30°C treatment of the bacteria in PBS completely abolished the bactericidal activity of ciprofloxacin, the lowest survival being 36.25% at 15 or 50µgml^{-1}. Survival at the OBC in PBS was 42.50% which was approximately 65-fold higher than that in nutrient broth. Thus ciprofloxacin once again only seemed to exhibit bactericidal mechanism A at 25°C.

Figure 36: Bactericidal activity of ciprofloxacin against
Staph. epidermidis at 30°C after 3 hours.

Figure 37: Bactericidal activity of ciprofloxacin against
Staph. epidermidis at 25°C after 3 hours.
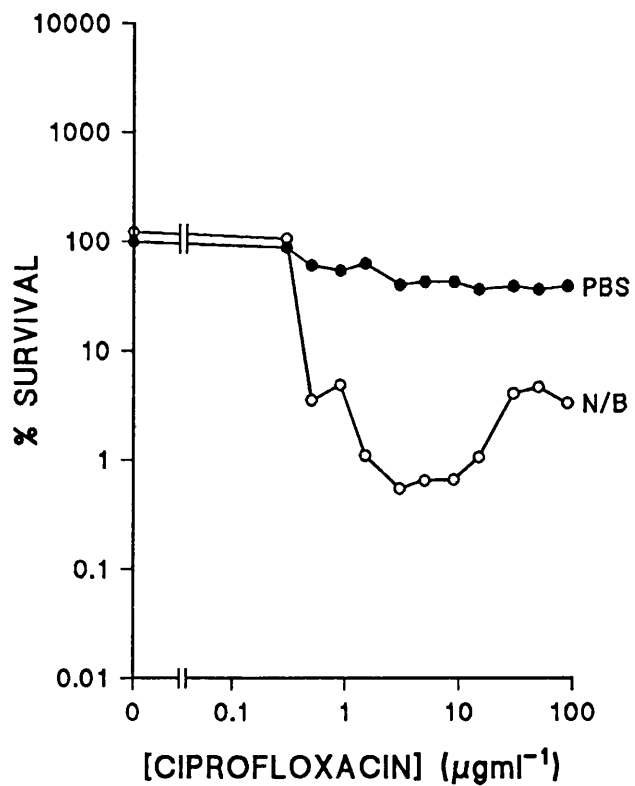
Figure 38 shows the bactericidal profiles of ciprofloxacin against *Staph. epidermidis* in nutrient broth and in PBS at 20°C. Ciprofloxacin exhibited a weak biphasic response in nutrient broth with 5.75% survival at the OBC ($9\mu gml^{-1}$) which itself was higher than those observed at the three higher temperatures studied (Table 12). When the bacteria were treated in PBS the bactericidal activity of ciprofloxacin was virtually abolished, with survival ranging from 62.32% at $1.5\mu gml^{-1}$ to 32.61% at $15\mu gml^{-1}$. Survival at the OBC in PBS was 35.51% which was approximately 6-times higher than that seen in nutrient broth.

Figure 38: Bactericidal activity of ciprofloxacin against *Staph. epidermidis* **at 20°C after 3 hours.**
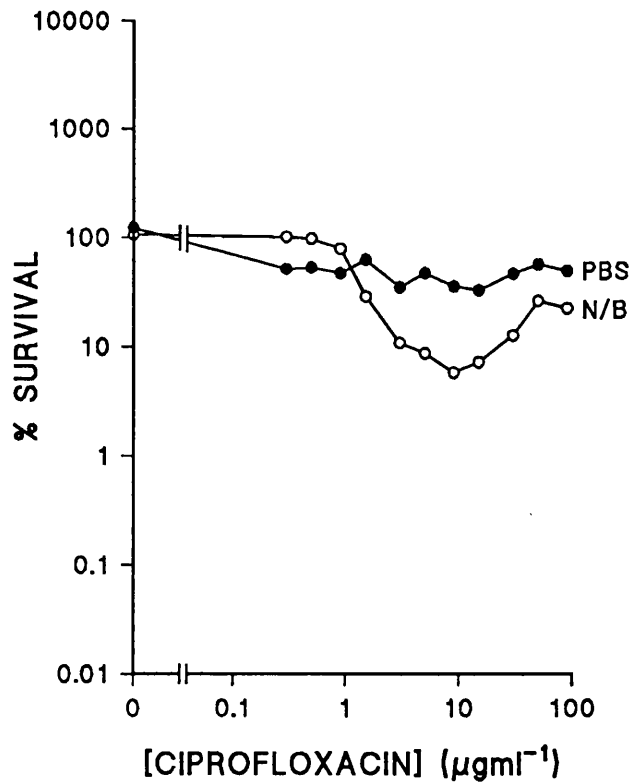
Figure 39 shows the profiles of ciprofloxacin in nutrient broth at 20, 25, 30 and 37°C derived from Figures 38, 37, 36 and 35, respectively. Overall, it appears that the bactericidal activity of ciprofloxacin at 25 or 30°C was similar with its activity at 20°C being lower and its activity at 37°C being higher than those at 25 or 30°C. It is interesting that at 25, 30 or at 37°C, at ciprofloxacin concentrations of 30-90µgml⁻¹ the survival was similar.

Decreasing the temperature of incubation from 37 to 20°C reduced the activity of ciprofloxacin by about 82-fold at the OBCs.

Figure 39: Bactericidal activity of ciprofloxacin in nutrient broth against *Staph. epidermidis* at 20-37°C after 3 hours.
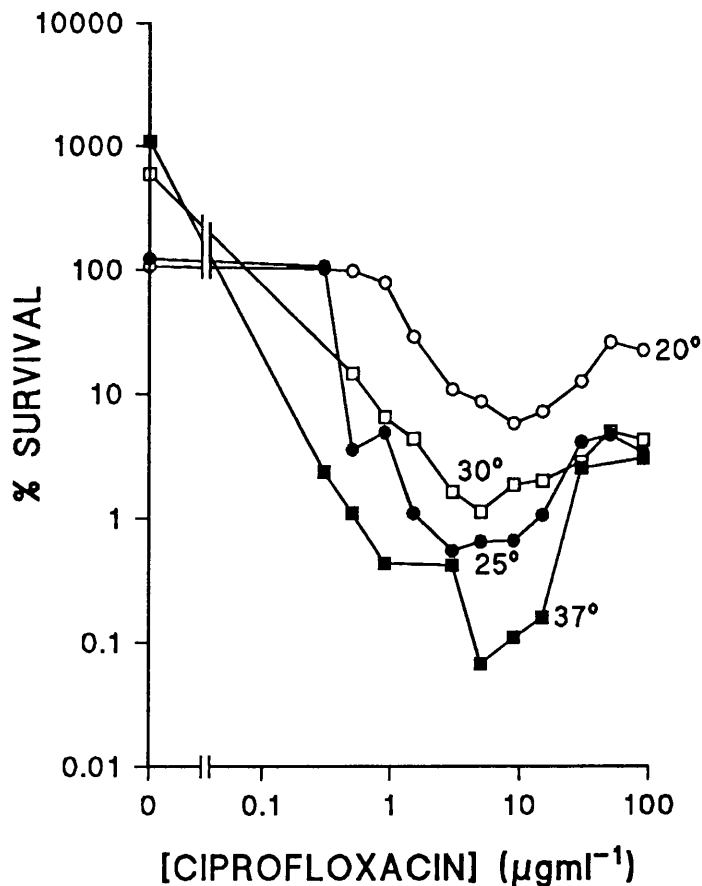
Figure 40 shows the profiles of ciprofloxacin in PBS at 20,

25, 30 and 37°C.   At 20, 25 or 30°C increasing the

concentration of ciprofloxacin had little effect on the

survival of *Staph. epidermidis*, whereas at 37°C there was a

marginal increase in activity as the concentration was

increased.   Thus, ciprofloxacin was effectively inactive in

PBS because it did not appear to possess either bactericidal

mechanism B or C against *Staph. epidermidis*.

Figure 40: **Bactericidal activity of ciprofloxacin in PBS
against *Staph. epidermidis* at 20-37°C after 3 hours.**
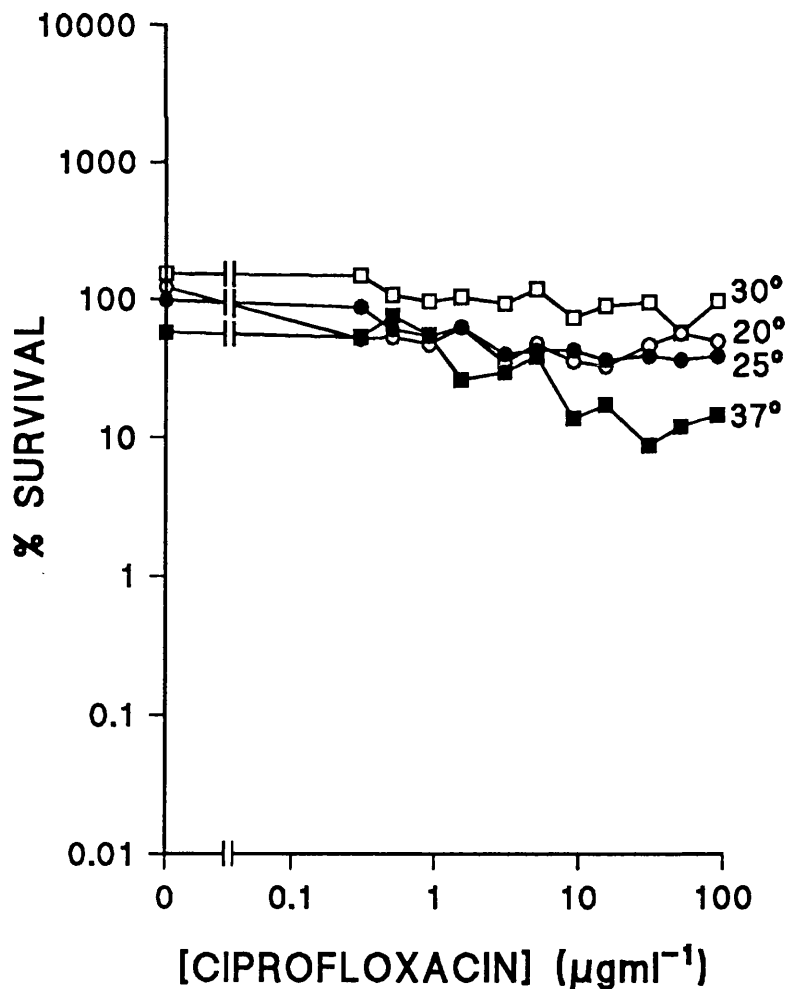
Bactericidal activity of DR-3355 against *Staph. epidermidis*:

Figure 41 shows the bactericidal profiles of DR-3355 against *Staph. epidermidis* in nutrient broth and in PBS at 37°C. DR-3355 was highly active in nutrient broth and survival at the OBC (5µgml^{-1}) was 0.02% (Table 13). Indeed, at 37°C the survival of *Staph. epidermidis* was lower than the survival observed at the OBCs of either ciprofloxacin or DR-3355 against *E. coli* or of ciprofloxacin against *Staph. aureus* or *Staph. epidermidis* (compare Tables 8, 9, 10, 12 and 13). Only DR-3355 against *Staph. aureus* was more bactericidal at 37°C than DR-3355 against *Staph. epidermidis* at 37°C (compare Tables 11 and 13). In PBS the activity of DR-3355 was greatly reduced at all concentrations tested: at the OBC (in nutrient broth) survival was 7.80% which was 390-fold higher than that in nutrient broth, and about 1.5-times lower than the survival of *Staph. aureus* treated with DR-3355 in PBS at 37°C (Tables 10 and 13).

Table 13: Effect of temperature on bactericidal activity of DR-3355 against *Staph. epidermidis*.

Medium	Nutrient Broth		PBS
Temp. (°C)	OBC (µgml^{-1})	% Survival	% Survival
20	3	0.38	1.66
25	3	0.37	1.19
30	3	0.03	0.58
37	5	0.02	7.80

Figure 41: Bactericidal activity of DR-3355 against *Staph. epidermidis* at 37°C after 3 hours.
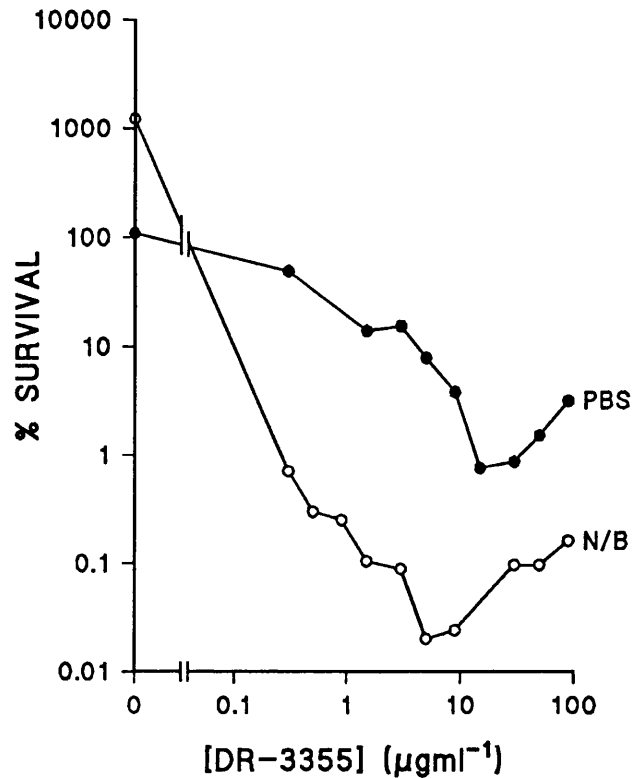
There was also a minor biphasic response in PBS with an OBC of 15µgml^{-1} and survival at the OBC being 0.76%: thus, perhaps PBS did not completely inhibit mechanism A of DR-3355 at 37°C. DR-3355 would seem to exhibit mechanisms A and either B or C against *Staph. epidermidis*: from these results it cannot be said whether bactericidal mechanism B or C was operating. This was because, as mentioned earlier, the bactericidal activity could not be determined in the presence of chloramphenicol since preliminary experiments found that the activity of chloramphenicol was highly variable with this species. However, it was found previously by Lewin and Amyes (1989) that DR-3355 exhibited mechanism B against *Staph. epidermidis* at 37°C.

Figure 42 shows the bactericidal profiles of DR-3355 against *Staph. epidermidis* in nutrient broth and in PBS at 30°C. There was a biphasic response in nutrient broth with an OBC of 3µgml^{-1} which was lower than at 37°C, and survival at the OBC was 0.03% which was slightly higher than that at 37°C (Table 13). In PBS the bactericidal activity of DR-3355 was greater than that observed at 37°C in PBS and at the OBC the survival (0.58%) was approximately 19-times higher than that seen in nutrient broth at 30°C. However, at DR-3355 concentrations greater than 15µgml^{-1} survival in PBS was similar to that in nutrient broth.

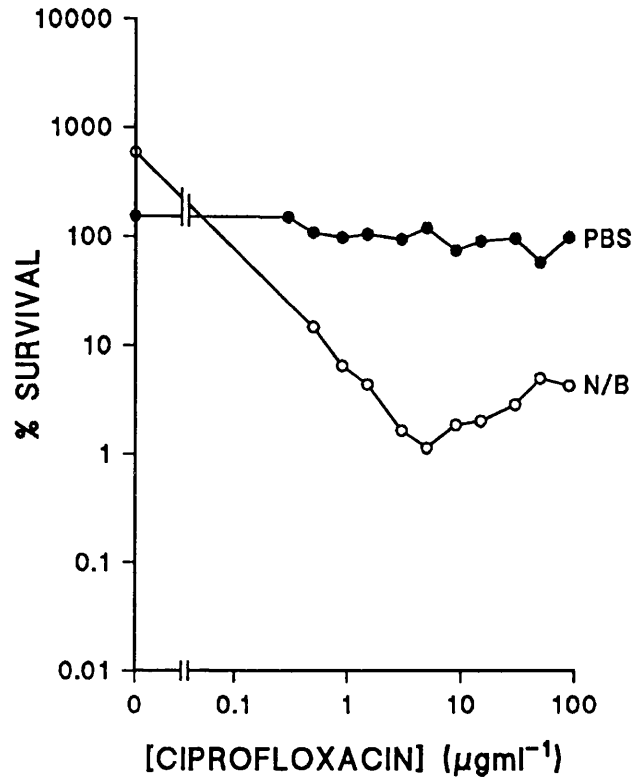**Figure 42: Bactericidal activity of DR-3355 against *Staph. epidermidis* at 30°C after 3 hours.**
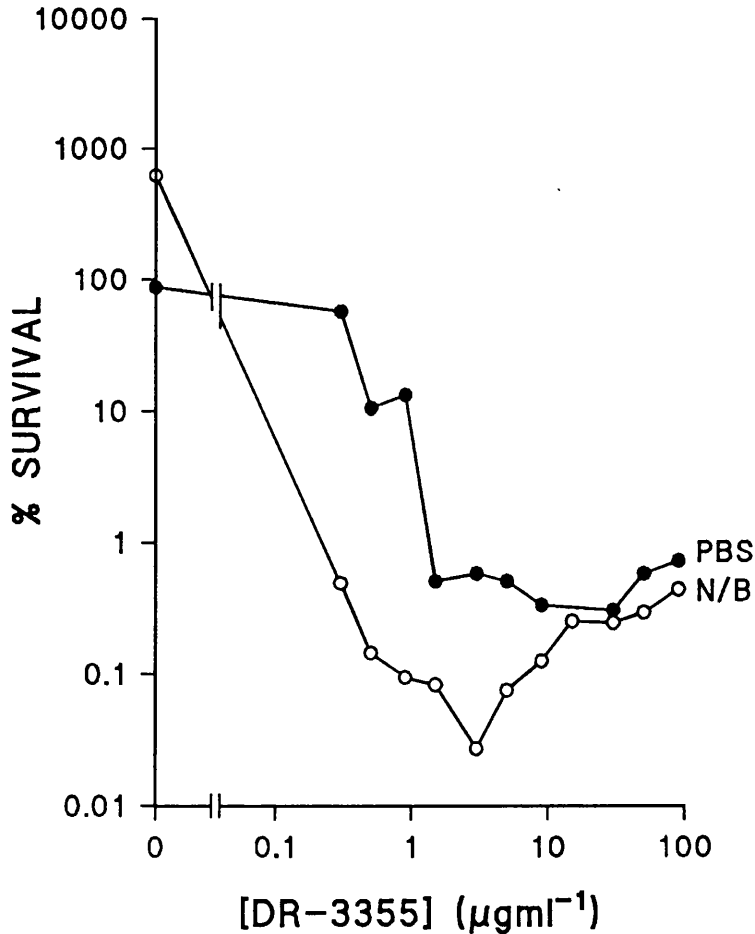
Figure 43 shows the bactericidal profiles of DR-3355 against
Staph. epidermidis in nutrient broth and in PBS at 25°C.
There was a minor biphasic response in nutrient broth. The
OBC was $3\mu gml^{-1}$ of DR-3355 and survival at the OBC was 0.37%,
increasing to 1.39% at $90\mu gml^{-1}$. When the bacteria were
treated in PBS the survival tended to become closer to that
in nutrient broth as the concentration of DR-3355 was
increased, and the survival between 30 and $90\mu gml^{-1}$ DR-3355
was virtually identical in either media. At the OBC in PBS
the survival was 1.19% which was about 3-fold higher than
that seen in nutrient broth.

**Figure 43: Bactericidal activity of DR-3355 against *Staph.
epidermidis* at 25°C after 3 hours.**
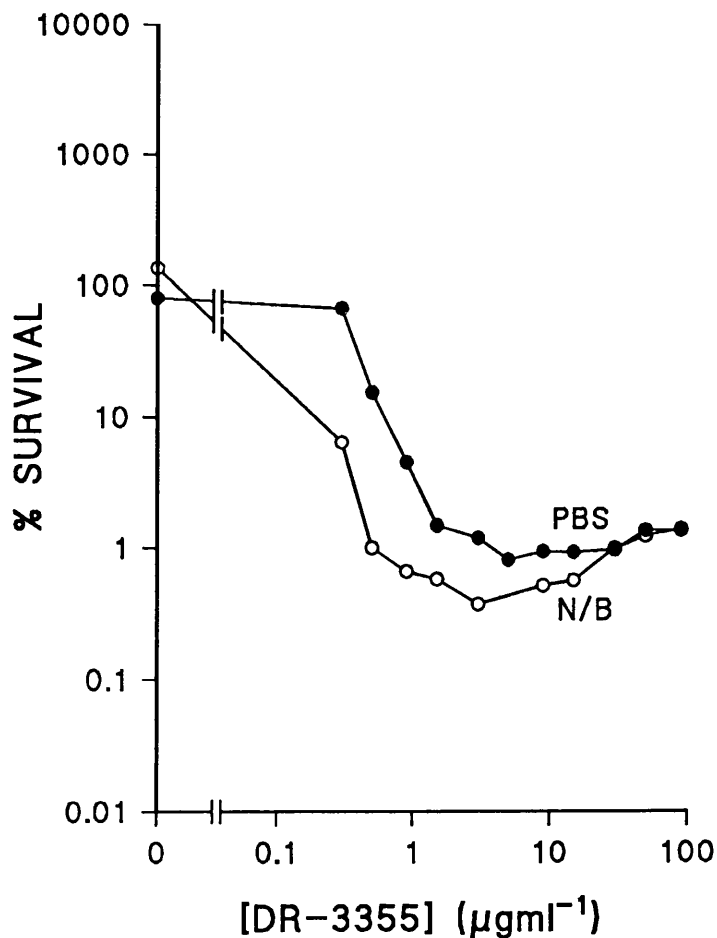
Figure 44 shows the bactericidal profiles of DR-3355 against

Staph. epidermidis in nutrient broth and in PBS at 20°C.

There was a biphasic response in nutrient broth wih an OBC

of 3µgml^{-1} and survival at the OBC was 0.38% (Table 13). In

PBS the shape of the profile was similar to that in nutrient

broth but the biphasic response was less pronounced and at

the highest concentration of DR-3355 tested (90µgml^{-1}) the

survival in PBS was virtually identical to that in nutrient

broth. At the OBC survival in PBS was 1.66% which was about

4-fold higher than in nutrient broth.

Figure 44: **Bactericidal activity of DR-3355 against** *Staph.*

epidermidis **at 20°C after 3 hours.**
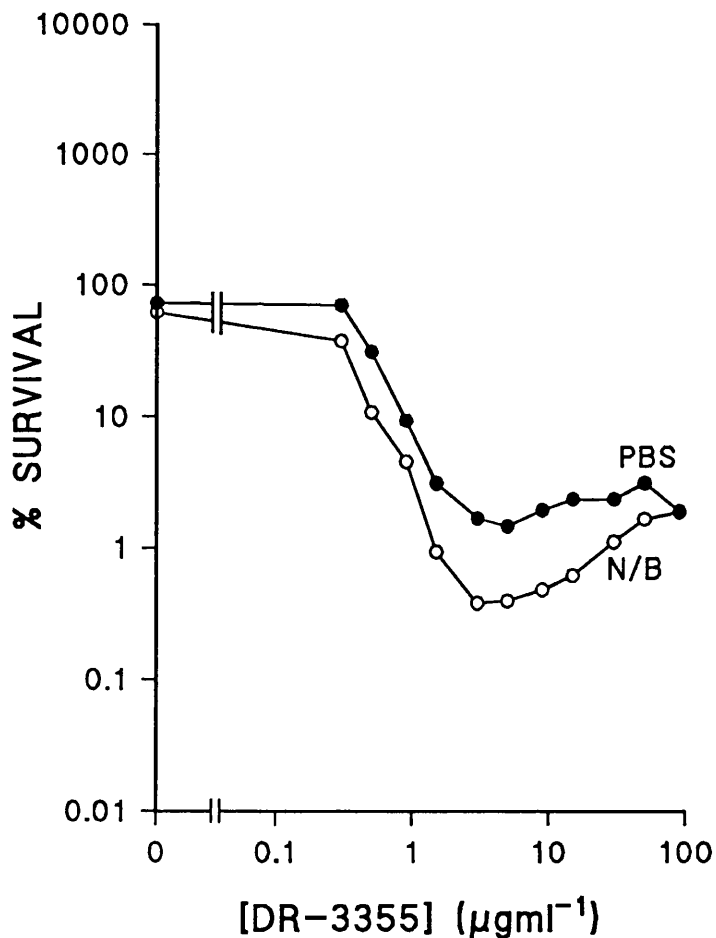
Figure 45: Bactericidal activity of DR-3355 in nutrient broth against *Staph. epidermidis* at 20-37°C after 3 hours.
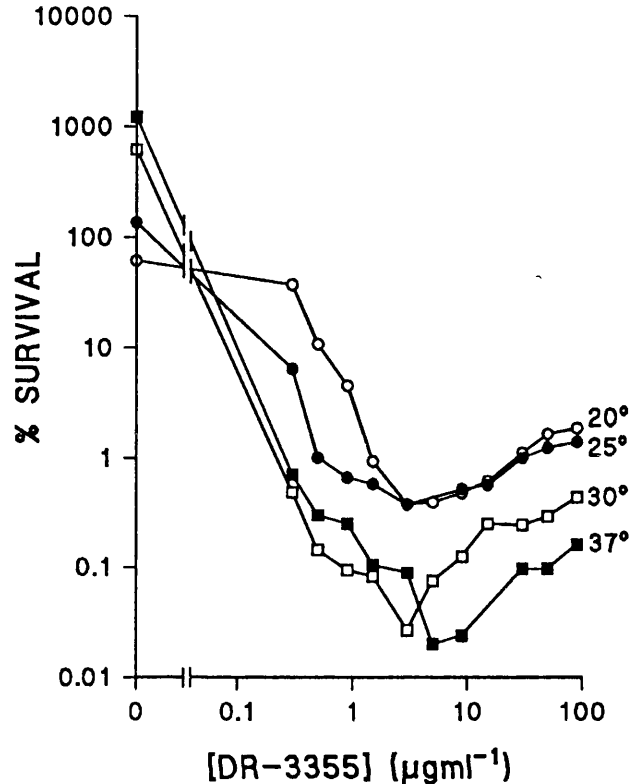
Figure 45 shows the profiles of DR-3355 in nutrient broth at 20, 25, 30 and 37°C. Although the activity of DR-3355 in nutrient broth at its respective OBC values was excellent at all four temperatures studied, decreasing the temperature of incubation from 37 to 20°C reduced the activity by 19-fold. As was found with *Staph. aureus* this relative reduction was much less (about 4-fold) than that observed with ciprofloxacin. At 30 or 37°C the activity of DR-3355 was similar at concentrations of 0.3 to 1.5µgml⁻¹ but above 15µgml⁻¹ the survival was quite different at these two temperatures. Figure 46 shows the bactericidal profiles of DR-3355 in PBS at 20, 25, 30 and 37°C. The shape of the profile at 37°C was quite different to the other profiles in

that there was a biphasic response and the survival at 1.5-9μgml^{-1} was higher than at the three lower temperatures studied. However, reducing the incubation temperature from 30 to 20°C decreased the activity of DR-3355 by approximately 3-fold at the OBCs. DR-3355 exhibited similar or lower OBCs than ciprofloxacin against *Staph. epidermidis* but the survival at the OBCs was lower with DR-3355 at any temperature (Tables 12 and 13). In PBS the activity of DR-3355 was much higher than that of ciprofloxacin because ciprofloxacin only exhibited bactericidal mechanism A against Staphylococci whereas DR-3355 exhibited bactericidal mechanisms A and B (and/or C).

Figure 46: Bactericidal activity of DR-3355 in PBS against *Staph. epidermidis* at 20-37°C after 3 hours.
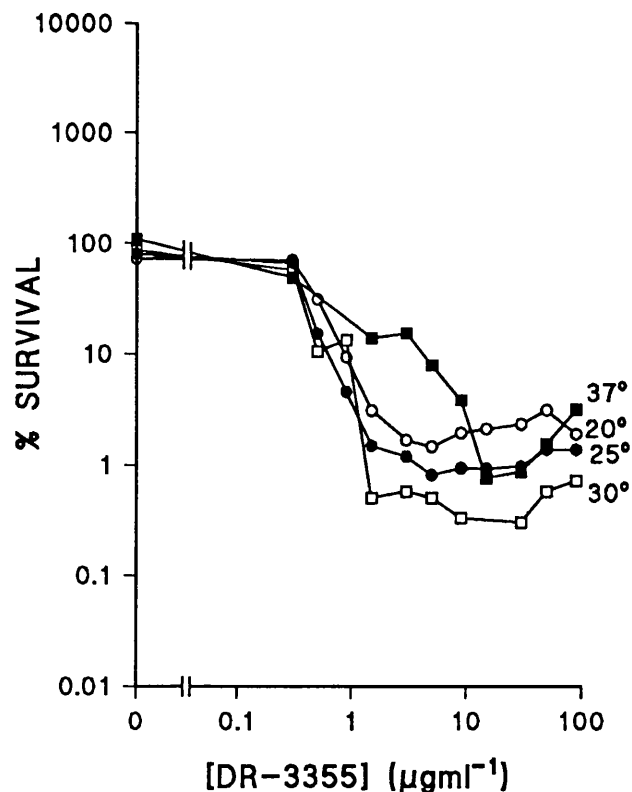
Of the bacteria tested *Ps. aeruginosa* was the most susceptible to either ciprofloxacin or to DR-3355, and this bacterium was unusual in that neither 4-quinolone exhibited a biphasic response. In nutrient broth *E. coli* was more susceptible than either *Staph. aureus* or *Staph. epidermidis* in that the OBCs of either ciprofloxacin or DR-3355 were much lower against the Gram-negative species. However, with the odd exception, at the OBCs for each 4-quinolone against these three bacteria the survival was quite similar at any given temperature. Ciprofloxacin and DR-3355 exhibited bactericidal mechanisms A and B (and/or C) against *E. coli*. Ciprofloxacin seemed to exhibit solely mechanism A against the two Staphylococci, while DR-3355 exhibited mechanism A and mechanism B (and/or C) against these two cocci. Decreasing the temperature of incubation from 37 to 20°C markedly increased the survival of all four of the bacteria tested at the OBCs of either 4-quinolone in nutrient broth. However, reducing the temperature of incubation had a much smaller effect on the activity of the 4-quinolones when *E. coli*, *Staph. aureus* or *Staph. epidermidis* were treated in PBS. This suggests that mechanism A was much more affected by temperature than mechanisms B or C. The temperature of incubation also affected the OBCs of ciprofloxacin and DR-3355 but differences were highly variable and did not follow any trend.

SECTION 5:

Thermodynamic aspects of the growth and the death of bacteria treated with 4-quinolones:

The effects of temperature on the growth of bacteria can be described by the simple Arrhenius equation. This equation was originally devised to describe the effect of temperature on the rate of hydrolysis of sucrose:

$$v = A\, e^{-E_a / RT} \qquad (4)$$

where v is the velocity of the reaction, R is the ideal gas constant (1.9862 calories mole^{-1} K^{-1}), T is the absolute temperature (K), A is a constant known as the pre-exponential factor and E_a is the apparent activation energy (calories). Taking the logarithm$_{10}$ of the above equation:

$$\log v = -E_a / 2.303 \mathrm{x} RT \qquad (5)$$

it is apparent that when $\log_{10} v$ is plotted against $1/T$ a straight line will be obtained with slope of $E_a/2.303\mathrm{x}RT$. Thus the apparent activation energy is the slope x $2.303R$. As the temperature of a reaction is increased the proportion of molecules that possess the kinetic energy (E_a) required to react is increased. Chemical catalysts and enzymes increase reaction rate by lowering (typically by half) the apparent activation energy of a reaction i.e. increase the proportion of molecules with sufficient energy to react (Atkin, 1986). The magnitude of the apparent activation

energy of a reaction is related to the nature of the reaction i.e. whether it is chemical, biological etc. First and second order chemical reactions typically have E_a values from 0 to 90,000 calories, whereas enzyme-catalysed reactions usually have values for E_a around 10,000 calories (Gutfreund, 1972; Atkin, 1986). The apparent activation energy for growth of bacteria can be calculated by replacing v with the specific growth rate (μ) (Pirt, 1975).

The effects of temperature on the specific growth rates of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* in nutrient broth at 20, 25, 30 and 37°C were studied. The specific growth rate (μ) is the slope of the straight line (i.e. exponential) portion of a plot of the natural logarithm (ln) of viable count in nutrient broth against time (Pirt, 1975). The specific growth rate can be expressed more conveniently as the time taken for the viable count to double (t_d), where:

$$t_d = \ln 2 \, / \, \mu \qquad\qquad (6)$$

The growth rates of the bacteria were determined at each temperature as described in the "Materials and Methods" section. The slope of the straight line portion of each growth curve was calculated using the "lreg" (simple linear regression) computer program on Unix at the School of Pharmacy.

Effects of temperature on the growth of _E. coli_:

Figure 47 shows the growth curves in nutrient broth for _E. coli_ over 4 hours at 37°C and over 5 hours at 20, 25 and 30°C. At zero time at 37°C the viable count was 7.20×10^6 colony-forming units (cfu) ml^{-1} and there was a short lag phase of approximately 0.5 hours before multiplication started, after which the bacteria multiplied rapidly over the next 1.5 hours, doubling every 20.4 minutes (Table 14). The value of 20.4 minutes for the doubling time of _E. coli_ agrees well with previous findings (Pirt, 1975). After 2 hours incubation the growth rate started to decrease and after 3 hours the viable count remained fairly constant at approximately 2.20×10^{-8} cfu ml^{-1}.

Figure 47: Multiplication of _E. coli_ in nutrient broth at 20-37°C.
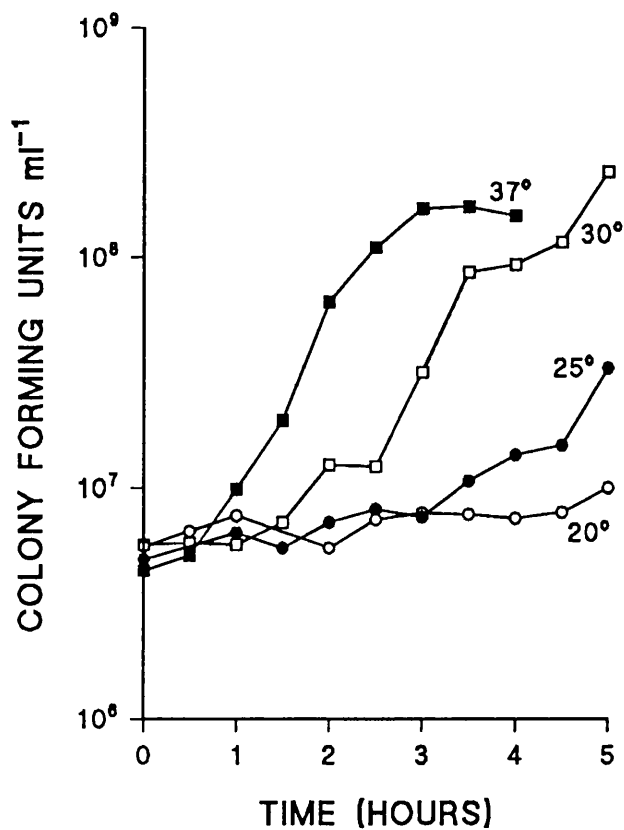
Table 14: culture doubling time (t_d) in minutes for multiplication of bacteria in nutrient broth:

BACTERIA	20°C	25°C	30°C	37°C
E. coli	203.8	72.8	31.0	20.4
Ps. aeruginosa	136.7	99.7	72.3	56.3
Staph. aureus	39.0	35.6	23.4	13.7
Staph. epidermidis	-	50.1	31.2	44.1

-= no multiplication

At 30°C the initial viable count was 6.85×10^6 cfu ml^{-1} and there was a lag of approximately 1.5 hours. After the lag period growth started more slowly than that observed at 37°C with a doubling time of 31.0 minutes for 2 hours. Between 3.5 and 5 hours the viable count increased more slowly to a final viable count of approximately 2.42×10^8 cfu ml^{-1} which was higher than the final count at 37°C after 4 hours. At 25°C the initial viable count was 3.95×10^6 cfu ml^{-1} and after a lag of about 2 hours the viable count doubled every 72.8 minutes to a viable count of 3.75×10^7 cfu ml^{-1} at 5 hours. At 20°C the viable count at time zero was 5.60×10^6 cfu ml^{-1}. Over the following 1.5 hours the viable count increased slightly and then decreased to 5.50×10^6 cfu ml^{-1} at 2 hours. After 2 hours incubation the culture grew slowly with a doubling time of 203.8 minutes to 1.00×10^7 cfu ml^{-1}. Overall, as the temperature of incubation increased both the lag period and the culture doubling time decreased (Table 14).

Effects of temperature on the growth of *Ps. aeruginosa*:

Figure 48 shows the growth curves for *Ps. aeruginosa* in nutrient broth at 20, 25, 30 and 37°C over 5 hours. The main respect in which they differed from the growth curves of *E. coli* (Figure 47) is that a lag phase did not occur at any of the temperatures studied. At 37°C the culture grew exponentially from an initial inoculum of 4.50×10^6 cfu ml^{-1} at time zero for 3.5 hours with a doubling time of 56.3 minutes, nearly three times that found with *E. coli* (Table 14). For the next 1.5 hours the growth rate decreased slightly and the viable count after 5 hours was 1.90×10^8 cfu ml^{-1}. At 30°C the culture grew exponentially from an initial

Figure 48: Multiplication of *Ps. aeruginosa* in nutrient broth at 20-37°C.
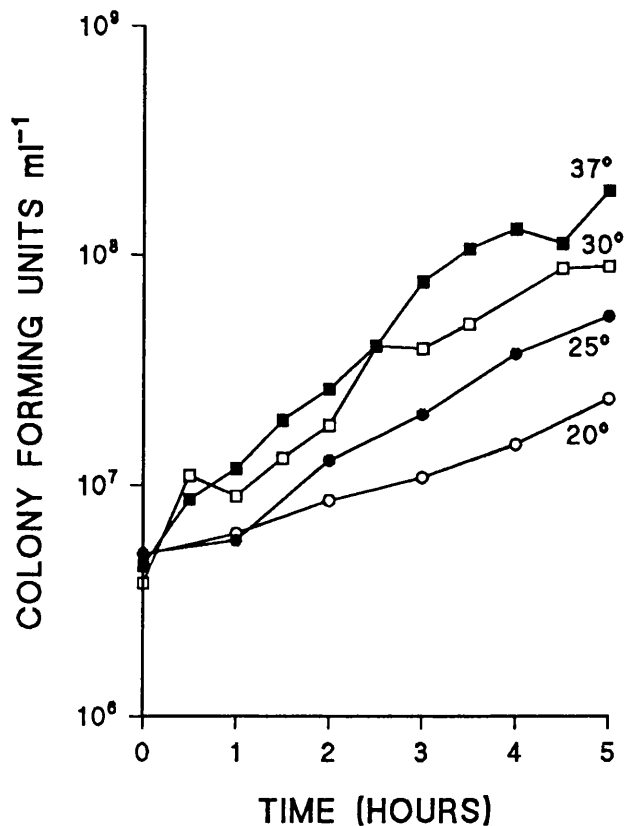
viable count of 3.80×10^6 cfu ml^{-1} to 8.90×10^7 cfu ml^{-1} at 5 hours, although there were some deviations from the straight line. The doubling time was 72.3 minutes, which was longer than that observed with *E. coli* at 30°C but virtually the same as that found at 25°C with *E. coli* (Table 14). At 25°C the viable count at time zero was 5.10×10^6 cfuml^{-1} and increased a little over the first hour of incubation. Between 1 and 5 hours incubation, growth was exponential with a doubling time of 99.7 minutes to a final viable count of 5.40×10^7 cfu ml^{-1}. At 20°C the culture grew exponentially with a doubling time of 136.7 minutes from an initial viable count of 5.00×10^6 cfu ml^{-1} to 2.37×10^7 cfu ml^{-1} after 5 hours. Thus, *Ps. aeruginosa* grew more rapidly than *E. coli* at 20°C (Table 14). As was found with *E. coli* increasing the temperature of incubation increased the growth rate of *Ps. aeruginosa*.

Effects of temperature on the growth of *Staph. aureus*:
Figure 49 shows the growth curves for *Staph. aureus* in nutrient broth at 20, 25, 30 and 37°C over 4 hours. At 37°C the viable count at time zero was 4.40×10^5 cfu ml^{-1} and it increased slowly to 7.80×10^5 cfu ml^{-1} after 1 hour of incubation. Over the next 1.5 hours the culture multiplied very rapidly with a doubling time of 13.7 minutes to 7.70×10^7 cfu ml^{-1}, which was more rapid than the growth of either *E. coli* or *Ps. aeruginosa* at 37°C (Table 14). Between 2.5 and 4 hours incubation there was a slight decrease in the viable count of the culture. At 30°C the initial viable count was 6.30×10^5 cfu ml^{-1} rising to 1.21×10^6

Figure 49: Multiplication of *Staph. aureus* **in nutrient broth at 20-37°C.**
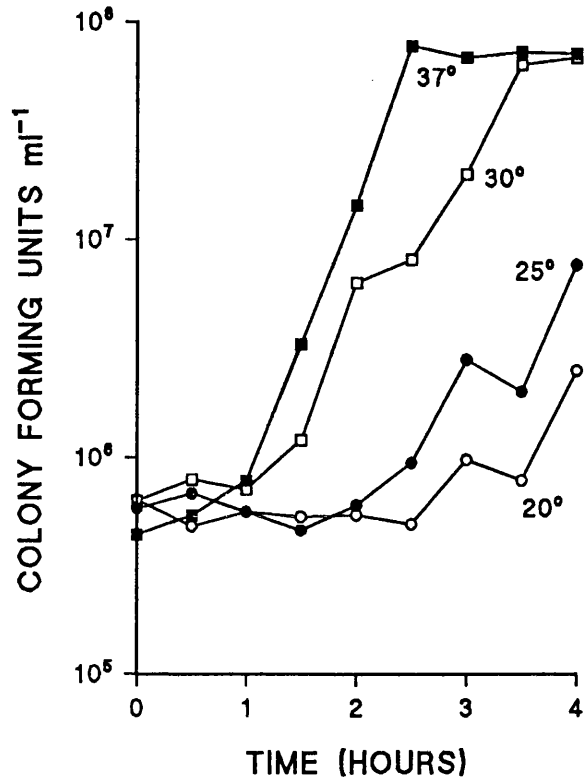
cfu ml^{-1} after 1.5 hours. From 1.5 to 3.5 hours incubation the viable count of the culture doubled every 23.4 minutes to a density of 6.30x10^7 cfu ml^{-1} after which the growth rate decreased, the viable count increasing to 6.80x10^7 cfu ml^{-1} at 4 hours. Thus, the maximum viable counts of the cultures grown at either 37 or 30°C were similar. At 25°C there was a lag phase of about 2 hours and from 2 to 4 hours of incubation the viable count increased from 6.00x10^5 to 7.60x10^6 cfu ml^{-1} which represents a doubling time of 35.6 minutes. At 20°C there was an extended lag phase of 2.5 hours during which there was a net decrease in the viable count from 6.40x10^5 to 4.90x10^5 cfu ml^{-1}. However, over the following 1.5 hours of incubation the viable count of the culture doubled every 39.0 minutes to a density of 2.50x10^6

cfu ml^{-1}. The doubling time and the length of the lag phase

before growth of *Staph. aureus* increased as the temperature

of incubation was decreased. *Staph. aureus* multiplied more

rapidly in nutrient broth than *E. coli* at every temperature

of incubation tested (Table 14). Although *Staph. aureus*

grew more slowly at 20°C compared to the three higher

temperatures studied, it grew more rapidly at 20°C than *Ps.*

aeruginosa at 20, 25, 30 or 37°C (Table 14).

Effects of temperature on the growth of *Staph. epidermidis*:

Figure 50 shows the growth curves for *Staph. epidermidis* in

nutrient broth at 20, 25, 30 and 37°C over 5 hours. At 37°C

there was no lag phase and the viable count doubled every

44.1 minutes from 9.20×10^5 cfu ml^{-1} at time zero to 1.50×10^7

cfu ml^{-1} after 3 hours of incubation. From 3 to 5 hours

incubation the growth rate decreased and the viable count

increased to 3.80×10^7 cfu ml^{-1}. At 30°C the initial viable

count was 7.40×10^5 cfu ml^{-1} increasing to 1.49×10^6 cfu ml^{-1}

after 1.5 hours. Between 1.5 and 4 hours incubation the

viable count doubled every 31.2 minutes to 2.50×10^7 cfu ml^{-1}.

Hence, the maximium specific growth rate was higher at 30°C

than at 37°C and virtually identical to that of *E. coli* at

30°C (Table 14). At 25°C the initial viable count was

8.00×10^5 cfu ml^{-1} and there was a lag of one hour. The

viable count then increased to 1.69×10^6 cfu ml^{-1} at 2 hours

and decreased again to 1.50×10^6 cfu ml^{-1} after 3 hours

incubation. Between 3 and 5 hours the viable count of the

culture increased exponentially with a doubling time of 50.1

Figure 50: Multiplication of *Staph. epidermidis* in nutrient broth at 20-37°C.
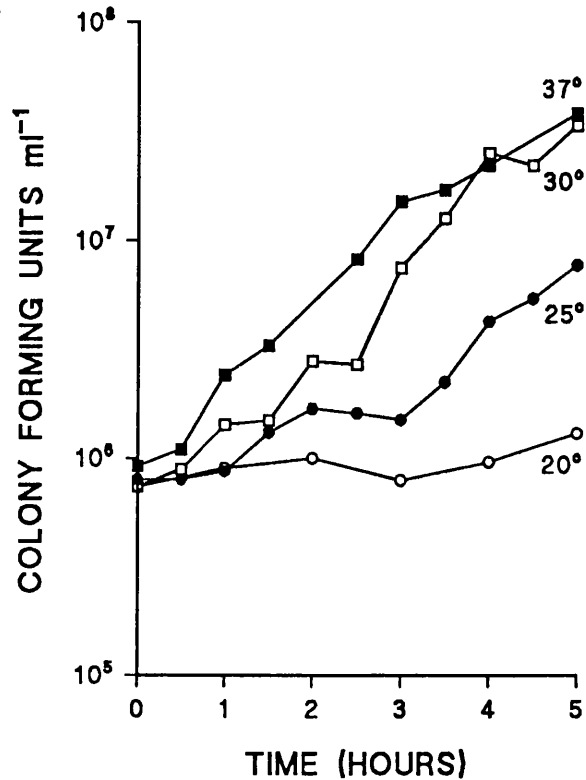
minutes to 7.70×10^6 cfu ml^{-1} (Table 14). At 20°C the initial viable count was 7.35×10^5 cfu ml^{-1} and increased to 1.12×10^6 cfu ml^{-1} after 2 hours. The viable count then decreased to 7.90×10^5 cfu ml^{-1} and then increased to 1.30×10^6 cfu ml^{-1} after 5 hours incubation. Thus, there was little or no multiplication of the bacteria after 5 hours incubation at 20°C. The optimum growth temperature of *Staph. epidermidis* was 30°C and it multiplied more slowly than *Staph. aureus* at all four temperatures studied, but more rapidly than *Ps. aeruginosa* at 37, 30 or 25°C (Table 14). All bacterial species are well known to exhibit an optimum growth temperature which is usually at temperatures exceeding 37°C (in mesophilic bacteria) and so the result with *Staph. epidermidis* was unexpected. Could this observation be

related to the rôle of *Staph. epidermidis* as a skin

commensal? Possible reasons for an optimum growth

temperature include thermolability of enzymes or structural

proteins, lower activity of certain enzymes, and additional

nutrient requirements at elevated temperatures (Ingraham,

1962; Herendeen *et al*, 1979). Interestingly, the

observation that the mutation frequency of *Staph.*

epidermidis to resist ciprofloxacin, ofloxacin or DR-3355

always decreased as the temperature of incubation was

reduced from 37 to 25°C (Table 4, Figure 8), would seem to

suggest that temperature had a direct effect on the mutation

rate, rather than *via* an indirect effect caused by the rate

of growth of the bacteria. Another respect in which *Staph.*

epidermidis was unusual was that mutants resistant to

ofloxacin or DR-3355 could be isolated at 25°C but were

unstable and could not be subcultured onto the same

concentration of the 4-quinolone.

Apparent activation energies for growth of the bacteria:

As described earlier the apparent activation energy (E_a) can

be determined over a range of growth temperatures by

substituting the specific growth rate (μ) for v in equation

(5). The apparent activation energy is the slope of the

Arrhenius plot of log μ (min^{-1}) against $1/T$ (K^{-1}). Figure 51

shows the Arrhenius plots for the growth of *E. coli*, *Ps.*

aeruginosa, *Staph. aureus* and *Staph. epidermidis* in nutrient

broth using the specific growth rates derived from the

growth curves described above (Figures 47, 48, 49 and 50,

Figure 51: Arrhenius plot for multiplication of bacteria in nutrient broth.
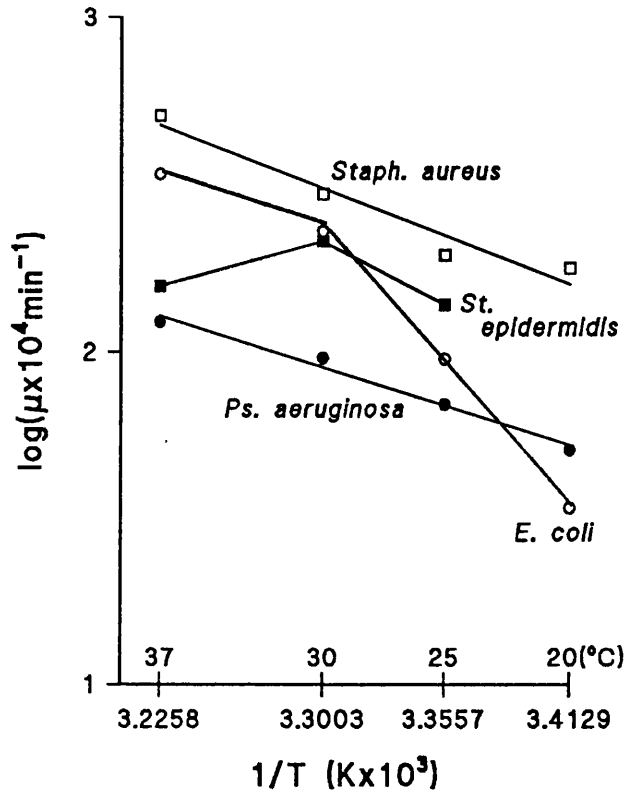
respectively) at 20, 25, 30 and 37°C. On the X-axis the

values for the temperature in °C are also shown for clarity.

The value for the slope of each line was calculated by

simple linear regression ("lreg" computer program) and in

addition, the "lreg" computer program determines how well

the data fit the straight line by calculating the

correlation coefficient (r) between the X- and the Y-data.

A value for r of +/-0.9000 or more indicates a reasonable

correlation between the data, and when the line is between

only two data points the value for r is +/-1.0000. Except

for *Staph. epidermidis* between 37 and 30°C the slopes of the

lines had negative values (and the value of r was also

negative) because the growth rates decreased as the

reciprocal of the temperature increased. With *E. coli* the

Arrhenius plot was not linear over the whole temperature range, being biphasic with the two phases being between 20 and 30°C and between 30 and 37°C. The values for the apparent activation energies were 33,197 for 20-30°C ($r= -0.9992$) and 10,995 calories for 30-37°C ($r= -1.0000$) (see Table 15).

Table 15: apparent activation energies (E_a) for multiplication of bacteria in nutrient broth.

BACTERIA	APPARENT ACTIVATION ENERGY (E_a) IN CALORIES			
	20-30°C	25-30°C	30-37°C	20-37°C
E. coli	33,197	N/A	10,995	N/A
Ps. aeruginosa	------------------------>			9,459
Staph. aureus	------------------------>			11,555
Staph. epidermidis	N/A	16,943	N/A	N/A

N/A= Not applicable

These results compare reasonably well with previous findings for the growth rates of *E. coli* whereby the apparent activation energy from 12 to 26°C was 28,600 calories and from 26 to 37°C was 16,200 calories (Pirt, 1975). It is thought that changes in the apparent activation energy indicate that differences in the rate-controlling reactions or in metabolic processes occur (Ingraham, 1962; Pirt, 1975). With *Ps. aeruginosa* the Arrhenius plot was an excellent straight line ($r= -0.9917$) between 20 and 37°C and

the apparent activation energy was 9,459 calories (Table 15). This value for the apparent activation energy compares reasonably well with a value of 12,600 calories for a psychrophilic pseudomonad grown in a rich medium between 12 and 30°C (Pirt, 1975). As was found with *Ps. aeruginosa* the Arrhenius plot for the growth of *Staph. aureus* was linear (*r*= -0.9561) between 20 and 37°C and the apparent activation energy over that temperature range was 11,555 calories (Table 15). The values for the apparent activation energies for *E. coli* between 30 and 37°C, and for *Ps. aeruginosa* or *Staph. aureus* between 20 and 37°C were very similar (≈9,000–11,500 calories, see Table 15). Could this reflect a growth rate-limiting step which is common to these three bacterial species? With *Staph. epidermidis* the Arrhenius plot was quite different to that of the other three bacteria tested in that the slope was positive between 37 and 30°C and negative between 30 and 25°C (E_a= 16,943 calories, *r*= -1.0000). As mentioned earlier *Staph. epidermidis* did not grow at 20°C in nutrient broth. Since the growth of *Staph. epidermidis* from 30 to 37°C did not obey the Arrhenius law (possible reasons for this were discussed above) this value for the apparent activation energy (*minus* 9,091 calories) was disregarded. With *E. coli* between 20 and 30°C and with *Staph. epidermidis* between 25 and 30°C the values for the apparent activation energies were higher than 11,500 calories (33,197 and 16,943 calories, respectively) which suggests that these bacterial species have two quite different growth rate-limiting steps at lower temperatures.

Effects of temperature on the death of bacteria treated with ciprofloxacin or DR-3355:

In addition the effects of temperature on the specific death rates of the four bacteria treated with ciprofloxacin or DR-3355 in nutrient broth were determined at 20, 25, 30 and 37°C. As described in the "Materials and Methods" section such bactericidal tests were determined essentially by the same method as that used to determine the growth curves but the reaction mixture was modified to include the 4-quinolone. The specific death rate (μ_q) is the slope of the initial portion of a plot of ln viable count in nutrient broth plus 4-quinolone versus time. Similarly, the time taken for the viable count to halve (t_h) can be calculated by replacing μ with μ_q in equation (6) above.

Effects of temperature on the death of *E. coli* treated with ciprofloxacin:

Figure 52 shows the death curves for *E. coli* treated with the OBC ($0.15\mu gml^{-1}$, determined at 37°C, Table 8) of ciprofloxacin in nutrient broth over 4 hours at 20, 25, 30 and 37°C. At 37°C the viable count halved every 8.5 minutes from 4.21×10^6 cfu ml^{-1} to 3.20×10^4 cfu ml^{-1} in the first hour of incubation (Table 16). Thus, no lag phase before death was observed, a phenomenon which was found with *E. coli* treated with nalidixic acid, ciprofloxacin, or ofloxacin (Smith, 1984a; Carret *et al*, 1991). After the first hour the death rate became progressively slower and after 4 hours incubation the viable count was 3.10×10^3 cfu ml^{-1}. At 30°C

Figure 52: Death of *E. coli* treated with the OBC[37] of ciprofloxacin at 20-37°C.
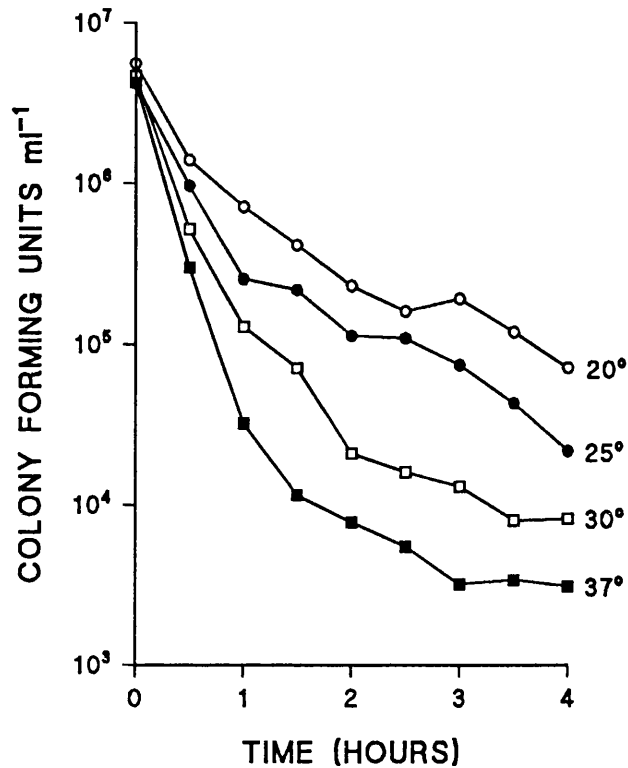
the viable count halved every 11.6 minutes from 4.66×10^6 cfu ml^{-1} at time zero to 1.29×10^5 cfu ml^{-1} after 1 hour. As was found at 37°C after the first hour the death rate progressively decreased and the viable count after 4 hours was 8.20×10^3 cfu ml^{-1}. The "tailing" of the death curves of *E. coli* treated with 4-quinolones has been reported (Smith, 1984a; Carret *et al*, 1991) and is probably due to induced, pre-existent or experimental heterogeneity in the bacterial population (Cerf, 1977). Carret *et al* (1991) also found that the bacteria from the second phase of the curve did not have higher minimum inhibitory concentrations (MICs) than the original culture, so tailing cannot be explained by the presence of resistant mutants. At 25°C in the first hour of incubation the viable count halving time was 14.9 minutes as

Table 16: culture halving time (t_h) in minutes for bacteria treated with ciprofloxacin in nutrient broth.

BACTERIA	20°C	25°C	30°C	37°C
E. coli	20.2	14.9	11.6	8.5
Ps. aeruginosa	2.9	1.6	0.8	0.4
Staph. aureus	53.0	31.5	14.8	7.9
Staph. epidermidis	57.6	34.7	18.7	8.8

the viable count decreased from 4.17×10^6 to 2.54×10^5 cfu ml^{-1}, after which the viable count fell more slowly to 2.16×10^4 cfu ml^{-1}. At 20°C the viable count halved every 20.2 minutes in the first hour of incubation to 7.10×10^5 cfu ml^{-1} and then decreased by 1 log to 7.10×10^4 cfu ml^{-1} after 4 hours. In agreement with the bactericidal profiles described earlier, the specific death rate decreased and hence the culture halving time increased (by more than 2-fold) as the incubation temperature was reduced from 37 to 20°C (Table 16).

Effects of temperature on the death of *Ps. aeruginosa* treated with ciprofloxacin:

Figure 53 shows the death curves for *Ps. aeruginosa* treated with 3µgml^{-1} ciprofloxacin in nutrient broth at 20, 25, 30 and 37°C. As mentioned earlier, preliminary experiments showed that this organism was killed extremely rapidly so that the bactericidal effects of ciprofloxacin or DR-3355 were determined by sampling every 5 minutes over 30 minutes

Figure 53: Death of *Ps. aeruginosa* treated with 3μgml⁻¹ ciprofloxacin at 20-37°C.
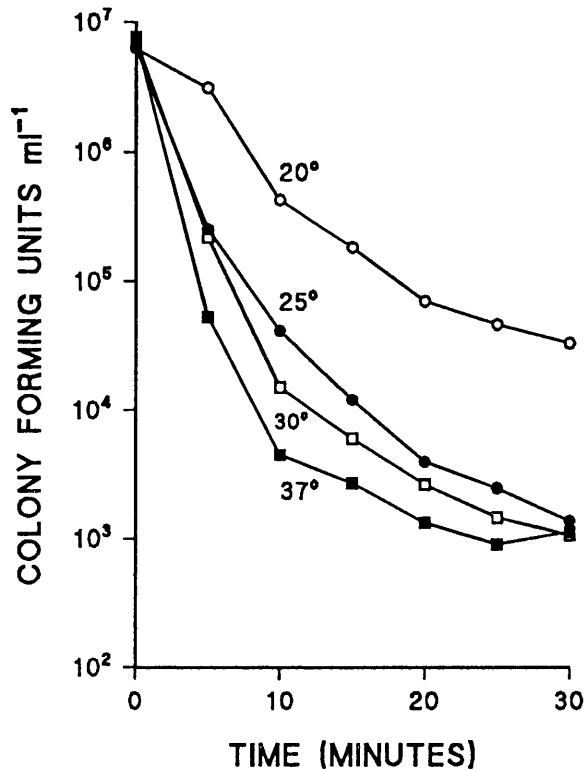
as described in the "Materials and Methods" section. The concentration of ciprofloxacin chosen was $3\mu gml^{-1}$ because it is the peak serum concentration achieved after a single oral dose of 500mg (Hooper and Wolfson, 1985) and it is close to the OBCs of ciprofloxacin or DR-3355 against either *Staph. aureus* or *Staph. epidermidis*. It should be noted that 4-quinolones do not exhibit an OBC with *Ps. aeruginosa* because at bactericidal concentrations they solely exert bactericidal mechanism B (Morrissey and Smith, 1990). At 37°C the initial viable count was 7.71×10^6 cfu ml⁻¹ and it halved every 0.4 minutes to 5.30×10^4 cfu ml⁻¹, which was approximately 20-times more rapid than the death of *E. coli* treated with the OBC of ciprofloxacin (Table 16). From 5 to 10 minutes the viable count decreased more slowly to

4.50×10^3 cfu ml^{-1} and more slowly to 9.00×10^2 cfu ml^{-1} after

25 minutes. The viable count then increased to 1.13×10^3 cfu

ml^{-1} after 30 minutes. At 30°C the viable count at time zero

was 7.50×10^6 cfu ml^{-1} decreasing with a halving time of 0.8

minutes to 1.50×10^4 cfu ml^{-1} after 10 minutes (Table 16).

The viable count then decreased more slowly to 1.06×10^3 cfu

ml^{-1} over the following 20 minutes. At 25°C the viable count

halved every 1.6 minutes over the first 10 minutes of

incubation from 6.42×10^6 to 4.12×10^4 cfu ml^{-1}. Thus, the

initial culture halving time at 25°C was two- or four-times

higher than that observed at 30 or 37°C, respectively (Table

16). At 25°C the viable count decreased more slowly to

1.36×10^3 cfu ml^{-1} after 30 minutes which was a similar viable

count to that seen at either 30 or 37°C after 30 minutes

incubation. The similarity of the viable counts after 30

minutes incubation may be explained by the "tailing" of the

death curves, which was also observed with *E. coli* (see

Figure 52). Although the initial death rate at 37°C was 4-

times greater than that observed at 25°C, in the second

phase of the death curves the death rate at 25°C was greater

than that observed at 37°C causing the curves to converge.

Furthermore, the bactericidal profiles of ciprofloxacin

against *Ps. aeruginosa* (Figure 21) showed that after 30

minutes incubation the survival of the bacteria treated with

$3\mu gml^{-1}$ at 30 or 37°C was similar at each temperature, but

that the survival of the bacteria at 25°C was slightly

higher than that observed at 30 or 37°C. At 20°C the viable

count halved every 2.9 minutes from 6.36×10^6 to 7.00×10^4 cfu

ml^{-1} between 0 and 20 minutes, and the viable count then decreased more slowly to 3.29x10^4 cfu ml^{-1} after 30 minutes. Therefore, as was found with *E. coli* decreasing the temperature of incubation increased the culture halving time, but the relative increase in t$_h$ from 37 to 20°C was approximately 7-fold which was greater than that found with *E. coli* (Table 16).

Effects of temperature on the death of *Staph. aureus* treated with ciprofloxacin:

Figure 54 shows the death curves for *Staph. aureus* treated with the OBC (5µgml^{-1}, determined at 37°C, Table 10) of ciprofloxacin in nutrient broth at 20, 25, 30 and 37°C. At 37°C the initial viable count of 4.17x10^6 cfu ml^{-1} decreased to 2.20x10^4 cfu ml^{-1} after 1 hour. The culture halving time was 7.9 minutes which was similar to that observed with *E. coli*, but about 20-times greater than that of *Ps. aeruginosa* (Table 16). The viable count of *Staph. aureus* then decreased at a constant lower rate to 6.00x10^2 cfu ml^{-1} after 4 hours. Therefore, as was found with *E. coli* at 30 or 37°C, and with *Ps. aeruginosa* at 25, 30 or 37°C, there was "tailing" of the death curves of *Staph. aureus* treated with ciprofloxacin at 37°C. At 30°C the shape of the death curve was quite different to the death curve at 37°C in that at 30°C there appeared to be a lag of 1 hour during which the viable count decreased slowly from 2.80x10^6 cfu ml^{-1} to 1.67x10^6 cfu ml^{-1}. Then from 1 to 2 hours the viable count halved every 14.8 minutes to 1.00x10^5 cfu ml^{-1} and the death

Figure 54: Death of *Staph. aureus* treated with the OBC[37] of

ciprofloxacin

at 20–37°C.
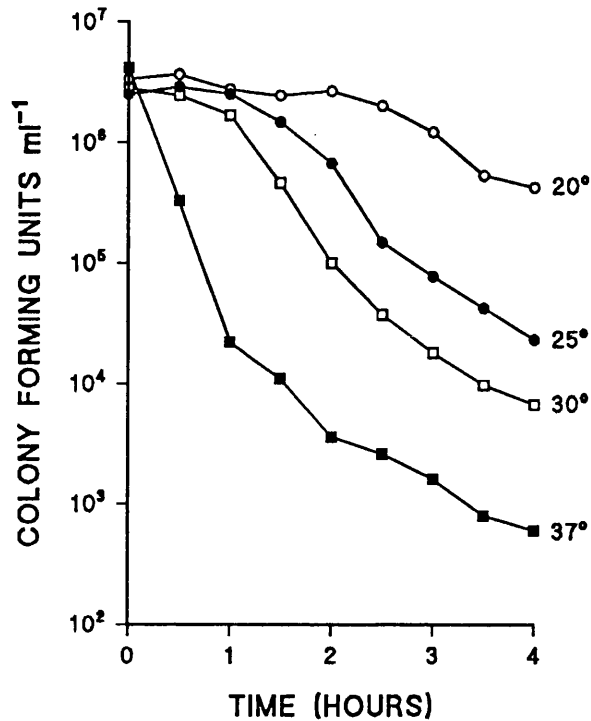
rate became progressively less as the viable count decreased

to 6.70×10^3 cfu ml^{-1} after 4 hours. As mentioned earlier,

the appearance of a lag phase before death has been reported

previously for *E. coli* treated with nalidixic acid,

pefloxacin, ciprofloxacin or ofloxacin (Smith, 1984a; Carret

et al, 1991), although none was found with *E. coli* in this

thesis (see Figure 52). At 25°C the death curve had a

similar shape to that observed at 30°C: at 25°C again there

was a lag of 1 hour during which the viable count slightly

increased from 2.49×10^6 to 2.51×10^6 cfu ml^{-1}. From 1 to 2

hours the viable count halved every 31.5 minutes to 6.70×10^5

cfu ml^{-1}, and then decreased to 2.30×10^4 cfu ml^{-1} after 4

hours incubation. At 20°C there was a lag of 2 hours which

was longer than that observed at either 25 or 30°C. The

viable count after 2 hours was 2.65×10^6 cfu ml^{-1} and it decreased with a halving time of 53.0 minutes to 1.21×10^6 cfu ml^{-1} after 3 hours. The death rate of the culture then began to decrease and the viable count after 4 hours was 4.20×10^5 cfu ml^{-1}. Therefore *Staph. aureus* was killed less rapidly than *E. coli* at 20, 25 or 30°C but both bacterial species were killed at similar rates at 37°C (Table 16). Decreasing the temperature of incubation from 37 to 20°C increased the culture halving time by approximately 7-fold, which was a similar relative increase to that observed with *Ps. aeruginosa* (Table 16).

Effects of temperature on the death of *Staph. epidermidis* treated with ciprofloxacin:

Figure 55 shows the death curves for *Staph. epidermidis* treated with the OBC (5µgml^{-1}, determined at 37°C, Table 12) of ciprofloxacin in nutrient broth at 20, 25, 30 and 37°C. At 37°C the initial viable count was 8.00×10^5 cfu ml^{-1} and it halved every 8.8 minutes to 7.00×10^3 cfu ml^{-1} after 1 hour. Thus, at 37°C the death rate of *Staph. epidermidis* was similar to that observed with either *Staph. aureus* or *E. coli* (Table 16). Between 1 and 4 hours the viable count decreased more slowly to 1.00×10^2 cfu ml^{-1}. At 30°C the culture viable count halved every 18.7 minutes from 8.50×10^5 cfu ml^{-1} at time zero to 9.20×10^4 cfu ml^{-1} after 1 hour. The viable count then decreased at a constant lower rate until after 4 hours the viable count was 2.00×10^3 cfu ml^{-1}. At 25°C the viable count halved every 34.7 minutes from time

Figure 55: Death of *Staph. epidermidis* treated with the OBC[37] of ciprofloxacin at 20-37°C.
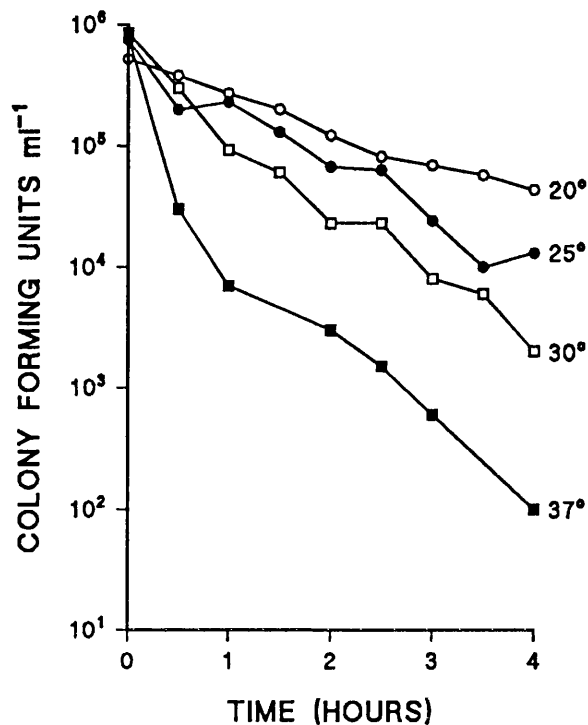
zero to 6.70×10^4 cfu ml^{-1} after 2 hours, and the viable count then continued to decline at a slightly lower rate to 1.30×10^4 cfu ml^{-1} after 4 hours. At 20°C the death curve was again biphasic as the viable count halved every 57.6 minutes to 1.22×10^5 cfu ml^{-1} after 2.5 hours and then at a slightly lower rate to 4.30×10^4 cfu ml^{-1} after 4 hours incubation. The values for the culture halving times of *Staph. epidermidis* at each individual temperature were higher than those for *Staph. aureus*, and as the temperature of incubation was reduced from 37 to 20°C the culture halving time increased about 7-fold, which was the same relative increase as that observed either with *Staph. aureus* or with *Ps. aeruginosa* (Table 16) but different from the results with *E. coli*.

**Apparent activation energies for the death of bacteria
treated with ciprofloxacin:**

Figure 56 shows the Arrhenius plots for the death of *E.
coli*, *Ps. aeruginosa*, *Staph. aureus* and *Staph. epidermidis*
treated with ciprofloxacin in nutrient broth, using the
initial specific death rates (μ_q) derived from Figures 52,
53, 54 and 55, respectively. As was done with the Arrhenius
plot for growth of the bacteria (Figure 51) the temperature
in °C is shown for clarity. The slopes of the lines and
their correlation coefficients (*r*) were calculated using the
"lreg" computer program, and the apparent activation energy
(E_a) was calculated as before. Each of the Arrhenius plots
for death of the bacteria (Figure 56) had a negative value

**Figure 56: Arrhenius plot for death of bacteria treated with
ciprofloxacin.**
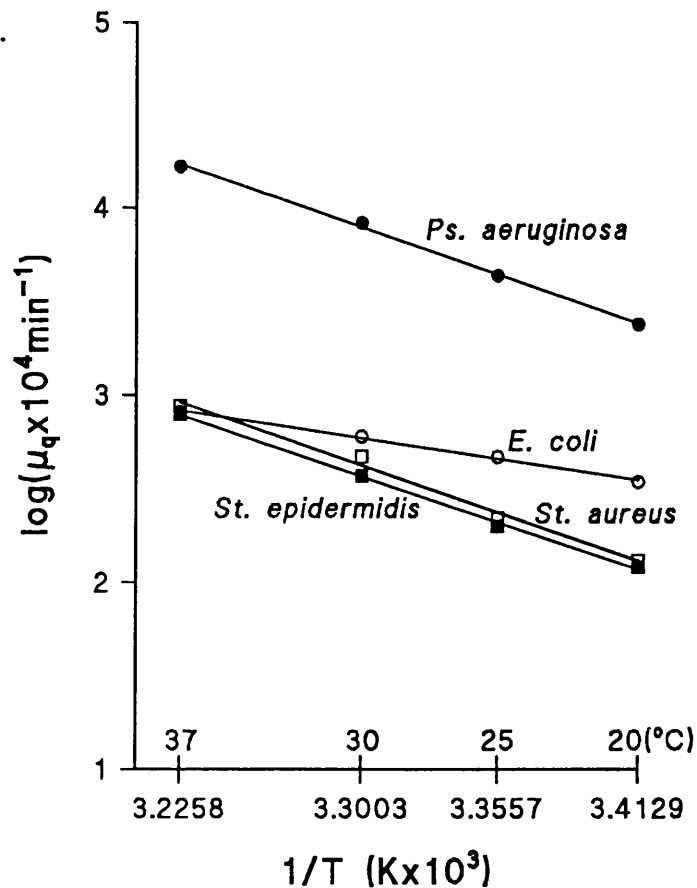
for the slope because in each case reducing the temperature
of incubation reduced the specific death rate (and increased
the culture halving time, see Table 16). With *E. coli* the
Arrhenius plot was an excellent straight line (r= -0.9982)
and the value for the apparent activation energy was 9,067
calories (Table 17).

**Table 17: apparent activation energy (E_a) for death of
bacteria treated with ciprofloxacin or DR-3355 between 20
and 37°C.**

BACTERIA	CONC.	APPARENT ACTIVATION ENERGY (E_a) IN CALORIES		
		CIPRO.	DR-3355	
		20-37°C	20-25°C	25-37°C
Ps. aeruginosa	3µgml^{-1}	20,696	46,159	6,939
E. coli	OBC	9,067	<---11,841--->	
Staph. aureus	OBC	20,588	<---18,456--->	
Staph. epidermidis	OBC	20,075	<---11,337--->	

CONC.= Concentration of 4-quinolone; CIPRO.= Ciprofloxacin;
OBC= optimum bactericidal concentration.

With *Ps. aeruginosa* again the Arrhenius plot was also an
excellent straight line (r= -0.9712) which was quite
separate from that of the other three bacterial species
studied because *Ps. aeruginosa* was killed much more rapidly
by ciprofloxacin (Table 16). The value for the apparent

activation energy was 20,696 calories which was more than

twice the value found for *E. coli* (Table 17). With *Staph.*

aureus or *Staph. epidermidis* the Arrhenius plots were also

excellent straight lines (*r*= -0.9955 or -0.9995,

respectively) and virtually parallel to each other and also

to that of *Ps. aeruginosa*. Therefore, the values for the

apparent activation energies for *Staph. aureus* or *Staph.*

epidermidis being 20,588 or 20,075 calories, respectively,

were similar to the value found for *Ps. aeruginosa* (Table

17). It is interesting that *E. coli* exhibited a lower value

for the MIC than that obtained with the other three

bacterial species studied, and a lower value for its OBC

than that obtained with *Staph. aureus* or *Staph. epidermidis*,

and the value for the apparent activation energy was also

much lower for *E. coli* than the values found for the other

three bacteria. In addition *E. coli* differed from the other

3 species in having two apparent activation energies for

growth (see Table 15).

Effects of temperature on the death of *E. coli* treated with
DR-3355:

Figure 57 shows the death curves of *E. coli* treated with the

OBC (0.3µgml^{-1}, determined at 37°C, Table 9) of DR-3355 in

nutrient broth at 20, 25, 30 and 37°C. At 37°C the initial

viable count was 5.90x10^6 cfu ml^{-1} and it fell with a halving

time of 5.6 minutes to 1.46x10^5 cfu ml^{-1} after 30 minutes

(Table 18). The viable count continued to decline more

slowly to 6.60x10^3 cfu ml^{-1} after 2.5 hours, and then at an

Figure 57: Death of *E. coli* treated with the OBC[37] of DR-3355 at 20-37°C.
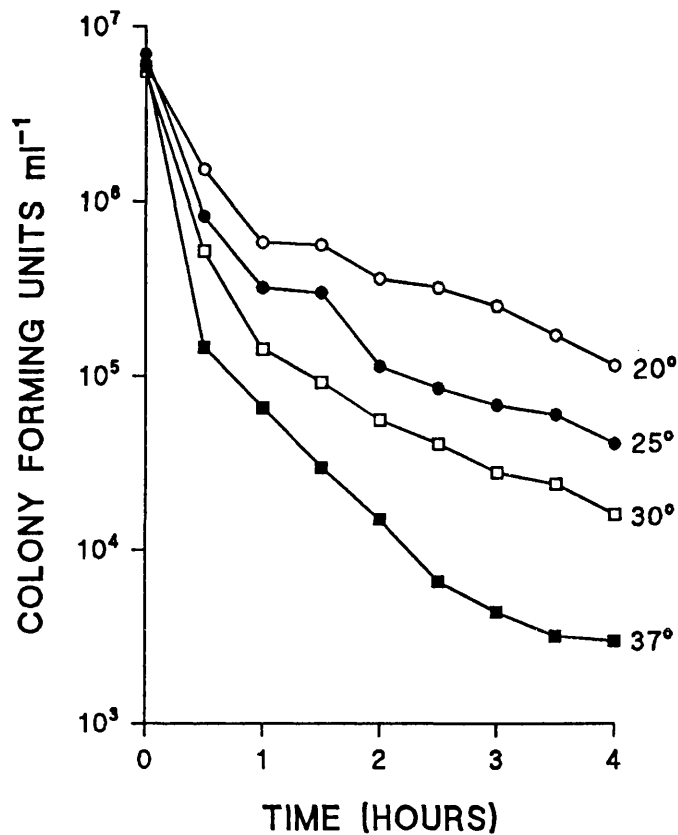
even lower rate for a further 1.5 hours to 3.10×10^3 cfu ml^{-1}. Thus at 37°C the initial death rate of *E. coli* treated with DR-3355 was more rapid than that observed when the same bacteria were treated with ciprofloxacin (Table 16). At 30°C the viable count of the culture halved every 11.4 minutes from an initial count of 5.50×10^6 cfu ml^{-1} to 1.42×10^5 cfu ml^{-1} after 1 hour, a rate which was virtually identical to that observed with ciprofloxacin at 30°C (Table 16). The viable count then continued to decrease at a virtually constant slower rate to 1.60×10^4 cfu ml^{-1} after 4 hours incubation. At 25°C the initial viable count was 6.90×10^6 cfu ml^{-1} and decreased with a halving time of 13.5

Table 18: culture halving time (t_h) in minutes for bacteria treated with DR-335 in nutrient broth.

BACTERIA	20°C	25°C	30°C	37°C
E. coli	17.8	13.5	11.4	5.6
Ps. aeruginosa	3.2	0.9	0.7	0.5
Staph. aureus	29.0	13.2	7.4	5.0
Staph. epidermidis	18.4	14.4	10.8	6.3

minutes to 3.20×10^5 cfu ml^{-1} after 1 hour, which again was more rapid than the halving time observed with ciprofloxacin at 25°C (Table 16). The viable count then continued to decrease at a lower rate to a viable count of 4.10×10^4 cfu ml^{-1} after 4 hours. At 20°C the initial viable count decreased from 6.00×10^6 cfu ml^{-1} to 5.80×10^5 cfu ml^{-1} after 1 hour with a halving time of 17.8 minutes, and continued to decline at a lower rate to 1.14×10^5 cfu ml^{-1} after 4 hours. Thus, as was found at the three higher temperatures studied, at 20°C the culture halving time was less when *E. coli* was treated with the OBC of DR-3355 (Table 18) than when it was treated with the OBC of ciprofloxacin (Table 16). These findings are in agreement with the results from the bactericidal profiles described earlier whereby the survival at the OBCs was higher with ciprofloxacin than with DR-3355 (compare Tables 8 and 9, respectively). Also, the shape of the death curves was similar with either ciprofloxacin or DR-3355 in that the initial rapid death phase was followed by a second phase during which the bacteria were killed more

slowly. As mentioned earlier such biphasic death curves have been described for *E. coli* treated with nalidixic acid, pefloxacin, ciprofloxacin or ofloxacin (Smith, 1984a; Carret *et al*, 1990). Overall, reducing the incubation temperature from 37 to 20°C decreased the death rate by approximately 3-fold, which was a higher relative reduction than seen with ciprofloxacin (2-fold).

Effects of temperature on the death of *Ps. aeruginosa* treated with DR-3355:

Figure 58 shows the death curves for *Ps. aeruginosa* treated with $3\mu gml^{-1}$ of DR-3355 at 20, 25, 30 and 37°C in nutrient broth. At 37°C the viable count at time zero was 4.70×10^6 cfu ml^{-1} and it decreased rapidly with a halving time of 0.5 minutes to 5.00×10^3 cfu ml^{-1}, which was only marginally slower than the initial death of *Ps. aeruginosa* treated with ciprofloxacin ($t_h = 0.4$ minutes, Table 16). The death rate became progressively less and the viable count decreased to 3.30×10^2 cfu ml^{-1} after 25 minutes. The viable count then decreased again to 3.30×10^1 cfu ml^{-1} at 30 minutes. At 30°C the initial viable count was 3.80×10^6 cfu ml^{-1} and decreased with a halving time of 0.7 minutes to 2.60×10^4 cfu ml^{-1}, which was slightly less than the value of t_h found for ciprofloxacin at 30°C (Table 16). As was observed at 37°C, the viable count continued to decrease at a progressively lower rate and again there was a sharp drop in the last 5 minutes of incubation from 5.70×10^2 to 1.00×10^2 cfu ml^{-1}. At 25°C the viable count at time zero was 4.70×10^6 cfu ml^{-1} and

Figure 58: Death of *Ps. aeruginosa* treated with 3μgml⁻¹ DR-3355 at 20-37°C.
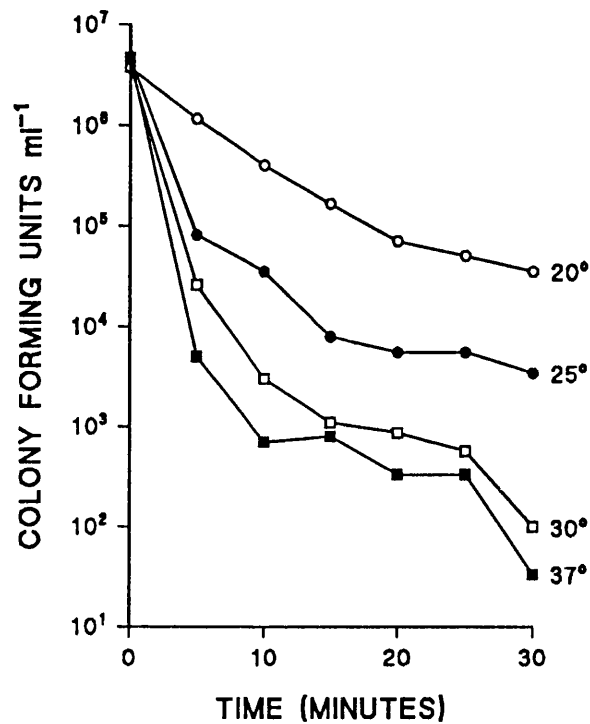
it decreased with a halving time of 0.9 minutes to 8.10×10^4 cfu ml⁻¹ after 5 minutes, which was nearly twice the death rate observed with ciprofloxacin at 25°C (Table 16), and continued to decrease at a progressively lower rate to 3.40×10^3 cfu ml⁻¹ after 30 minutes. The death curves for *Ps. aeruginosa* treated with DR-3355 at 25, 30 or 37°C (Figure 58) did not tend to converge to the extent observed with the ciprofloxacin death curves (Figure 53) at the same temperatures, which is in agreement with the results from the bactericidal profiles of DR-3355 described earlier (Figure 22). At 20°C the viable count decreased at a constant rate (t_h= 3.2 minutes) for the first 20 minutes of incubation to 7.00×10^4 cfu ml⁻¹, which was about 3.5-times lower than the death rate at 25°C, and slightly lower than

that observed with ciprofloxacin at 20°C (Table 16). The viable count then decreased at a constant lower rate for a further 10 minutes to 3.50×10^4 cfu ml^{-1}. Overall, the relative increase in the culture halving time was about 6-fold as the temperature was reduced from 37 to 20°C, which was slightly lower than the increase seen with ciprofloxacin.

Effects of temperature on the death of *Staph. aureus* treated with DR-3355:

Figure 59 shows the death curves for *Staph. aureus* treated with the OBC (3µgml^{-1}, determined at 37°C, Table 11) of DR-3355 at 20, 25, 30 and 37°C in nutrient broth. At 37°C the initial viable count was 2.40×10^6 cfu ml^{-1} and it decreased with a halving time of 5.0 minutes to 6.00×10^2 cfu ml^{-1}, which was a shorter t_h value than that observed with ciprofloxacin at 37°C, and also shorter than the halving times for *E. coli* treated with either ciprofloxacin (Table 16) or DR-3355 (Table 18) at 37°C. The viable count continued to decline at a progressively lower rate to 6.70×10^1 cfu ml^{-1} after 2.5 hours, and then increased 3-fold to 2.00×10^2 cfu ml^{-1} after 4 hours. At 30°C the shape of the death curve was quite different to that observed with ciprofloxacin (Figure 54) in that no lag phase was seen with DR-3355 (Figure 59). The viable count at time zero was 1.72×10^6 cfu ml^{-1} which decreased with a t_h value of 7.4 minutes to 6.00×10^3 cfu ml^{-1} after 1 hour incubation. Thus the initial death rate of *Staph. aureus* at 30°C with DR-3355

Figure 59: Death of *Staph. aureus* treated with the OBC[37] of
DR-3355 at 20-37°C.
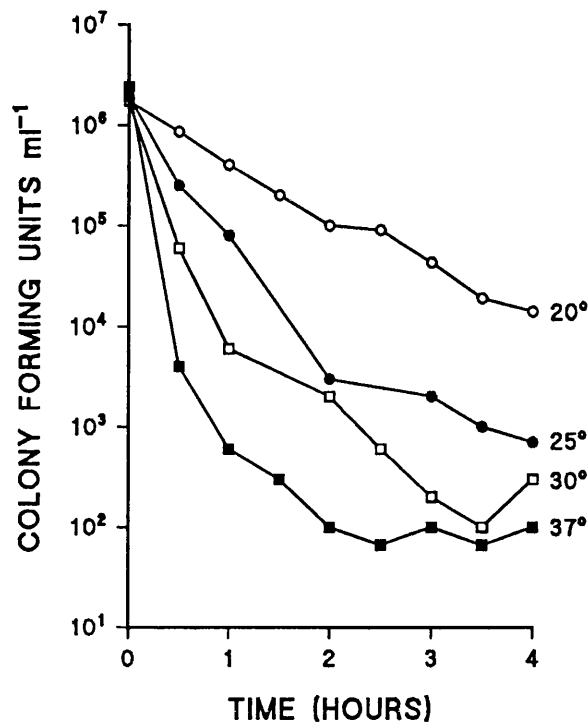
was twice that observed with ciprofloxacin (Tables 18 and 16, respectively). The viable count continued to decline at a lower rate to 1.00×10^2 cfu ml^{-1} after 3.5 hours, and as was found at 37°C, the viability then increased 3-fold to 3.00×10^2 cfu ml^{-1}. As was found at 30°C the death curve for DR-3355 at 25°C did not exhibit a lag phase (which was observed with ciprofloxacin, Figure 54) and the viable count halved every 13.2 minutes to 3.00×10^3 cfu ml^{-1} after 2 hours. From 2 to 4 hours incubation the viability decreased at a lower rate to 7.00×10^2 cfu ml^{-1}. At 20°C again there was no lag phase as had been found with ciprofloxacin and the viable count halved every 29.0 minutes to 1.00×10^5 cfu ml^{-1} after 2 hours. From 2 to 4 hours the viable count decreased at a lower rate to 1.40×10^4 cfu ml^{-1}. Could the lag phase

observed with the death curves at 20, 25 or 30°C with

ciprofloxacin (Figure 54) be because ciprofloxacin possesses

only bactericidal mechanism A against *Staph. aureus* (Lewin

and Smith, 1989a), whilst DR-3355, which did not exhibit a

lag phase, possesses both mechanisms A and B (and/or C)

against *Staph. aureus* (Lewin and Amyes, 1989)? The relative

increase in the value of t_h with DR-3355 as the incubation

temperature was reduced from 37 to 20°C was approximately 6-

fold, which was similar to that seen with *Ps. aeruginosa*

(Table 18) but slightly lower than that observed with

ciprofloxacin (Table 16).

Effects of temperature on the death of *Staph. epidermidis*

treated with DR-3355:

Figure 60 shows the death curves for *Staph. epidermidis*

treated with the OBC ($5\mu gml^{-1}$, determined at 37°C, Table 13)

of DR-3355 at 20, 25, 30 and 37°C in nutrient broth. At

37°C the initial viable count was 3.70×10^5 cfu ml^{-1} and

decreased with a halving time of 4.3 minutes for the first

30 minutes of incubation to 3.00×10^3 cfu ml^{-1} which was more

than twice the death rate found with ciprofloxacin (Table

16). Between 0.5 and 1.5 hours incubation the viability of

the culture declined at a progressively lower rate to

3.33×10^2 cfu ml^{-1}, and for the remaining 2.5 hours there was

a small net decrease in the viable count to 2.00×10^2 cfu ml^{-1}. The shape of the death curve with DR-3355 at 37°C was

quite different to that observed with ciprofloxacin at 37°C

(Figure 55) in that with DR-3355 most of the bacteria were

Figure 60: Death of *Staph. epidermidis* treated with the OBC[37] of DR-3355 at 20-37°C.
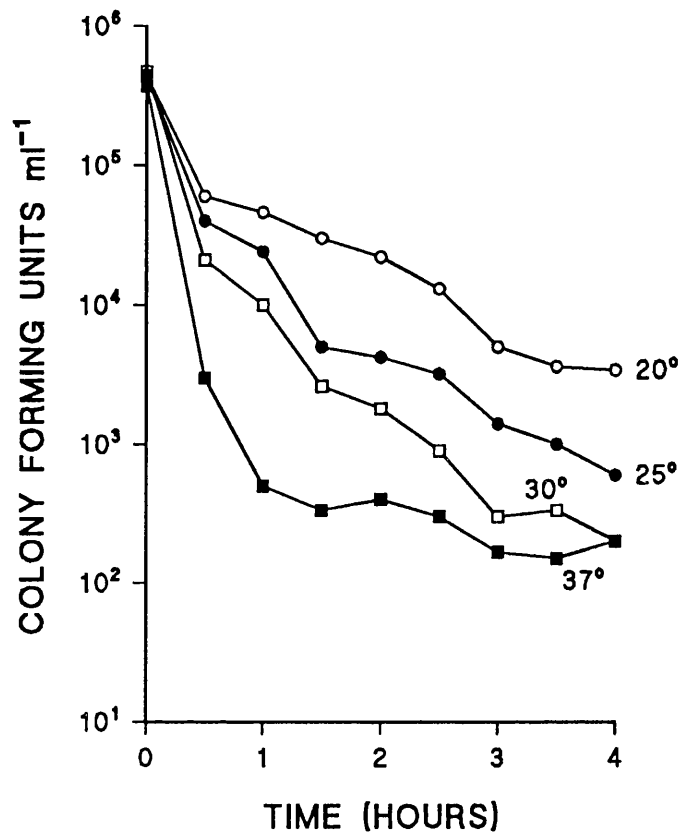
killed in the first hour of incubation, whereas with ciprofloxacin the initial death rate was lower but the bacteria continued to be killed at a high rate for the remaining time. At 30°C the viable count decreased from 4.70×10^5 cfu ml^{-1} with a t_h value of 6.7 minutes to 2.10×10^4 cfu ml^{-1} after 30 minutes, which was nearly 3-times the death rate with ciprofloxacin at 30°C (Table 16). The viable count continued to decrease at a near-constant lower rate to 3.00×10^2 cfu ml^{-1} after 3 hours and then to 2.00×10^2 cfu ml^{-1} after 4 hours. Thus the death curves at 30 and 37°C converged after the initial, most rapid, phase of the death. At 25°C the initial viable count was 4.30×10^5 cfu ml^{-1} and

fell with a t_h value of 8.8 minutes to 4.00×10^4 cfu ml^{-1}

after 30 minutes which was approximately 4-times faster than

the initial death rate seen with ciprofloxacin at 25°C

(Table 16). The viable count then decreased at a lower rate

to 5.00×10^3 cfu ml^{-1} after 1.5 hours and then at an even

lower rate to 6.00×10^2 cfu ml^{-1} after 4 hours. At 20°C the

initial viable count of 4.40×10^5 cfu ml^{-1} decreased with a

halving time of 10.4 minutes to 6.10×10^4 cfu ml^{-1} after 30

minutes, which was approximately 5.5-times less than the

value found with ciprofloxacin at 20°C (Table 16). The

death rate then remained constant at a lower rate for the

next 2 hours to 1.30×10^4 cfu ml^{-1} and the viability then

decreased to 3.40×10^3 cfu ml^{-1} after 4 hours.

The death rates for *Staph. epidermidis* treated with DR-3355

(Table 18) were higher than those with ciprofloxacin (Table

16) at the same temperature. These findings are in

agreement with the results from the bactericidal profiles

whereby the % survival at the OBCs was lower with DR-3355

than with ciprofloxacin at the same temperature (Tables 13

and 12, respectively). The relative increase in the value

for t_h as the incubation temperature was reduced from 37 to

20°C was just over 2-fold, which was smaller than the

increase seen with ciprofloxacin (Tables 18 and 16,

respectively). The main respects in which the death curves

for *Staph. epidermidis* treated with DR-3355 (Figure 60)

differed from those with ciprofloxacin (Figure 55) were that

with DR-3355 the initial death rates were much higher and

the actual length of the initial death phase was shorter. Could these differences in the death curves with the two 4-quinolones be due to the possession of different mechanisms of action? As was described earlier, ciprofloxacin possessed solely bactericidal mechanism A against *Staph. epidermidis* whereas DR-3355 exerted mechanisms A and B (and/or C) against this organism (Lewin and Amyes, 1989).

As was described in the section "Effects of temperature on the bactericidal activities of 4-quinolones" the relative reduction in the activity of ciprofloxacin or DR-3355 at their OBCs (against *E. coli* and the two Staphylococci) was on average about 60-fold as the temperature of incubation was reduced from 37 to 20°C. However, the relative reduction in the initial death rates was much smaller (2 to 7-fold) as the incubation temperature was decreased from 37 to 20°C. This discrepancy in the magnitude of the effect of temperature can be explained by the shape of the death curves, such that the death curves, with the odd exception, tended to diverge as the temperature of incubation was reduced. Since the profiles determined the survival of the bacteria after 3 hours (or 30 minutes in the case of *Ps. aeruginosa*) and the initial death rates are by definition from the early part of the death curves, the size of the relative reductions in bactericidal activity were quite different by each method.

Apparent activation energies for the death of bacteria treated with DR-3355:

Figure 61 shows the Arrhenius plots for the death of *E. coli*, *Ps. aeruginosa*, *Staph. aureus* and *Staph. epidermidis* treated with DR-3355 in nutrient broth, using initial specific death rates derived from Figures 57, 58, 59 and 60, respectively. As was done with the other Arrhenius plots (Figures 51 and 56) the temperature in °C is shown for clarity. The slopes of the lines and their correlation coefficients (*r*) were calculated using the "lreg" computer program, and the apparent activation energies were calculated as before. As was the case with ciprofloxacin

Figure 61: Arrhenius plot for death of bacteria treated with DR-3355.
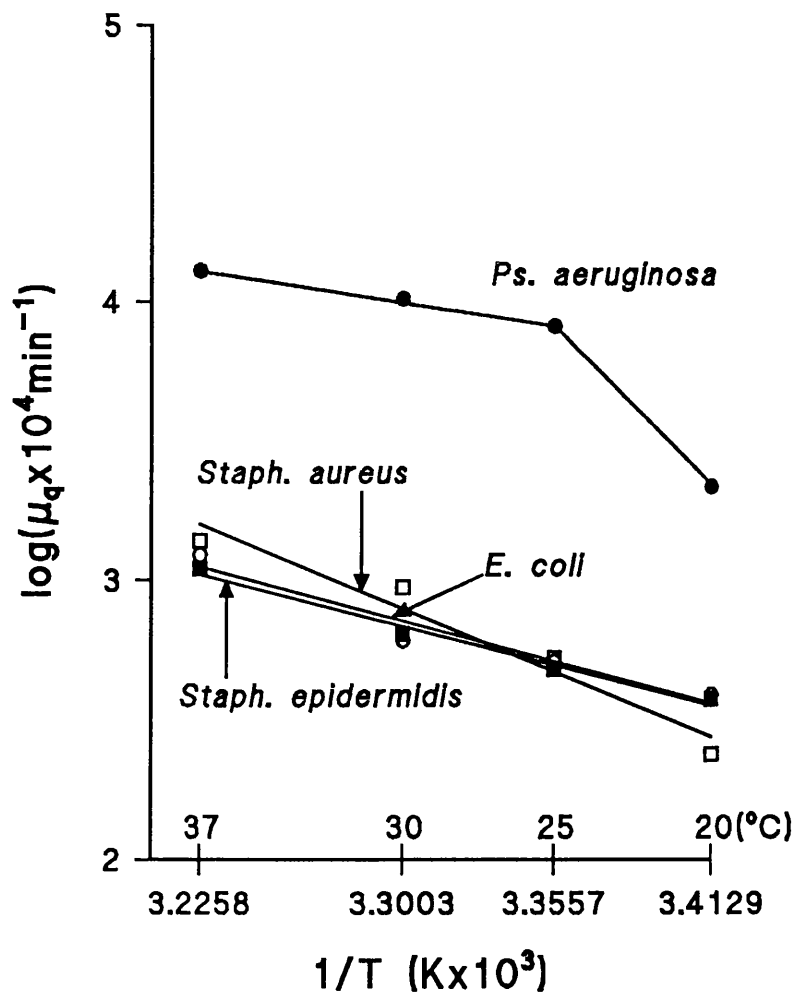
(Figure 56) the Arrhenius plots for DR-3355 had negative slopes because reducing the incubation temperature decreased the specific death rate). With *E. coli* the Arrhenius plot was an excellent straight line ($r=$ -0.9728) and the value for the apparent activation energy was 11,841 calories, which was similar to the value found for ciprofloxacin (Table 17). With *Ps. aeruginosa*, the Arrhenius plot was unusual in that there was a well defined break, with the two phases being between 20 and 25°C ($r=$ -1.0000) and between 25 and 37°C ($r=$ -0.9978). The value for the apparent activation energy from 20 to 25°C was 46,159 calories which was much greater than any value found for the death of any of the other bacteria treated with either ciprofloxacin or DR-3355 (Table 17). The value for the E_a from 25 to 37°C was 6,939 calories which was the lowest value found for any of the 4-quinolone/bacterial combinations. This would seem to suggest that the death rate-limiting step of *Ps. aeruginosa* treated with DR-3355 at 20°C was quite different to that between 25 and 37°C. DR-3355 is more hydrophobic than ciprofloxacin (Chapman and Georgopapadakou, 1988) and so DR-3355 may be more dependent on diffusion through the lipid bilayer (as opposed to water-filled porin channels) as a route of entry to the cytoplasm. If diffusion through the lipid bilayer was significantly reduced at 20°C (possibly through increased membrane "viscosity", Christensen, [1975]), which seems a reasonable assumption, this may explain the biphasic Arrhenius plot. With *Staph. aureus* the Arrhenius plot was an excellent straight line ($r=$ -0.9754)

with the slope corresponding to a value for the apparent
activation energy of 18,456 calories, which was quite
similar to the value found for ciprofloxacin (Table 17).
With *Staph. epidermidis* the Arrhenius plot was linear ($r = -0.9925$) and virtually parallel to that of *E. coli* and the
value for the apparent activation energy was therefore
similar, being 11,337 calories (Table 17). Thus, with
Staph. epidermidis the value for the E_a with DR-3355 was
about half the value observed with ciprofloxacin. Could
this be due to the greater hydrophobicity of DR-3355
compared to ciprofloxacin? Although little is known about
the uptake of 4-quinolones into Gram-positive bacteria, it
may be that accumulation of DR-3355 by *Staph. epidermidis* is
less affected by temperature. Alternatively, this
observation may be due to the possession of bactericidal
mechanisms A and B (and/or C) by DR-3355 (Lewin and Amyes,
1989), whereas ciprofloxacin exerts solely mechanism A
against Staphylococci (Lewin and Smith, 1989b). However,
the possession of an additional mechanism of action by DR-3355 compared to ciprofloxacin did not seem to significantly
affect the values for the apparent activation energies of
these 4-quinolones against *Staph. aureus* (see Table 17).

For *Staph. aureus* or *Staph. epidermidis* the magnitude of the
values of the E_a were related to the killing rate since the
initial halving times with DR-3355 were lower than with
ciprofloxacin (compare Tables 18 and 16, respectively) and
the values for the E_a were also lower with DR-3355 than with

ciprofloxacin (Table 17). It should be noted that the
difference in the values for the E_a with *Staph. aureus* may
not be significant. With *E. coli* the death rates with
ciprofloxacin or DR-3355 (Tables 16 or 18, respectively)
were similar and the apparent activation energies were also
similar (Table 17). With *Ps. aeruginosa* there was little or
no relationship between the killing rate and the apparent
activation energies for ciprofloxacin from 20 to 37°C or for
DR-3355 from 25 to 37°C because the differences in the
initial death rates with either 4-quinolone were very small
(compare Tables 16 and 18). *Ps. aeruginosa* was killed much
more rapidly by ciprofloxacin or DR-3355 than the other
three bacteria studied, yet the values for the E_a for
ciprofloxacin or for DR-3355 from 25 to 37°C were broadly
similar to the values for the other bacterial species (Table
17). This finding is consistent with there being a common
mode of action of 4-quinolones against both Gram-positive
and Gram-negative bacterial species and inhibition of DNA
gyrase could well be a candidate for this action.

With the exception of *Ps. aeruginosa* between 25 and 37°C the
magnitude of the apparent activation energies for each
bacterial species also seemed to be directly related to the
susceptibility of the bacteria to the 4-quinolone as
determined by the minimum inhibitory concentration (MIC)
tests at 37°C with the lowest inoculum size. In other words
higher MIC values were associated with greater apparent
activation energies as follows: DR-3355 exhibited an MIC of

$0.015\mu gml^{-1}$ against *E. coli* at 37°C (Table 3) whilst the MIC

of ciprofloxacin was $0.0075\mu gml^{-1}$ (Table 1), and the apparent

activation energies of the two drugs were 11,841 and 9,067

calories, respectively (Table 17). With *Staph. aureus* the

MICs of ciprofloxacin or DR-3355 were 0.15 or $0.075\mu gml^{-1}$,

respectively (see Tables 1 and 3), and the values for E_a

were 20,588 or 18,456 calories, respectively (Table 17).

With *Staph. epidermidis* the MIC of ciprofloxacin was

$0.2\mu gml^{-1}$ (Table 1) and the MIC of DR-3355 was $0.1\mu gml^{-1}$

(Table 3), whilst the values for E_a were 20,075 and 11,337

calories, respectively (Table 17). Thus, for *E. coli*,

Staph. aureus or *Staph. epidermidis* the lower the MIC value

the lower the apparent activation energy for death of the

bacteria treated with the OBC of the 4-quinolone. With *Ps.*

aeruginosa the MIC of DR-3355 was $0.2\mu gml^{-1}$ (Table 3) and

that of ciprofloxacin was $0.075\mu gml^{-1}$ (Table 1), whilst the

values for E_a were 46,159 and 20,696 calories, respectively,

between 20 and 25°C. On the other hand at temperatures

between 25 and 37°C with *Ps. aeruginosa* the opposite seemed

to be the case since the MICs of ciprofloxacin or DR-3355

were 0.075 or $0.2\mu gml^{-1}$, respectively (Tables 1 or 3,

respectively), while the values for the apparent activation

energies were inversely related to these MIC values, being

20,696 calories or 6,939 calories, respectively (Table 17).

SECTION 6:

Discussion:

The effects of temperature on bacterial multiplication in drug-free medium, on bacterial 4-quinolone susceptibility and on the frequency of mutation of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* to resist 4-quinolones were investigated *in vitro*. It was found that the temperature of incubation not only affected bacterial multiplication, but also the frequency of mutation to 4-quinolone resistance, the minimum inhibitory concentrations (MICs) of 4-quinolones, and the bactericidal activities of the 4-quinolones against the four bacterial species studied.

With the exception of *Staph. epidermidis* increasing the temperature of incubation from 20 to 37°C progressively increased the specific growth rates of the bacteria tested (Table 14). Of all the bacteria tested *Staph. aureus* multiplied most rapidly at any one temperature, and the overall ranking of growth rates at 37°C was *Staph. aureus* > *E. coli* > *Staph. epidermidis* > *Ps. aeruginosa* (Table 14). However, at 20°C *Ps. aeruginosa* multiplied more rapidly than *E. coli*. The effect of temperature on the multiplication of *Staph. epidermidis* was quite different to that observed with the other three bacterial species. *Staph. epidermidis* did not multiply at all at 20°C, and it exhibited a temperature optimum for growth of 30°C because its rate of

multiplication at 25 or at 37°C was less than that observed at 30°C (Table 14). Could the rather narrow temperature range of *Staph. epidermidis* be related to its rôle as a commensal of skin and mucous membranes? As was discussed earlier bacteria are well known to exhibit temperature optima for growth for various reasons, including temperature lability of enzymes or structural proteins, reduced enzyme activity or increased nutritional requirements at elevated temperatures (Ingraham, 1962; Herendeen *et al*, 1979).

The apparent activation energy (E_a) is the kinetic energy required by molecules to react together. The number of molecules which possess E_a usually increases as temperature increases, and the value for the E_a is the slope of the Arrhenius plot ($\log_{10}$ reaction rate *vs* 1/T). The apparent activation energies for growth of the four bacterial species between 20 and 37°C were determined from their specific growth rates (μ) in nutrient broth. With *Ps. aeruginosa* or *Staph. aureus* the Arrhenius plots were linear between 20 and 37°C and their slopes corresponded to E_a values of 9,459 and 11,555 calories, respectively (Figure 51, Table 15). With *E. coli* the Arrhenius plot was biphasic, indicating a change in the growth rate-limiting step, which has also been observed by Ingraham (1962) and Pirt (1975). In the experiments reported here the two phases were from 20 to 30°C (E_a= 33,197 calories) and from 30 to 37°C (E_a= 10,995 calories). Thus it seems possible that for *E. coli* between 30 and 37°C and for *Ps. aeruginosa* or *Staph. aureus* between

20 and 37°C that there may be a common growth rate-limiting step. *Staph. epidermidis* did not obey the Arrhenius law between 30 and 37°C because the growth curve exhibited a temperature optimum which was 30°C (Figure 50, Table 14). It did not grow at 20°C and the apparent activation energy between 25 and 30°C was 16,943 calories (Table 15).

The susceptibilities of the four bacterial species to 4-quinolones as determined by the MIC tests were different. The MIC values of ciprofloxacin (Table 1), determined at 37°C with the lowest inoculum size against *E. coli*, *Ps. aeruginosa*, *Staph. aureus* or *Staph. epidermidis* were 0.0075, 0.075, 0.15 or 0.2μgml^{-1}, respectively. Ofloxacin (Table 2) was generally less potent than ciprofloxacin, with the ranking of the MIC values being different because *Ps. aeruginosa* was least susceptible. The MIC values were 0.03, 0.15, 0.2 or 0.4μgml^{-1} for *E. coli*, *Staph. aureus*, *Staph. epidermidis* or *Ps. aeruginosa*, respectively. DR-3355 (Table 3) was twice as potent as ofloxacin, which is not surprising because it is the active isomer in the racemic mixture termed ofloxacin (Tanaka *et al*, 1990). As expected the ranking of susceptibility was the same as that for ofloxacin with the MIC values being 0.015, 0.075, 0.1 or 0.2μgml^{-1} for *E. coli*, *Staph. aureus*, *Staph. epidermidis* or *Ps. aeruginosa*, respectively.

When the effect of temperature on the MIC values was tested it was found that reducing the temperature of incubation

from 37 to 30°C, or from 30 to 25°C either had no effect or reduced the MIC values of ciprofloxacin, ofloxacin or DR-3355 (Tables 1, 2 or 3, respectively). The main exception to this trend was *Staph. epidermidis* tested with ciprofloxacin whereby the MIC value increased as the temperature was reduced from 37 to 30°C or from 30 to 25°C (Table 1). However, the overall changes in the MIC values were small being 1.5- to 4-fold.

The effect of the selection temperature used on the expression of mutational resistance to 5 times the MIC values (determined individually at each temperature with the largest inoculum size) of 4-quinolones was also investigated. In agreement with Smith (1986a) and Odell and Crumplin (1990) it was found that *E. coli* exhibited a temperature optimum for mutation to resist ciprofloxacin which was 30°C (Figure 2, Table 4). It was also found that *Staph. aureus* exhibited a similar temperature optimum for mutation to resist ciprofloxacin (Figure 2). However, no such mutation rate temperature relationships were observed either with ofloxacin or with DR-3355 for these three bacterial species (Figures 2 or 3, respectively). The mutation frequencies of *Ps. aeruginosa* to resist ciprofloxacin (Figure 2), *Staph. aureus* to resist ofloxacin (Figure 3) and for *E. coli* to resist DR-3355 (Figure 4) increased progressively as the temperature of selection was reduced from 37 to 25°C. On the other hand with *E. coli* and ofloxacin (Figure 3) or with *Ps. aeruginosa* and either

ofloxacin (Figure 3) or DR-3355 (Figure 4) the opposite
response to selection temperature was seen. Thus, in
agreement with Smith (1990b), it seems that prediction of *in
vitro* mutation rates is not possible from the susceptibility
of the bacteria to the 4-quinolones as determined by MIC
tests, which merely reflect the bacteriostatic potencies of
4-quinolones (Smith, 1984a).

It is interesting that with the exception of *Staph.
epidermidis*, the shape of the mutation frequency temperature
relationships with ofloxacin or DR-3355 were so different
from each other considering that DR-3355 is the active
(S-[-]-) isomer in the racemic mixture termed ofloxacin
(Tanaka *et al*, 1990). Indeed, the most unusual and
unexpected mutation rate temperature profile was that of
Staph. aureus and DR-3355 (Figures 4), because the mutation
rates at 25 or 37°C were much higher than those observed at
30°C (Table 4). Thus, *Staph. aureus* exhibited a temperature
minimum of 30°C for selection of mutants resistant to DR-
3355 which was the opposite trend to that found with
ciprofloxacin (Figure 7). It seems possible that the
inactive (R-[+]-) isomer present in racemic ofloxacin, i.e.
DR-3354, may interact with the 4-quinolone binding site
without actually interfering with DNA gyrase activity and
could influence selection of resistant mutants. On the
other hand uptake may be a chiral process (Mitscher *et al*,
1990) in which case DR-3354 may affect the uptake of DR-3355
by the bacteria. It would be interesting to determine the

mutation frequency temperature profiles of DR-3354 to see whether there is a relationship with the mutation frequency to resist DR-3355 or ofloxacin at each temperature. However, our attempts to obtain DR-3354 for this purpose were unsuccessful.

Coagulase activity is an important pathogenicity factor in *Staph. aureus* infection and it is generally accepted that acquisition of resistance to 4-quinolones may reduce the pathogenicity of many bacterial species (Dalhoff and Döring, 1985; Ravizzolla *et al*, 1987; Smith, 1990b; Crumplin, 1990). Loss of coagulase activity by *Staph. aureus* upon acquisition of ofloxacin resistance has been reported by Smith (1990ab) in 71% of mutants isolated *in vitro*. In this thesis approximately 6% of mutants which became resistant to DR-3355 lost their coagulase activity, whereas all of the mutants of this species that were tested were resistant to either ciprofloxacin or ofloxacin remained coagulase-positive.

With *Staph. epidermidis* there was a quite different temperature profile with respect to its frequency of mutation to resist each of the 4-quinolones tested, whereby the mutation rate decreased significantly at each incremental temperature reduction (Figure 8). Moreover, three quarters of mutants that were resistant to ciprofloxacin at 25°C could not be subcultivated on the same concentration of ciprofloxacin, so were unstable. Also,

none of the mutants resistant to ofloxacin or DR-3355 that were selected at 25°C, could be subcultivated on the concentration of the same 4-quinolone from which they were selected, so all of the mutants tested were unstable. The instability of these resistant colonies and the unusual temperature profiles may be related to the rather narrow growth range (with respect to temperature) of *Staph. epidermidis* which itself may be a reflection of this species being a skin commensal.

That these *in vitro* mutation frequency results were highly variable is perhaps not surprising when the different mechanisms of resistance available to the bacteria are considered (Lewin *et al*, 1990). As was described in the "Introduction" section resistance to 4-quinolones can occur by mutations affecting the DNA gyrase A or B subunits (Miller and Scurlock, 1983; Hooper *et al*, 1986; Takahata and Nishino, 1988; Yoshida *et al*, 1988; 1990a; 1991a), the uptake mechanism(s) (Hirai *et al*, 1987; Chamberland *et al*, 1989; Legakis *et al*, 1989; Fukuda *et al*, 1990; Michea-Hamzehpour *et al*, 1991ab) or the efflux mechanism(s) for the 4-quinolones (Cohen *et al*, 1988 ; Ubukata *et al*, 1989; Yoshida *et al*, 1990c; 1991b). Although the mutants isolated in this thesis were "one-step" mutants (because they were isolated after a single exposure to the drug) and were therefore unlikely to possess more than one mechanism of resistance, clinical isolates of *E. coli*, *Ps. aeruginosa* or *Staph. aureus* exhibiting DNA gyrase *and* accumulation

mutations have been reported (Aoyama *et al*, 1987; Masecar *et al*, 1990; Moniot-Ville *et al*, 1991; Nakanishi *et al*, 1991b; Diver *et al*, 1991). Another factor which should be considered is the induction of the SOS response by 4-quinolones, since DNA damage (caused by U.V. irradiation or 4-quinolones, for example) which is repaired by the SOS system is error-prone and therefore potentially mutagenic (Little and Mount, 1982). Howard and Smith (1991) found that the SOS response in *E. coli* was responsible for repair of damage caused by bactericidal mechanism B. They suggested that 4-quinolones which possess mechanism B may be more prone to produce mutants than 4-quinolones which possess solely bactericidal mechanism A (for example nalidixic acid) the damage of which is repaired solely by the *rec*A system and not by processes of the SOS response. However, since all three of the 4-quinolones tested in this thesis possess both mechanisms A and B against *E. coli*, the proposal of Howard and Smith (1991) cannot be confirmed. Mutagenic DNA repair mechanisms such as those involved in the SOS response in *E. coli* have not been investigated in the Staphylococci. On the assumption that in Staphylococci analagous processes exist for repairing DNA damage caused by bactericidal mechanisms A and B as those in *E. coli*, it seems that with *Staph. epidermidis* possession of mechanism B by ofloxacin or DR-3355 (Lewin and Smith, 1988; Lewin and Amyes, 1989) may increase mutation since the mutation rates to resist these two drugs were higher than that for ciprofloxacin (Figure 8) which possesses solely mechanism A

(Lewin and Smith, 1988). The situation with *Staph. aureus* is even less clear since the ranking of mutation rates to resist ofloxacin, DR-3355 or ciprofloxacin was different at each temperature (Figure 7).

With *E. coli*, *Ps. aeruginosa* or *Staph. aureus* it seems possible that development of resistance to ciprofloxacin *in vivo* may be increased at sites of infection (or even remote from the infection site) which are at lower-than-body temperature, i.e. <37°C. Such sites could include skin surfaces and structure, soft tissues, and the upper respiratory tract. Resistance to antibacterials may also occur at sites remote to the infection, such as on mucous membranes (including anterior nares) or in the gut, and then cause pulmonary or urinary tract infection (Kresken and Wiedemann, 1986). Elderly people or people with peripheral vascular disease or diabetes mellitus have poor blood circulation, especially at their extremities, and thus low skin temperatures. Indeed, it is at such sites of infection and most often in patients with underlying chronic disease that resistance development by *Ps. aeruginosa* or *Staph. aureus* seems to occur most readily (Wood and Logan, 1986; Fass, 1986; Licitra *et al*, 1987; Ball, 1990; Parry *et al*, 1989; Chin *et al*, 1989; Lentino *et al*, 1991). Furthermore, Scully *et al* (1986) reported that "in soft tissue infections, a *surface culture* [my italics] of a healing wound often grew a resistant Pseudomonas during or at the end of therapy as the wound continued to heal uneventfully".

Presumably, such resistant bacteria could also prevent healing of the wound but would not normally be differentiated from a subsurface infection. With *Staph. epidermidis*, on the other hand, 4-quinolone resistance seems to be less likely to develop at lower-than-body temperatures since the *in vitro* mutation rates decreased significantly as the temperature of incubation was reduced from 37°C (Figure 8). However, resistance in coagulase-negative Staphylococci such as *Staph. epidermidis* is becoming a problem in certain types of infection, especially infections of indwelling catheters and other prostheses (Peters, 1988; Archer, 1988), which may be closer to the core body temperature. The reason for the failures may, however, be due to production of extracellular slime substance (ESS) which facilitates attachment to polymer surfaces and interferes with host defence mechanisms (Peters, 1988). Layers of ESS may reduce diffusion of antibacterial agents and cause anaerobic conditions which have been shown to abolish the bactericidal activity of 4-quinolones *in vitro* (Lewin *et al*, 1989c; Morrissey *et al*, 1990; Lewin *et al*, 1991b). It has been proposed that where alternative chemotherapy is available for Staphylococcal infections, for example ceftazidime or cefotaxime, it should be used in preference to 4-quinolones therapy so as to limit emergence of resistance (Ball, 1990).

Aside from the considerations of development of resistance at specific sites, perhaps the most sinister finding from the mutation studies was the generally high mutation rates

observed, normally in the range 10^{-8} to 10^{-7}, and with *Ps.*
aeruginosa the mutation frequency to resist ciprofloxacin
was even higher, being greater than 10^{-7} at all three
temperatures tested (Table 4). In patients with cystic
fibrosis, oral treatment with ciprofloxacin on an out-
patient basis has revolutionised the therapy of infective
exacerbations of lung abscesses caused by *Ps. aeruginosa*
(Scully *et al*, 1987; Ball, 1990). Before the development of
ciprofloxacin these infections had to be treated solely with
parenteral combinations of ß-lactams and aminoglycosides,
which by definition had to be administered in hospital,
which is costly, uncomfortable to the patients and often
non-efficacious (Ball, 1990). However, development of
resistance to ciprofloxacin during therapy is becoming a
problem in the treatment of *Ps. aeruginosa* in cystic
fibrosis (Rubio and Shapiro, 1986; Scully *et al*, 1987;
Shalit *et al*, 1987) so the high *in vitro* mutation rates
reported here agree well with the clinical findings. If *in
vitro* mutation frequencies to resist 4-quinolones do reflect
the liklehood of resistance development *in vivo*, as was
proposed by Smith (1990b), it seems that the prevalence of
4-quinolone resistance in clincial isolates will increase as
this class of drugs is used more widely and more
indiscriminately. The findings of surveys of clinical
resistance are often conflicting, and they vary from centre
to centre. Increases in clinical resistance amongst
methicillin-sensitive and methicillin-resistant *Staph.*
aureus have been reported (Blumberg *et al*, 1989; Shalit *et*

al, 1989). In the ex-Federal Republic of Germany, West Berlin, Austria and Switzerland from 1975 to 1986 there was little change in the prevalence of nalidixic acid resistance amongst clinical isolates, with 2% resistance in *E. coli*, 0% in *Ps. aeruginosa* or *Staph. aureus*, but 8% amongst *Klebsiella* sp. (Kresken and Wiedemann, 1986; Wiedemann and Zühlsdorf, 1989). Since the clinical introduction of norfloxacin, ciprofloxacin and ofloxacin in the 1980's excepting *Ps. aeruginosa* there has been no general change in resistance to 4-quinolones (Kresken and Wiedemann, 1988). From 1983 to 1986 Wiedemann and Zühlsdorf (1989) reported either an overall one-step decrease or a one-step increase in distribution of MIC values of ciprofloxacin or ofloxacin, respectively, amongst all isolates of *Ps. aeruginosa*. More importantly, however, the number of strains whose MIC value was greater than the breakpoint of 2µgml^{-1} of ciprofloxacin, ofloxacin or enoxacin increased from about 3 to 10% with *Ps. aeruginosa* from 1983 to 1986 in the same study (Kresken and Wiedemann, 1988). Resistance development has not been limited exclusively to the species mentioned but has occurred with other species. The commonest bacterial enteropathogens *Campylobacter jejuni* and *Campylobacter coli* have exhibited significant increases in 4-quinolone resistance between 1978-80 and 1990 in Finland (Rautelin *et al*, 1991). Norfloxacin resistance in these two species increased from 4 to 11% and resistance to ciprofloxacin increased from 0 to 9%. Over the same period resistance to erythromycin, gentamicin or doxycycline remained unchanged

at 3, 0 or ≈16%, respectively. A study in Holland found similar increases in the occurence of 4-quinolone resistance in *Campylobacter* spp. isolates of both human and poultry origin, which paralleled the increasing use of 4-quinolones in human and veterinary medicine, respectively (Endtz, *et al*, 1991). This suggests that veterinary use of 4-quinolones may increase the emergence of resistance to these drugs, and that erythromycin should be the antimicrobial agent of choice for Campylobacteriosis since resistance to this drug has not increased with time (Rautelin *et al*, 1991).

In contrast to the results of bacteriostatic (MIC) tests, ciprofloxacin or DR-3355 invariably exhibited progressively less bactericidal activity as the temperature of incubation was reduced from 37 to 20°C (see Tables 8, 9, 10, 11, 12, 13, 16 and 18, and Figures 13, 19, 21, 22, 27, 33, 39, 45, 52, 53, 54, 55, 57, 58, 59 and 60). Two different types of bactericidal assay were employed in this thesis, both of which were described in the "Materials and Methods" section. The actual magnitude of the reduction in bactericidal activity depended on whether the initial death rates at a single 4-quinolone concentration or the bactericidal profiles obtained for several different concentrations for a fixed time period were considered. The reduction in the initial death rates was about 2- to 7-fold as the temperature was decreased from 37 to 20°C whereas the mean increase in % survival of *E. coli* or the two Staphylococci

was about 60-fold in nutrient broth (at the optimum

bactericidal concentrations [OBC] after 3 hours) with the

same temperature reduction (see Tables 8, 9, 10, 11, 12 and

13 and Figures 13, 19, 27, 33, 39, and 45). As was

described earlier this difference was because the death

curves were biphasic and tended to diverge as incubation

proceeded, thereby progressively increasing the relative

difference in survival as the temperature was reduced. A

biphasic response (Crumplin and Smith, 1975) was exhibited

by ciprofloxacin or DR-3355 against *E. coli* and both

Staphylococci in nutrient broth. Bactericidal mechanism A

can only kill bacteria that are capable of division and are

also able to synthesise protein and RNA (Smith, 1984a).

Mechanism B has none of the prerequisites of mechanism A and

so can kill bacteria in which protein or RNA synthesis is

inhibited by chloramphenicol or rifampicin, respectively, or

bacteria that are suspended in phosphate-buffered saline

(PBS) to prevent multiplication. Mechanism C also operates

in PBS but differs from mechanism B in that protein and RNA

synthesis is essentially required for its activity

(Ratcliffe and Smith, 1985). In this thesis mechanisms B or

C could not be resolved because the experiments were

performed in nutrient broth and PBS only, for reasons

described earlier. However, mechanism C has been shown to

be possessed by norfloxacin or enoxacin against *E. coli*, and

these two drugs do not possess mechanism B (Lewin *et al*,

1989a; Ratcliffe and Smith, 1985). DR-3355 exhibited

mechanisms A and B (and/or C) against *E. coli*, *Staph. aureus*

or *Staph. epidermidis*, and ciprofloxacin exhibited

mechanisms A and B (and/or C) against *E. coli* but only

mechanism A against the two Staphylococci. When the

bactericidal profiles of the 4-quinolones were determined in

PBS to inhibit bactericidal mechanism A, the relative

reduction in the activity of ciprofloxacin against *E. coli*

or DR-3355 against *E. coli, Staph. aureus* or *Staph.*

epidermidis was much less (2- to 3-fold) than the reduction

seen in nutrient broth as the temperature was reduced from

37 to 20°C (see Tables 8, 9, 11 and 13). This may have been

because bactericidal mechanism A, which requires dividing

bacteria (Smith, 1984a) was more affected by the reduced

multiplication rate of the bacteria at lower temperatures,

as was described in the section "Thermodynamic aspects of

the growth and death of bacteria treated with 4-quinolones".

However, as was described above, the multiplication rate of

Staph. epidermidis was slightly less at 37°C compared to

that found at 30°C, so it would seem that the activity was

related directly to the temperature and not the growth rate.

In agreement with Morrissey and Smith (1990) ciprofloxacin

or DR-3355 did not exhibit a biphasic response against *Ps.*

aeruginosa because 4-quinolones possess solely mechanism B

against this organism at bactericidal concentrations (see

Figures 21 and 22).

The values for the OBCs of ciprofloxacin or DR-3355 were

affected differently by reducing the temperature of

incubation from 37 to 20°C. With *E. coli* treated with

either ciprofloxacin or DR-3355 there was little or no
change in the values for the OBC as temperature was reduced
from 37°C (0.15 to 0.3μgml^{-1} ciprofloxacin or 0.3μgml^{-1} DR-
3355, Tables 8 and 9, respectively). With *Staph. aureus*
treated with ciprofloxacin the OBC increased progressively
from 5 to 15μgml^{-1} as the temperature was reduced from 37 to
20°C (Table 10). The peak serum concentration of
ciprofloxacin after a single 500mg oral dose is about 3μgml^{-1}
(Hooper and Wolfson, 1985). Assuming that the OBC value in
serum is identical to that observed in nutrient broth, then
the OBC of ciprofloxacin would be unattainable in the serum
at 37°C and would become even further out of reach as the
temperature was reduced to 20°C. Could the observed
increase in the OBCs of ciprofloxacin have a bearing its the
failure in the treatment of *Staph. aureus* infections? On
the other hand, the effect of temperature on the OBCs may be
offset by higher concentrations (often much greater than peak
serum level) of ciprofloxacin achieved in tissues such as
macrophages, polymorphonulcear leukocytes and lungs (Easmon
and Crano, 1985; Hoogkamp-Korstanje and Klein, 1986). This
is germane to infections caused by *Staph. aureus*, which is
well known to survive ingestion by phagocytes. With *Staph.*
aureus treated with DR-3355 the OBCs were 5μgml^{-1} at 37 and
20°C or 3μgml^{-1} at 25 and 30°C (Table 11). Thus, the OBCs of
DR-3355 were attainable in serum because the peak serum
concentration of DR-3355 is approximately twice that of
ciprofloxacin (Nakashima *et al*, 1988). With *Staph.*

epidermidis treated with ciprofloxacin the values for the

OBCs were variable and greater than the peak serum

concentration at 37, 30 or 20°C, being 5, 5 or 9µgml^{-1},

respectively (Table 12). At 25°C the value for the OBC was

3µgml^{-1}. With *Staph. epidermidis* temperature had little

effect on the OBCs of DR-3355, being 5µgml^{-1} at 37°C or

3µgml^{-1} at the three lower temperatures tested, so these OBCs

were achievable in the serum at each of the temperatures

(Table 13).

Why does decreasing the temperature of incubation reduce the

bactericidal activity of the 4-quinolones? There are

several possible reasons for this, the most obvious being

that chemical and biological reaction rates (including

growth rates) generally increase with increasing temperature

because the reacting molecules possess more kinetic energy

(Atkin, 1986). Susceptibility of bacteria to antimicrobial

agents is well known to be related to the growth rate (Brown

et al, 1990) and the growth rate was affected by

temperature. Reduction in growth rate generally decreases

susceptibility to antimicrobial agents by alterations in the

cell envelope components such as phospholipid, outer

membrane porin, lipopolysaccharide or cation levels (Brown

et al, 1990). From these experiments it is not possible to

distinguish between the effects of temperature on the

bactericidal activity from the effect that growth rate has

on bactericidal activity. To determine the effects of

growth rate on activity of the 4-quinolones it would be
preferable to study death kinetics in a chemostat in which
the steady-state growth rate can be precisely controlled at
a constant temperature (Pirt, 1975; Brown *et al*, 1990).
Although it is generally accepted that DNA gyrase is the
primary target for 4-quinolones, the actual mechanisms by
which 4-quinolones kill bacteria have not been fully
elucidated. It seems that at low concentrations death is
protein synthesis-dependent and caused by filamentation
(involving peptidoglycan synthesis) followed much later by
lysis (Vincent *et al*, 1991). As the 4-quinolone
concentration is increased both DNA synthesis and protein
synthesis are progressively inhibited, and filamentation or
lysis are increasingly inhibited (Crumplin and Smith, 1975;
Georgopapadakou and Bertasso, 1991; Vincent *et al*, 1991).
It is reasonable to presume that at low or high
concentrations the rates of filamentation/lysis or DNA
synthesis inhibition, respectively, are temperature-
dependent. Also, increased accumulation of ^{14}C-
ciprofloxacin has been demonstrated in *E. coli* as
temperature was increased from 0 to 42°C by Diver *et al*
(1990) who suggested that temperature may affect membrane
fluidity or cell metabolism associated with drug uptake. It
seems possible that reduced accumulation of 4-quinolones at
lower temperatures may adversely affect the activity of the
drugs. However, there may be other reasons for the effect
of temperature on activity of 4-quinolones to do with the
supercoiling of DNA. Decreasing the temperature from 39 to

17°C was found to increase supercoiling of plasmid DNA by increasing the helical pitch (θ) of the DNA helix (Wang, 1969b; Depew and Wang, 1975). The larger the plasmid the greater the effect of temperature on supercoiling. In the bacterial cell it is thought that a constant level of supercoiling is maintained over the normal growth range (17-37°C) by balancing the effect of temperature on helical pitch by altering the linking number (α) of the DNA (see "Introduction" section) (Goldstein and Drlica, 1984). In *E. coli* changes in the linking number are thought to be controlled homeostatically by topoisomerase I and DNA gyrase, which remove negative supercoils (reduce α) or add negative supercoils (increase α), respectively (Pruss *et al*, 1982; Menzel and Gellert, 1983; Wang, 1991). At lower temperatures the activity of DNA gyrase will be temporarily reduced relative to that of topoisomerase I. If the temperature of incubation is increased the opposite process would occur. Thus, the reduced activity of the 4-quinolones at lower temperatures could well be due, in part, to transiently lowered activity of their target enzyme, DNA gyrase (Gellert *et al*, 1977). Alternatively, since the effects of temperature on DNA gyrase activity are thought to be temporary, a more plausible proposal is that 4-quinolones may bind less efficiently to the DNA gyrase-DNA complex at lower temperatures because of steric effects of increased helical pitch of the DNA. If this were the case, could chloroquine (an intercalating agent which introduces positive superhelical turns) offset the effect of

temperature on θ and thereby decrease the fold-reduction in the bactericidal activity of 4-quinolones as temperature is reduced from 37°C?

That the temperature of incubation had quite different effects on the bacteriostatic and the bactericidal activities of the 4-quinolones tested emphasises the differences between these two activities. When the magnitudes of the MIC values and the death rates are compared it is apparent that there was little or no relationship between the two tests. For example, with *Ps. aeruginosa* the MIC values of ciprofloxacin (Table 1) were similar to those found with *Staph. aureus* yet the hypersusceptibility of *Ps. aeruginosa* to 4-quinolones (Morrissey and Smith, 1990) meant that the bactericidal tests with this organism had to be determined over 30 minutes, but the bactericidal tests with the other three bacterial species were determined over 3 hours. Also, with *E. coli* the MIC values were much less than those found for the other three bacterial species (Tables 1, 2 and 3), but the death rates with ciprofloxacin or DR-3355 were similar to those found with *Staph. aureus* or *Staph. epidermidis* (Tables 16 and 18, respectively).

In general the mutation frequencies to resist 4-quinolones also seemed to be unrelated to the bactericidal activity of the 4-quinolone, since lowering the temperature of incubation from 37°C had variable effects on the mutation

frequency but always reduced the bactericidal activity of
the drugs. The exception to this was *Staph. epidermidis*
where its mutation rates were directly proportional to the
bactericidal activity at 37, 30 or 25°C, which was
unexpected. Also unexpected was the finding that *Ps.
aeruginosa*, which was killed extremely rapidly at all
temperatures tested, exhibited mutation frequencies to
resist ciprofloxacin, ofloxacin or DR-3355 at 37, 30 or 25°C
higher than those of the other three bacterial species
(Table 4), even though their death rates when treated with
4-quinolones were much less (Tables 16 and 18). This
observation agrees with the proposal that treatment of *Ps.
aeruginosa* with 4-quinolones may select inherently resistant
clones within a population of bacteria (Scully *et al*, 1986;
Ball, 1990), and may also explain the shape of the death
curves described in this thesis (Figures 53 and 58). Thus,
in agreement with Smith (1990b) it seems that prediction of
in vitro mutation rates is not possible from the
susceptibility of the bacteria to the 4-quinolones as
determined by either bacteriostatic or bactericidal tests.

The apparent activation energies (E_a) for the death of
bacteria treated with 4-quinolones between 20 and 37°C were
determined from the slopes of the Arrhenius plots of their
initial specific death rates (μ_q) in nutrient broth (Figures
56 and 61). Ciprofloxacin or DR-3355 were found to have
broadly similar values for the apparent activation energies
for the death of *E. coli, Ps. aeruginosa, Staph. aureus* or

Staph. epidermidis (Table 17). Although there was considerable variation in the values for the E_a with *Ps. aeruginosa* or *Staph. epidermidis* the values fell in the range of 7,000 to 21,000 calories. The exception to this was *Ps. aeruginosa* treated with DR-3355 between 20 and 25°C where E_a was about 46,000 calories, which may have been due to this drug being more affected by diffusion through the phopholipid bilayer since it is more hydrophobic than ciprofloxacin (Chapman and Gerogopapadakou, 1988). The possession of mechanism B (and/or C) by DR-3355 against the Staphylococci (Lewin and Amyes, 1989) seemed to reduce the apparent activation energy compared to that for ciprofloxacin (Table 17). This is because temperature affected the initial death rates with ciprofloxacin to a greater degree than the death rates with DR-3355, and this finding was supported by the fold-increase in % survival at the OBCs of both drugs as the temperature was reduced from 37 to 20°C. With *Staph. aureus* the fold-increase in survival after 3 hours was 86-fold with ciprofloxacin (Table 10) and 46-fold with DR-3355 (Table 11) while the values for E_a were 20,588 and 18,456 calories, respectively (Table 17). With *Staph. epidermidis* the fold-increase in survival after 3 hours was 82-fold with ciprofloxacin (Table 12) but only 19-fold with DR-3355 (Table 13) and the values for E_a were 20,075 and 11,337 calories, respectively (Table 17). The possession of more than one mechanism of action by DR-3355 together with its activity against the Staphylococci being less temperature-dependent would tend to indicate DR-3355 in

preference to ciprofloxacin. Also, with the sole exception of *Ps. aeruginosa* between 25 and 37°C, for each bacterial species higher MIC values were always associated with greater E_a values for kill with ciprofloxacin or DR-3355. The magnitudes of the values for E_a are consistent with an enzyme activity being the rate-limiting step in the bactericidal activity of 4-quinolones. Possible candidates for the rate-limiting step are inhibition of DNA gyrase, enzyme activities involved in peptidoglycan synthesis, DNA degradation, or induction of the SOS response. Interestingly, Slilaty *et al* (1986) found that the rate of hydrolysis of the lexA protein, which is the repressor for the SOS system that repairs DNA damage caused by bactericidal mechansim B (Howard and Smith, 1991), was temperature-dependent and the apparent activation energy for its hydrolysis at 25°C was approximately 15,300 calories. This value is of a broadly similar magnitude to E_a values observed for the death of the bacteria treated with ciprofloxacin or DR-3355 found in this thesis (Table 17).

Do the effects of temperature of incubation *in vitro* have any relevance to the clinical milieu? Firstly, in terms of the mutation rates to resist 4-quinolones it seems that even though reducing the temperature from 37 to 25°C had highly variable effects on the selection of resistant mutants, selection of mutants was generally more frequent at lower-than-body temperatures than was observed at 37°C. This also means that resistance could develop on fomites which are at

room temperature in hospitals, such as in sinks and drains, or on surfaces where drug residues may occur. Furthermore, development of resistance on fomites may partially explain the isolation of 4-quinolone-resistant methicillin-resistant *Staph. aureus* isolates from patients that had never received 4-quinolone therapy (Peterson *et al*, 1989; Smith *et al*, 1990), although nasal carriage by other patients and hospital staff is probably a greater source of such strains (Casewell and Hill, 1986). Nevertheless the anterior nares are at temperatures less than 37°C. Reducing the temperature from 37°C increased the mutation rates of *E. coli* to resist ciprofloxacin or DR-3355 (Figure 5), *Ps. aeruginosa* to resist ciprofloxacin (Figure 6), and *Staph. aureus* to resist ciprofloxacin or ofloxacin (Figure 7; Table 4). Reducing the temperature almost invariably lessened the bactericidal activity, thereby increasing the survival of treated bacteria. Any factor which improves survival potential of bacteria in the clinical scene inevitably predisposes to the likelyhood of resistance development. In the particular instances mentioned above perhaps a combination of greater survival together with increased mutation frequency act synergistically to increase treatment failure. On the other hand, the effect of lower skin temperatures may be counterbalanced to an extent by the warmth generated by the inflammatory response to infections.

Secondly, clinical or bacteriological failure with 4-quinolones is not always associated with resistance

development during therapy, as determined by MIC tests of isolates before, during and after therapy (Fass, 1986; Wood and Logan, 1986; Righter, 1987). The bactericidal studies in this thesis would seem to suggest that a possible contributory factor in clinical failure (in the absence of resistance development) at sites of infection which may have lower-than-body temperatures is the reduced bactericidal activity of 4-quinolones. Other contributory factors may be low redox conditions (Lewin *et al*, 1989c; Lewin *et al*, 1991b) which is encouraged by the presence of large numbers of bacteria or by poor wound drainage (Ball, 1990). Indeed, large numbers of bacteria have been shown to abolish the bactericidal activity of 4-quinolones *in vitro* because of reduced oxygen levels (Lewin *et al*, 1989c; Morrissey *et al*, 1990; Lewin *et al*, 1991b). Poor tissue penetration of antibacterials may also affect bacteriological response at abscess cavities or in the central nervous system (Ball, 1990; Nix *et al*, 1991).

It would be interesting to determine the effect of topical heating of skin lesions such as ulcers or abscesses in patients undergoing 4-quinolone therapy for *Ps. aeruginosa* or Staphylococcal infection. Heat could perhaps be applied to the infected areas *via* electrical pads. Ideal candidates for this treatment would be those from whom bacterial isolates with elevated MICs were not detected, but in whom the infection was recalcitrant to 4-quinolone therapy. If this technique proved to be of therapeutic value it could

perhaps be applied to other classes of antibacterial agent where the activity may also be less at lower-than-body temperatures. Perhaps the old usage of hot bread poultices for skin infections was ahead of its time!

REFERENCES:

Ambler, J. E., Drabu, Y. J., Blakemore, P. H., Pinney, R. J.

Journal of Pharmacy and Pharmacology, 1991, **43**

Supplement, 17P.

Aoyama, H., Sato, K., Fujii, T., Fujimaki, K., Inoue, M.,

Mitsuhashi, S.

Antimicrobial Agents and Chemotherapy, 1988a, **32,**

104-109.

Aoyama, H., Fujimaki, K., Sato, K., Fujii, T., Inoue, M.,

Hirai, K., Mitsuhashi, S.

Antimicrobial Agents and Chemotherapy, 1988b, **32,**

922-924.

Aoyama, H., Sato, K., Kato, T., Hirai, K., Mitsuhashi, S.

Antimicrobial Agents and Chemotherapy, 1987, **31,**

1640-1641.

Archer, G. L.

Journal of Antimicrobial Chemotherapy, 1988, **21,**

Supplememt C, 133-138.

Atkin, P. W.

In: *Physical Chemistry.* Oxford University Press, Third

edition, 1986, Chapter 28.

Bachmann, B. J.

Microbiological Reviews, 1990, **54,** 130-197.

Ball, P.

Journal of Antimicrobial Chemotherapy, 1990, **26,**

165-179.

Bauer, W., Vinograd, J.

> *Journal of Molecular Biology*, 1968, **33**, 141-171.

Bedard, J., Chamberland, S., Wong, S., Schollaardt, T., Bryan, L. E.

> *Antimicrobial Agents and Chemotherapy*, 1989, **33**, 1457-1464.

Bedard, J., Wong, S., Bryan, L.E.

> *Antimicrobial Agents and Chemotherapy*, 1987, **31**, 1348-1354.

Berrios, M., Osheroff, N., Fisher, P. A.

> *Proceedings of the National Academy of Sciences of the United States of America,* 1985, **82**, 4142-4146.

Blumberg, H. M., Rimland, D., Carroll, D. J., Terry, P., Wachsmuth, I. K.

> *Journal of Infectious Diseases*, 1991, **163**, 1279-1285.

Blumberg, H. H., Rimland, D. Wachsmuth, I. K.

> *29th Interscience Conference on Antimicrobial Agents and Chemotherapy*, 1989, Abstract 7.

Bosso, J. A., Black, P. G., Matsen, J. M.

> *American Journal of Medicine*, 1987, **82** Supplement 4A, 180-184.

Bouthier de la Tour, C., Portemer, C., Nadal, M., Stetter, K. O., Forterre, P., Duguet, M.

> *Journal of Bacteriology*, 1990, **172**, 6803-6808.

Brahms, J. G., Dargouge, O., Brahms, S., Ohara, Y., Vagner, V.

Journal of Molecular Biology, 1985, **181**, 455-465.

Brown, M. R. W., Collier, P. J., Gilbert, P.

Antimicrobial Agents and Chemotherapy, 1990, **34**, 1623-1628.

Brown, P. O., Peebles, C. L., Cozzarelli, N. R.

Proceedings of the National Academy of Sciences of the United States of America 1979, **76**, 6110-6114.

Bryan, L. E.

Journal of Antimicrobial Chemotherapy, 1988, **22** Supplement A, 1-15.

Bryan, L.E., Bedard, J.

European Journal of Clinical Microbiology and Infectious Disease, 1991, **10**, 232-239.

Burrington, M. G., Morgan, A. R.

Canadian Journal of Biochemistry, 1978, **56**, 123-128.

Carret, G., Flandrois, J.P., Lobry, J.R.

Journal of Antimicrobial Chemotherapy, 1991, **27**, 319-327.

Casewell, M. W., Hill, R. L. R.

Journal of Antimicrobial Chemotherapy, 1986, **18** Supplement A, 1-12.

Celesk, R. A., Robillard, N. J.

   Antimicrobial Agents and Chemotherapy, 1989, **33**,

   1921-1926.

Cerf, O.

   Journal of Applied Bacteriology, 1977, **42**, 1-19.

Chamberland, S., Bayer, A. S., Schollaardt, T., Wong, S. A.,

Bryan, L. E.

   Antimicrobial Agents and Chemotherapy, 1989, **33**,

   624-634.

Champoux, J. J.

   Journal of Biological Chemistry, 1981, **256**, 4805-4809.

Chapman, J.S., Georgopapadakou, N.H.

   Antimicrobial Agents and Chemotherapy, 1988, **32**,

   438-442.

Chaudhry, A. Z., Knapp, C. C., Sierra-Madero, J.,

Washington, J. A.

   Antimicrobial Agents and Chemotherapy, 1990, **34**,

   1843-1845.

Chin, N.-X., Clynes, N., Neu, H.C.

   American Journal of Medicine 1989, **87** Supplement 5A,

   28-31.

Chin, N.X., Gu, J.W., Yu, K.W., Zhang, Y.X., Neu, H.C.

   Antimicrobial Agents and Chemotherapy, 1991, **35**,

   567-571.

Christensen, H. N.

In: *Biological Transport*, Second Edition, W. A.

Benjamin, Inc., Massachusetts, 1975, Chapter 3.

Cohen, S. P., Hooper, D. C., Wolfson, J. S., Souza, K. S.,

McMurray, L. M., Levy, S.B.

Antimicrobial Agents and Chemotherapy, 1988 , **32**,

1187-1191.

Cook, T. M., Brown, K. G., Boyle, J. V., Goss, W. A.

Journal of Bacteriology, 1966, **92**, 1510-1514.

Cooper, M. A., Andrews, J. M., Ashby, J. P., Matthews, R.

S., Wise, R.

Journal of Antimicrobial Chemotherapy, 1990, **26**,

667-676.

Courvalin, P.

Antimicrobial Agents and Chemotherapy, 1990, **34**,

681-684.

Courvalin, P., Poyart-Salmeron, C., Derlot, E.

In: *The 4-quinolones: Antibacterial Agents* in vitro

(Ed. G. C. Crumplin) Springer-Verlag, London, 1990,

Chapter 16.

Crumplin, G. C.

In: *The 4-quinolones: Antibacterial Agents* in vitro

(Ed. G. C. Crumplin) Springer-Verlag, London, 1990,

257-268.

Crumplin, G. C., Smith, J. T.

Nature, 1976, **260**, 643-645.

Crumplin, G. C., Smith, J. T.

Antimicrobial Agents and Chemotherapy, 1975, **8**,

251-261.

Cullen, M. E., Wyke, A. W., Kuroda, R., Fisher, L. M.

Antimicrobial Agents and Chemotherapy 1989, **33**,

886-894.

D'Arpa, P., Beardmore, C., Liu, L. F.

Cancer Research, 1990, **50**, 6919-6924.

D'Arpa, P., Liu, L.F.

Biochimica Biophysica Acta, 1989, **989**, 163-177

Dalhoff, A., Döring, G.

In: *The Influence of Antibiotics on the Host-Parasite*

Relationship **II**, (Eds. D. Adam, H. Hahn, and W.

Opterkuch), Springer-Verlag, Berlin Heidelberg, 1985,

246-255.

Dayn, A., Malkhosyan, S., Duzhy, D., Lyamichev, V.,

Panchenko, Y., Mirkin, S.

Journal of Bacteriology, 1991, **173**, 2658-2664.

Dechène, M., Leying, H., Cullmann, W.

Chemotherapy 1990, **36**, 13-23.

Depew, R. E., Wang, J. C.

   Proceedings of the National Academy of Sciences of the United States of America, 1975, **72**, 4275-4279.

DiNardo, S., Voelkel, K. A., Sternglanz, R.

   Cell, 1982, **31**, 43-51

Diver, J. M., Wise, R.

   Journal of Antimicrobial Chemotherapy, 1986, **18** Supplement D, 31-41.

Diver, J.M., Piddock, L.J.V., Wise, R.

   Journal of Antimicrobial Chemotherapy, 1990, **25**, 319-333.

Diver, J.M., Schollaardt, T., Rabin, H.R., Thorson, C., Bryan, L.E.

   Antimicrobial Agents and Chemotherapy, 1991, **35**, 1538-1546.

Drlica, K., Franco, R. J.

   Biochemistry, 1988, **27**, 2253-2259.

Drlica, K., Rouviere-Yaniv, J.

   Microbiological Reviews, 1987, **51**, 301-319.

Easmon, C. S. F., Crano, J. P.

   Journal of Antimicrobial Chemotherapy, 1985, **16**, 67-73.

Endtz, H. Ph., Rujis, G. J., van Klingeren, B., Jansen, W. H., van der Ryden, T., Mouton, R. P.

   Journal of Antimicrobial Chemotherapy, 1991, **27**, 199-208

Eron, L. J.

   American Journal of Medicine, 1987, **82** Supplement 4A, 224-226.

Fass, R. J.

    Antimicrobial Agents and Chemotherapy, 1983, **24**,

    568-574.

Fass, R. J.

    Journal of Antimicrobial Chemotherapy 1986, **18**

    Supplement D, 153-157.

Fass, R. J.

    Antimicrobial Agents and Chemotherapy, 1987, **31**,

    148-150.

Felmingham, D., Foxall, P., O'Hare, M. D., Webb, G., Ghosh,
G., Grünberg, R. N.

    Journal of Antimicrobial Chemotherapy, 1988, **22**

    Supplement C, 27-34.

Finch, J. T., Lutter, L. C., Rhodes, D., Brown, R. S.,
Rushton, B., Levitt, M., Klug, A.

    Nature, 1977, **269**, 29-36.

Fisher, L. M., Mizuuchi, K., O'Dea, M. H., Ohmori, H.,
Gellert, M.

    Proceedings of the National Academy of Sciences of the

    United States of America, 1981, **78**, 4165-4169.

Fong, I. W., Linton, W., Simbul, M., Thorup, R., McLaughlin,
B., Rahm, V., Quinn, P. A.

    American Journal of Medicine, 1987, **82** Supplement 4A,

    311-316.

Fukuda, H., Hosaka, M., Hirai, K., Iyobe, S.

    Antimicrobial Agents and Chemotherapy, 1990, **34**,

    1757-1761.

Fuller, F. B.

   Proceedings of the National Academy of Sciences of the United States of America, 1978, **75**, 3557-3561.

Gasser, T. C., Graversen, P. H., Madsen, P. O.

   American Journal of Medicine, 1987, **82** Supplement 4A, 278-279.

Geigy, J. R.

   Documenta Geigy Scientific Tables, Fifth Edition, Jesse Broad and Co. Ltd, 1955, 47-48

Gellert, M.

   Annual Reviews in Biochemistry, 1981, **50**, 879-910

Gellert, M., Mizuuchi, K., O'Dea, M. H., Nash, H. A.

   Proceedings of the National Academy of Sciences of the United States of America 1976a, **73**, 3872-3876.

Gellert, M., O'Dea, M. H., Itoh, T., Tomizawa,J.-I.

   Proceedings of the National Academy of Sciences of the United States of America, 1976b, **73**, 4474-4478.

Gellert, M., Mizuuchi, K., O'Dea, M.H., Itoh, T., Tomizawa, J.-I.

   Proceedings of the National Academy of Sciences of the United States of America, 1977, **74**, 4772-4776.

Georgopapadakou, N. H., Bertasso, A.

   Antimicrobial Agents and Chemotherapy, 1991, **35**, 2645-2648.

Gilbert, D. N., Tice, A. D., Marsh, P. K., Craven, P. C., Preheim, L. C.

   American Journal of Medicine, 1987, **82** Supplement 4A, 254-258.

Goldstein, E., Drlica, K.

Proceedings of the National Academy of Sciences of the United States of America, 1984, **81**, 4046-4050.

Gootz, T.D., Martin, B.A.

Antimicrobial Agents and Chemotherapy, 1991, **35**, 840-845.

Goss, W. A., Deitz, W. H., Cook, T. M.

Journal of Bacteriology, 1965, **89**, 1068-1074.

Gutfreund, H.

In: Enzymes: Physical Principles, Wiley-Interscience, London, 1972, Chapter 7.

Hallett, P., Maxwell, A.

Antimicrobial Agents and Chemotherapy, 1991, **35**, 335-340.

Hammond, G.G., Cassidy, P.J., Overbye, K.M.

Journal of Bacteriology, 1991, **173**, 5564-5567.

Hane, M.W., Wood, T.H.

Journal of Bacteriology, 1969, **99**, 238-241.

Harnett, N., Brown, S., Krishnan, C.

Antimicrobial Agents and Chemotherapy, 1991, **35**, 1911-1913.

Helgerson, S. L., Cramer, W. A.

Journal of Supramolecular Structure, 1976, **5**, 291-308.

Herendeen, S. L., VanBogelen, R. A., Neidhardt, F. C.

Journal of Bacteriology, 1979, **139**, 185-194.

Higgins, N. P., Peebles, C. L., Sugino, A., Cozzarelli, N. R.

   Proceedings of the National Academy of Sciences of the United States of America, 1978, **75**, 1773-1777.

Hirai, K., Aoyama, H., Irikura, T., Iyobe, S., Mitsuhashi, S.

   Antimicrobial Agents and Chemotherapy, 1986a, **29**, 535-538.

Hirai, K., Aoyama, H., Suazue, S., Irikura, T., Iyobe. S., Mitsuhashi, S.

   Antimicrobial Agents and Chemotherapy, 1986b, **30**, 248-253.

Hirai, K., Suzue, S., Irikura, T., Iyobe, S., Mitsuhashi, S.
   Antimicrobial Agents and Chemotherapy, 1987, **31**, 582-586.

Holmes, M.L., Dyall-Smith, M.L.
   Journal of Bacteriology, 1991, **173**, 642-648.

Hoogkamp-Korstanje, J. A. A., Klein, S. J.
   Journal of Antimicrobial Chemotherapy, 1986, **18**, 407-413.

Hooper, D. C., Wolfson, J. S.
   Antimicrobial Agents and Chemotherapy, 1985, **28**, 716-721.

Hooper, D. C., Wolfson, J. S., Souza, K. S., Tung, C., McHugh, G. L., Swartz, M. N.
   Antimicrobial Agents and Chemotherapy, 1986, **29**, 639-644.

Hooper, D. C., Wolfson, J. S., Tung, C., Souza, K. S., Swartz, M. N.

Journal of Bacteriology, 1989, **171**, 2235-2237.

Hopewell, R., Oram, M., Briesewitz, R., Fisher, L. M.

Journal of Bacteriology, 1990, **172**, 3481-3484.

Horowitz, D. S., Wang, J. C.

Journal of Biological Chemistry, 1987, **262**, 5339-5344.

Howard, B. M. A., Smith, J. T.

Journal of Pharmacy and Pharmacology, 1991, **43**

Supplement, 14P.

Huff, A., C., Kreuzer, K. N.

Third Conference on DNA Topoisomerases in Therapy,

1990, Abstract 10, 23.

Ingraham, J. L.

In: *The Bacteria.* (Eds. I. C. Gunsalus and R. Y.

Stanier). Academic Press, 1962, **IV**, 265-296.

Jensen, T., Pedersen, S. S., Nielsen, C. H., Hoilby, N., Koch, C.

Journal of Antimicrobial Chemotherapy, 1987, **20**,

585-594.

Kaguni, J. M., Kornberg, A.

Journal of Biological Chemistry, 1984, **259**, 8578-8583.

Kato, J.-I., Nishimura, Y., Imamaura, R., Niki, H., Hiraga, S., Suzuki, H.

Cell, 1990, **63**, 393-404.

Keller, W.

> *Proceedings of the National Academy of Sciences of the United States of America*, 1975, **72**, 4876-4880.

Kikuchi, A., Asai, K.

> *Nature*, 1984, **309**, 677-681.

Kirkegaard, K., Wang, J. C.

> *Nucleic Acids Research*, 1978, **5**, 3811-3820.

Kirkegaard, K., Wang, J. C.

> *Cell*, 1981, **23**, 721-729.

Klevan, L., Wang, J. C.

> *Biochemistry*, 1980, **19**, 5229-5234.

Kotera, Y., Watanabe, M., Yoshida, S., Inoue, M., Mitsuhashi, S.

> *Journal of Antimicrobial Chemotherapy*, 1991, **27**, 733-739.

Krawiec, S., Riley, M.

> *Microbiological Reviews*, 1990, **54**, 502-539.

Kresken, M., Wiedemann, B.

> *Journal of Antimicrobial Chemotherapy*, 1986, **18** Supplement C, 235-242.

Kresken, M., Wiedemann, B.

> *Antimicrobial Agents and Chemotherapy* 1988, **32**, 1285-1288.

Kreuzer, K. N., Cozzarelli, N.

> *Journal of Bacteriology*, 1979, **140**, 424-435.

Kung, T. C. Wang, J. C.

> *Journal of Biological Chemistry*, 1977, **252**, 5398-5402.

LeBel, M., Bergeron, M. G., Vallée, F., Fiset, C., Chassé, G., Bigonesse, P., Rivard, G.

    Antimicrobial Agents and Chemotherapy, 1986, **30**, 260-266.

LeBon, J. M., Kado, C. I., Rosenthal, L. J., Chirikjian, J. G.

    Proceedings of the National Academy of Sciences of the United States of America, 1978, **75**, 4097-4101.

Legakis, N. J., Tzouvelekis, L. S., Makris, A., Kotsifaki, H.

    Antimicrobial Agents and Chemotherapy, 1989, **33**, 124-127.

Lentino, J.R., Augustinsky, J.B., Weber, T.M., Pachucki, C.T.

    Chemotherapy, 1991, **37**, 70-76.

LePecq, J.-B., Paoletti, C.

    Journal of Molecular Biology, 1967, **27**, 87-106.

Lesse, A. J., Freer, C., Salata, R. A., Francis, J. B., Scheld, W. M.

    American Journal of Medicine, 1987, **82** Supplement 4A, 247-253.

Lewin, C. S., Smith, J. T.

   Journal of Antimicrobial Chemotherapy, 1988, **22**

   Supplement C, 1-8.

Lewin, C. S., Amyes, S. G. B.

   Journal of Medical Microbiology, 1989, **30**, 227-231.

Lewin, C. S., Amyes, S. G. B., Smith, J. T.

   European Journal of Clinical Microbiology and

   Infectious Diseases, 1989a, **8**, 731-733.

Lewin, C. S., Howard, B. M. A., Ratcliffe, N. T., Smith, J.
T.

   Journal of Medical Microbiology, 1989b, **29**, 139-144.

Lewin, C. S., Morrissey, I., Smith, J. T.

   Reviews of Infectious Diseases, 1989c, **11** Supplement 5,

   S913-S914.

Lewin, C. S., Smith, J. T., Hamilton-Miller, J. M. T.,
Brumfitt, W.

   Reviews of Infectious Diseases, 1989d, **11** Supplement 5,

   S970-S971.

Lewin, C. S., Allen, R. A., Amyes, S. G. B.

   Journal of Medical Microbiology, 1990, **31**, 153-161.

Lewin, C. S., Howard, B. M. A., Smith, J. T.

   Journal of Medical Microbiology, 1991a, **34**, 19-22.

Lewin, C. S., Morrissey, I., Smith, J. T.

   European Journal of Clinical Microbiology & Infectious

   Diseases, 1991b, **10**, 240-248.

Lewin, C. S., Amyes, S. G. B.

   Journal of Medical Microbiology, 1990, **32**, 83-86.

Lewin, C. S., Smith, J. T.

Journal of Antimicrobial Chemotherapy, 1989a, **24**, 84-85.

Lewin, C. S., Smith, J. T.

Journal of Chemotherapy, 1989b, Supplement 4, 151-153.

Lewin, C. S., Smith, J. T.

Journal of Medical Microbiology, 1990, **31**, 65-70.

Licitra, C. M., Brooks, R. G., Sieger, B. E.

Antimicrobial Agents and Chemotherapy, 1987, **31**, 805-807.

Lilley, D. M. J.

Biochemical Society Transactions, 1986, **14**, 489-493

Little, J. W., Mount, D. W.

Cell, 1982, **29**, 11-22.

Liu, L. F., Depew, R. E., Wang, J. C.

Journal of Molecular Biology, 1976, **106**, 439-452.

Liu, L. F., Wang, J. C.

Proceedings of the National Academy of Sciences of the United States of America, 1987, **84**, 7024-7027.

Liu, L. F., Wang, J. C.

Proceedings of the National Academy of Sciences of the United States of America, 1978, **75**, 2098-2012.

Lode, H., Höffken, G., Boeckk, M., Deppermann, N., Borner, K., Koeppe, P.

Journal of Antimicrobial Chemotherapy, 1990, **26** Supplement B, 41-59.

Lother, H., Lurz, R., Orr, E.

Nucleic Acids Research, 1984, **12**, 901–914.

Louarn, J., Bouche, J.-P., Patte, J., Louarn, J.-M

Molecular and General Genetics, 1984, **195**, 170–174.

Low, R. L., Kaguni, J. M., Kornberg, A.

Journal of Biological Chemistry, 1984, **259**, 4576–4581.

Lucain, C., Regamey, P., Bellido, F., Pechère, J. C.

Antimicrobial Agents and Chemotherapy, 1989, **33**, 937–943.

Masecar, B. L., Celesk, R. A., Robillard, N., J.

Antimicrobial Agents and Chemotherapy, 1990, **34**, 281–286.

Masecar, B.L., Robillard, N.J.

Antimicrobial Agents and Chemotherapy 1991, **35**, 898–902.

Maxwell, A.

Third Conference on DNA topoisomerases in therapy, 1990, Abstract 12, 23.

McEachern, F., Fisher, L. M.

Federation of European Biochemical Societies Letters, 1989, **253**, 67–70.

Mehtar, S., Blakemore, P. H., Ellis, K.

American Journal of Medicine, 1987, **82** Supplement 4A, 55–57.

Menzel, R., Gellert, M.

Cell, 1983, **34**, 105-113.

Menzel, R., Gellert, M.

Journal of Bacteriology, 1987a, **169**, 1272-1278.

Menzel, R., Gellert, M.

Proceedings of the National Academy of Sciences of the United States of America, 1987b, **84**, 4185-4189.

Michea-Hamzehpour, M., Lucain, C., Pechère, J.C.

Antimicrobial Agents and Chemotherapy, 1991a, 35, 512-518.

Michea-Hamzehpour, M., Furet, Y. X., Pechère, J.-C.

Antimicrobial Agents and Chemotherapy, 1991b, **35**, 2091-2097.

Miller, R. V., Scurlock, T. R.

Biochemical and Biophysical Research Communications, 1983, **110**, 694-700.

Mitscher, L. A., Devasthale, P. V., Zavod, R. M.

In: *The 4-quinolones: Antibacterial Agents* in vitro (Ed. G. C. Crumplin) Springer-Verlag, London, 1990, Chapter 9.

Mizuuchi, K., Fisher, L. M., O'Dea, M. H., Gellert, M.

Proceedings of the National Academy of Sciences of the United States of America, 1980, **77**, 1847-1851.

Moniot-Ville, N., Guibert, J., Moreau, N., Acar, J.F., Collatz, E., Gutmann, L.

Antimicrobial Agents and Chemotherapy 1991, 35, 519-523.

Moore, C. L., Klevan, L., Wang, J. C., Griffith, J. D.

Journal of Biological Chemistry, 1983, **258**, 4612-4617.

Morrison, A. L., Cozzarelli, N. R.

Proceedings of the National Academy of Sciences of the United States of America, 1981, **78**, 1416-1420

Morrison, A. L., Higgins, N. P., Cozzarelli, N. R.

Journal of Biological Chemistry, 1980, **255**, 2211-2219.

Morrissey, I., Lewin, C. S., Smith, J. T.

In: *The 4-quinolones: Antibacterial Agents* in vitro (Ed. G. C. Crumplin) Springer-Verlag, London, 1990, Chapter 3.

Morrissey, I., Smith, J. T.

Journal of Pharmacy and Pharmacology, 1990, **42** Supplement, 63P.

Munshi, M. H., Sack, D. A., Haider, K., Ahmed, Z. V., Rahaman, M. M., Morshed, M. G.

Lancet 1987, **ii**, 419-421.

Nadal, M., Jaxel, C., Portemer, C., Forterre, P., Mirambeau, G., Duguet

Biochemistry, 1988, **27**, 9102-9108.

Nakamura, S., Nakamura, M., Kojima, T., Yoshida, H.

Antimicrobial Agents and Chemotherapy, 1989, **33**, 254-255.

Nakanishi, N., Yoshida, S., Wakebe, H., Inoue, M., Mitsuhashi, S.

Antimicrobial Agents and Chemotherapy, 1991a, **35**, 1053-1059.

Nakanishi, N., Yoshida, S., Wakebe, H., Inoue, M., Yamaguchi, T., Mitsuhashi, S.

Antimicrobial Agents and Chemotherapy, 1991b, **35**, 2562-2567.

Nakashima, M., Uematsu, T., Kanamura, M.
28th Interscience Conference on Antimicrobial Agents and Chemotherapy, 1988, Abstract 951.

Nelson, E. M., Tewey, K. M., Liu, L. F.
Proceedings of the National Academy of Sciences of the United States of America, 1984, **81**, 1361-1365.

Neu, H. C., Davidson, S., Briones, F.
American Journal of Medicine 1989, **87** Supplement 5A, 209-212.

Nikaido, H.
Antimicrobial Agents and Chemotherapy, 1989, **33**, 1831-1836.

Nikaido, H.
Journal of Antimicrobial Chemotherapy, 1988, **22** Supplement A, 17-22.

Nikaido, H., Nikaido, K., Harayama, S.
Journal of Biological Chemistry, 1991, **266**, 770-779.

Nix, D. E., Goodwin, S. D., Peloquin, C. A., Rotella, D. L., Schentag, J. J.
Antimicrobial Agents and Chemotherapy, 1991, **35**, 1953-1959.

Odell, M., Crumplin, G. C.
In: *The 4-quinolones: Antibacterial Agents* in vitro (Ed. G. C. Crumplin) Springer-Verlag, London, 1990, Chapter 15.

Ogasawara, N., Seiki, M., Yoshikawa, H.

Nature, 1979, **281**, 702-704.

Oriel, J. D.

Journal of Antimicrobial Chemotherapy, 1986, **18**
Supplement D, 129-132.

Palu', G., Valisena, S., Peracchi, Palumbo, M.

Biochemical Pharmacology, 1988, **37**, 1887-1888.

Parry, M.F., Panzer, K.B., Yukna, M.E.

American Journal of Medicine 1989, **87** Supplement 5A,
12-16.

Pedersen, S. S., Jensen, T., Hvidberg, E. F.

Journal of Antimicrobial Chemotherapy, 1987, **20**,
575-583.

Perea, E.J.

Journal of Antimicrobial Chemotherapy, 1990, **26**, 55-60.

Peters, G.

Journal of Antimicrobial Chemotherapy, 1988, **21**
Supplement C, 139-148.

Phillips, I., Culebras, E., Moreno,F., Baquero, F.

Journal of Antimicrobial Chemotherapy, 1987, **20**,
631-638.

Piddock, L. J. V., Wise, R.

Federation of European Microbiology Societies
Microbiology Letters, 1987, **41**, 289-294.

Pirt, S. J.

In: *Principles of Microbe and Cell Cultivation,*
Blackwell Scientific Publications, Oxford, 1975.

Pruss, G. J., Manes, S. H., Drlica, K.

    Cell, 1982, **31**, 35-42.

Pruss, G., J., Drlica, K.

    Proceedings of the National Academy of Sciences of the United States of America, 1986, **83**, 8952-8956.

Pulleybank, D. E., Shure, M., Tang, D., Vinograd, J., Vosberg, H.-P.

    Proceedings of the National Academy of Sciences of the United States of America, 1975, **72**, 4280-4284.

Raoof, S., Wollschlager, C., Khan, F.

    Journal of Antimicrobial Chemotherapy, 1986, **18** Supplement D, 139-145.

Ratcliffe, N. T., Smith, J. T.

    Journal of Pharmacy and Pharmacology, 1985, **37** Supplement, 92P.

Rau, D. C., Gellert, M., Thoma, F., Maxwell, A.

    Journal of Molecular Biology, 1987, **193**, 555-569.

Rautelin, H., Renkonnen, O.-V., Kosunen, T. U.

    Antimicrobial Agents and Chemotherapy, 1991, **35**, 2065-2069.

Ravizzola, G., Pirali, F., Paolucci, A., Terlenghi, L., Peroni, L., Colombi, A., Turano, A.

    Journal of Antimicrobial Chemotherapy, 1987, **20**, 825-829.

Reece, R. J., Maxwell, A.

    Journal of Biological Chemistry, 1989, **264**, 19,648-19,653.

Reece, R.J., Maxwell, A.

Journal of Biological Chemistry, 1991, **266,**

3540-3546.

Renkonnen, O.-V., Sivonen, A., Visakorpi, R.

Antimicrobial Agents and Chemotherapy, 1987, **31,**

962-963.

Righter, J.

Journal of Antimicrobial Chemotherapy, 1987, **20,**

595-597.

Robillard, N.J., Scarpa, A.L.

Antimicrobial Agents and Chemotherapy, 1988, **32,**

535-539.

Rowe, T. C., Tewey, K. M., Liu, L. F.

Journal of Biological Chemistry, 1984, **259,** 9177-9181.

Rubio, T. T., Shapiro, C.

Journal of Antimicrobial Chemotherapy, 1986, **18**

Supplement D, 147-152.

Ryan, M. J.

Biochemistry, 1976, **15,** 3769-3777.

Ryan, M. J., Wells, R. D.

Biochemistry, 1976, **15,** 3778-3782.

Sato, K., Inoue, Y., Fujii, T., Aoyama, H., Mitsuhashi, S.

Infection, 1986, **14** Supplement 4, S226-S230.

Scully, B. E., Neu, H. C., Parry, M. F., Mandell, W.

Lancet, 1986, **i,** 819-822.

Scully, B. E., Nakatomi, M., Ores, C., Davidson, S., Neu, H. C.

American Journal of Medicine, 1987, **82** Supplement 4A, 196-201.

Self, P. L., Zeluff, B. A., Sollo, D., Gentry, L. O.
American Journal of Medicine, 1987, **82** Supplement 4A, 236-238.

Serour, F., Dan, M., Gorea, A., Yellin, A., Lieberman, Y., Berger, S.A.

Antimicrobial Agents and Chemotherapy, 1991, **35**, 380-381.

Shalit, I., Stutman, H. R., Marks, M. I., Chartrand, S. A., Hilman, B. C.

American Journal of Medicine 1987, **82** Supplement 4A, 189-195.

Shen, L. L., Pernet, A. G.

Proceedings of the National Academy of Sciences of the United States of America, 1985, **82**, 307-311.

Shen, L.

Biochemical Pharmacology, 1989, **38**, 2042-2044.

Shen, L. L., Kohlbrenner, W. E., Weigl, D., Baranowski, J.
Journal of Biological Chemistry, 1989a, **264**, 2973-2978.

Shen, L. L., Baranowski, J., Pernet, A. G.

Biochemistry, 1989b, **28**, 3879-3885.

Shen, L. L., Mitscher, L. A., Sharma, P. N., O'Donnell, T. J., Chu, D. W. T., Cooper, C. S., Rosen, T., Pernet, A. G.

Biochemistry 1989c, **28**, 3886-3894.

Sinden, R. R., Pettijohn, D. E.

Journal of Biological Chemistry, 1984, **259**,

6593-6600.

Slilaty, S. N., Rupley, J. A., Little, J. W.

Biochemistry, 1986, **25**, 6866-6875.

Smith, C. L., Kubo, M., Imamoto, F.

Nature, 1978, **275**, 420-423.

Smith, G. M., Leyland, M. J., Farrell, I. D., Geddes, A. M.

Journal of Antimicrobial Chemotherapy, 1986, **18**

Supplement D, 165-174.

Smith, J. T.

The Pharmaceutical Journal, 1984a, **233**, 299-305.

Smith, J. T.

Fortschritte der antimikrobiellen und

antineoplastischen Chemotherapie, 1984b, Band 3-5 S,

493-508.

Smith, J. T.

European Journal of Clinical Microbiology, 1984c, **3**,

347-350.

Smith, J. T.

Scandinavian Journal of Infectious Diseases, 1986a,

Supplement 49, 115-123.

Smith, J. T.

Journal of Antimicrobial Chemotherapy, 1986b, **18**

Supplement D, 21-29.

Smith, J.T.

Arzneimittel-Forschung/ Drug Research, 1990a, **40**,

65-68.

Smith, J. T.

In: *The 4-quinolones: Antibacterial Agents* in vitro
(Ed. G. C. Crumplin), Springer-Verlag, London, 1990b,
Chapter 14.

Smith, J. T., Ratcliffe, N. T.

Infection, 1986, **14** Supplement 1, 31-35.

Smith, S.M., Eng, R.H.K., Bais, P., Fan-Havard, P.,
Tecson-Tumang, F.

Journal of Antimicrobial Chemotherapy, 1990, **26,**
567-572.

Snyder, M., Drlica, K.

Journal of Molecular Biology, 1979, **131,** 287-302.

Sreedharan, S., Oram, M., Jensen, B., Peterson. L. R.,
Fisher, L. M.

Journal of Bacteriology, 1990, **172,** 7260-7262.

Sreedharan, S., Peterson, L. R., Fisher, L. M.

Antimicrobial Agents and Chemotherapy, 1991, **35,**
2151-2154.

Srivenugopal, K. S., Lockshon, D., Morris, D. R.

Biochemistry, 1984, **23,** 1899-1906.

Staudenbauer, W. L., Orr, E.

Nucleic Acids Research, 1981, **9,** 3589-3602.

Steck, T. R., Drlica, K.

Cell, 1984, **36,** 1081-1088.

Steen, H. J., Scott, E. M., Stevenson, M. I., Black, A. E.,
Redmond, A. O. B., Collier, P. S.

Journal of Antimicrobial Chemotherapy, 1989, **24,**
787-795.

Stein, D. C., Danaher, R. J., Cook, T. M.

Antimicrobial Agents and Chemotherapy, 1991, **35,**
622-626.

Sternglanz, R., DiNardo, S., Voelkel, K. A., Nishimura, Y.,
Hirota, Y., Becherer, K., Zumstein, L., Wang, J. C.

Proceedings of the National Academy of Sciences of the
United States of America, 1981, **78,** 2747-2751.

Sugino, A., Bott, K. F.

Journal of Bacteriology, 1980, **141,** 1331-1339.

Sugino, A., Higgins, N. H., Brown, P. O., Peebles, C. L.,
Cozzarelli, N. R.

Proceedings of the National Academy of Sciences of the
United States of America, 1978, **75,** 4838-4842.

Sugino, A., Peebles, C.L., Kreuzer, K.N., Cozzarelli, N.R.

Proceedings of the National Academy of Sciences of the
United States of America, 1977, **74,** 4767-4772.

Takahata, M., Nishino, T.

Antimicrobial Agents and Chemotherapy, 1988, **32,**
1192-1195.

Tanaka, M., Otsuki, M., Une, T., Nishino, T.

Journal of Antimicrobial Chemotherapy, 1990, **26,**
659-666.

Tennent , J. M., Young H. K., Lyon, B. R., Amyes, S. G. B.,
Skurray, R. A.

Journal of Medical Microbiology, 1988, **26,** 67-73.

Tolkoff-Rubin, N. E., Rubin, R. H.

American Journal of Medicine, 1987, **82** Supplement 4A,
270-277.

Tornaletti, S., Pedrini, A. M.

Biochimica et Biophysica Acta, 1988, **949**, 279-287.

Trexler Hessen, M., Ingerman, M. J., Kaufman, D. H., Weiner, P., Santoro, J., Korzeniowski, O. M., Boscia, J., Topiel, M., Bush, L. M., Kaye, D., Levison, M. E.

American Journal of Medicine, 1987, **82** Supplement 4A, 262-265.

Tse, Y. C., Wang, J. C.

Cell, 1980, **22**, 269-276.

Tse, Y. K., Kirkegaard, K., Wang, J.C.

Journal of Biological Chemistry, 1980, **255**, 5560-5565.

Tse-Dinh, Y.-C., Beran, R. K.

Journal of Molecular Biology, 1988, **202**, 735-742.

Ubukata, K., Itoh-Yamashita. N., Konno, M.

Antimicrobial Agents and Chemotherapy, 1989, **33**, 1535-1539.

Valainis, G.T., Pankey, G.A., katner, H.P., Cortez, L.M., Dalovisio, J.R.

American Journal of Medicine, 1987, **82** Supplement 4A, 230-232.

Vincent, S., Glauner, B., Gutmann, L.

Antimicrobial Agents and Chemotherapy, 1991, **35**, 1381-1385.

Vosberg, H.-P.

Current Topics in Microbiology and Immunology, 1985, **114**, 19-102.

Wahle, E., Kornberg, A.

   European Molecular Biology Organisation Journal, 1988,

   7, 1889-1895.

Walters, R. N., Piddock, L. J. V., Wise, R.

   Journal of Antimicrobial Chemotherapy, 1989, **24**,

   863-873.

Wang, J. C.

   Journal of Molecular Biology, 1969a, **43**, 263-272.

Wang, J.C.

   Journal of Molecular Biology, 1969b, **43**, 25-39.

Wang, J. C.

   Journal of Molecular Biology, 1971, **55**, 523-533.

Wang, J. C.

   Journal of Biological Chemistry, 1991, **266**, 6659-6662.

Wang, J. C., Liu, L. F.,

   In: *Molecular Genetics*, Part III (Ed. J. H. Taylor)

   Academic Press, New York, 1979, 65-88

Watanabe, M., Inoue, M., Mitsuhashi, S.

   Antimicrobial Agents and Chemotherapy, 1989, **33**,

   1837-1840.

Weisser, J., Wiedemann, B.

   Antimicrobial Agents and Chemotherapy, 1987, **31**,

   531-534.

Wiedemann, B., Zühlsdorff, M. T.

   American Journal of Medicine, 1989, **87** Supplement 5A,

   9-11.

Wigley, D.B., Davies, G.J., Dodson, E.J., Maxwell, A.,

Dodson, G.

   Nature, 1991, **351**, 624-629.

Wise, R., Andrews, J. M., Edwards, L. J.

Antimicrobial Agents and Chemotherapy, 1983, **23,**

559-564.

Wise, R., Donovan, I. A.

American Journal of Medicine, 1987, **82** Supplement 4A,

103-107.

Wise, R., Andrews, J. M., Ashby, J. P., Matthews, R. S.

Antimicrobial Agents and Chemotherapy, 1988, **32,**

617-622.

Wise, R., Lister, D., McNulty, C. A. M., Griggs, D.,
Andrews, J. M.

Journal of Antimicrobial Chemotherapy, 1986, **18**

Supplement D, 71-81.

Wolfson, J. S., Hooper, D. C., Swartz, M. N., McHugh, G. L.

Journal of Bacteriology, 1982, **152,** 338-344.

Wolfson, J. S., Hooper, D. C.

European Journal of Clinical Microbiology and

Infectious Diseases, 1991, **10,** 267-274

Wood, M. J., Logan, M. N.

Journal of Antimicrobial Chemotherapy, 1986, **18**

Supplement D, 159-164.

Worcel, A.

In: Mechanism and Regulation of DNA Replication. (Eds.

A. R. Kolber and M. Kohiyama), Plenum Press, New York,

1974, 201-224.

Wu, H.-Y., Shyy, S., Wang, J. C., Liu, L. F.

Cell, 1988, **53,** 433-440.

Yamano, Y., Nishikawa, T., Komatsu, Y.

Journal of Antimicrobial Chemotherapy, 1990, 26,

175-184.

Yang, Y., Ferro-Luzzi Ames, G.

Proceedings of the National Academy of Sciences of the

United States of America, 1988, **85**, 8850-8854.

Yoshida, H., Kojima, T., Yamagishi, J.-I., Nakamura, S.

Molecular and General Genetics, 1988, **211**, 1-7.

Yoshida, H., Bogaki, M., Nakamura, M., Nakamura, S.

Antimicrobial Agents and Chemotherapy, 1990a, **34**,

1271-1272.

Yoshida, H., Nakamura, M., Bogaki, M., Nakamura, S.

Antimicrobial Agents and Chemotherapy, 1990b, **34**,

1273-1275.

Yoshida, H., Bogaki, M., Nakamura, S., Ubukata, K., Konno,
M.

Journal of Bacteriology, 1990c, **172**, 6942-6949.

Yoshida, H., Bogaki, M., Nakamura, M., Yamanaka, L.M.,
Nakamura, S.

Antimicrobial Agents and Chemotherapy, 1991a, **35**,

1647-1650.

Yoshida, S., Kojima, T., Inoue, M., Mitsuhashi, S.

Antimicrobial Agents and Chemotherapy, 1991b, **35**,

368-370.

Zweerink, M. M., Edison, A.

Antimicrobial Agents and Chemotherapy, 1986, **29**,

598-601.

EFFECT OF TEMPERATURE ON BACTERIAL MUTATIONAL CIPROFLOXACIN RESISTANCE

A.C Parte and J.T. Smith, Microbiology Section, Department of Pharmaceutics, The School of Pharmacy, University of London, Brunswick Square, London WC1N 1AX.

The commonest mechanism causing clinical resistance of bacteria to antibiotics and chemotherapeutic agents is the possession of transferrable drug resistance plasmids. However, four exceptional antibacterials which do not suffer from plasmid-mediated resistance are polymyxin, nitrofurantoin, metronidazole and the 4-quinolones. With these four the only possible mechanism of clinical resistance for bacteria is chromosomal mutation. Consequently studies of the development of mutational resistance in vitro with such drugs have more clinical relevance than such studies with other antibacterials.

With E. coli mutational resistance to ciprofloxacin has been shown to occur more frequently at 30°C than at 25 or 37°C (Smith 1986). It was not known whether this effect was species-specific, so other bacteria were investigated as follows. Minimum inhibitory concentrations (MIC's) of ciprofloxacin were determined using 2 x 10^5-10^6 colony-forming units of Staph. aureus, Staph. epidermidis, Pseudomonas aeruginosa and E. coli inoculated on nutrient agar incubated at 25, 30 and 37°C. After 1 to 3 days (depending on the temperature) the lowest concentration of ciprofloxacin inhibiting colony formation was recorded. Bacterial cultures concentrated 20-fold by centrifugation were spread on nutrient agar containing 5 times the MIC of ciprofloxacin for each organism at each temperature and the plates incubated for up to 6 days. Colonies were counted and their frequency of occurrence used to calculate mutation rates.

	MUTATION RATES AT		
	25°C	30°C	37°C
Escherichia coli	1.75x10^{-8} (0.005)	2.88x10^{-8} (0.0075)	6.24x10^{-9} (0.02)
Staphylococcus aureus	2.96x10^{-8} (0.1)	4.80x10^{-8} (0.15)	1.57x10^{-8} (0.2)
Pseudomonas aeruginosa	1.42x10^{-7} (0.15)	1.38x10^{-7} (0.15)	1.07x10^{-7} (0.2)
Staphylococcus epidermidis	4.05x10^{-10} (0.4)	2.48x10^{-9} (0.3)	2.70x10^{-8} (0.2)

MIC's in mg/L are shown in parentheses

The results (Table) show that as before E. coli exhibited a temperature optimum for mutation to ciprofloxacin resistance as the frequency was greater at 30°C than at 25 or 37°C; a similar effect was seen with Staph. aureus. However, with Pseudomonas the mutation frequencies were similar at 25 and 30°C, but significantly higher than that at 37°C. With Staph. epidermidis quite different MIC results were seen and its mutation rates were also different being greatest at 37°C and progressively less at 30 and 25°C. Interestingly, mutants of all species isolated at 25 and 30°C grew at 37°C.

Skin surfaces exhibit temperatures less than 37°C and as mutational resistance occurred more frequently at such temperatures with three out of four species studied, perhaps ciprofloxacin may be more prone to therapeutic failure when used to treat skin infections caused by such bacteria. It may be relevant that the development of mutational ciprofloxacin resistance in patients occurs rarely with most bacteria but is significant with Staph. aureus and Pseudomonas aeruginosa, (Kresken and Weidmann 1988; Blumberg et al 1989) and both cause skin infections. It is also possible that mutational resistance may occur on fomites at room temperature.

Blumberg, H.M.,Rimland, D.,Wachsmuth, I.K. (1989) 29th ICAAC Abstract 7 page 102
Kresken, M., Weidemann, B. (1988) Antimicrob. Agents Chemother. 32:1285-1288
Smith, J.T. (1986) Scand J Inf Dis 49 (Suppl.):115-123

EFFECT OF TEMPERATURE ON BACTERIAL MUTATIONAL CIPROFLOXACIN RESISTANCE

A.C Parte and J.T. Smith, Microbiology Section, Department of Pharmaceutics, The School of Pharmacy, University of London, Brunswick Square, London WC1N 1AX.

The commonest mechanism causing clinical resistance of bacteria to antibiotics and chemotherapeutic agents is the possession of transferrable drug resistance plasmids. However, four exceptional antibacterials which do not suffer from plasmid-mediated resistance are polymyxin, nitrofurantoin, metronidazole and the 4-quinolones. With these four the only possible mechanism of clinical resistance for bacteria is chromosomal mutation. Consequently studies of the development of mutational resistance in vitro with such drugs have more clinical relevance than such studies with other antibacterials.

With E. coli mutational resistance to ciprofloxacin has been shown to occur more frequently at 30°C than at 25 or 37°C (Smith 1986). It was not known whether this effect was species-specific, so other bacteria were investigated as follows. Minimum inhibitory concentrations (MIC's) of ciprofloxacin were determined using 2 x 10^5-10^6 colony-forming units of Staph. aureus, Staph. epidermidis, Pseudomonas aeruginosa and E. coli inoculated on nutrient agar incubated at 25, 30 and 37°C. After 1 to 3 days (depending on the temperature) the lowest concentration of ciprofloxacin inhibiting colony formation was recorded. Bacterial cultures concentrated 20-fold by centrifugation were spread on nutrient agar containing 5 times the MIC of ciprofloxacin for each organism at each temperature and the plates incubated for up to 6 days. Colonies were counted and their frequency of occurrence used to calculate mutation rates.

	MUTATION RATES AT		
	25°C	30°C	37°C
Escherichia coli	1.75x10^{-8} (0.005)	2.88x10^{-8} (0.0075)	6.24x10^{-9} (0.02)
Staphylococcus aureus	2.96x10^{-8} (0.1)	4.80x10^{-8} (0.15)	1.57x10^{-8} (0.2)
Pseudomonas aeruginosa	1.42x10^{-7} (0.15)	1.38x10^{-7} (0.15)	1.07x10^{-7} (0.2)
Staphylococcus epidermidis	4.05x10^{-10} (0.4)	2.48x10^{-9} (0.3)	2.70x10^{-8} (0.2)

MIC's in mg/L are shown in parentheses

The results (Table) show that as before E. coli exhibited a temperature optimum for mutation to ciprofloxacin resistance as the frequency was greater at 30°C than at 25 or 37°C; a similar effect was seen with Staph. aureus. However, with Pseudomonas the mutation frequencies were similar at 25 and 30°C, but significantly higher than that at 37°C. With Staph. epidermidis quite different MIC results were seen and its mutation rates were also different being greatest at 37°C and progressively less at 30 and 25°C. Interestingly, mutants of all species isolated at 25 and 30°C grew at 37°C.

Skin surfaces exhibit temperatures less than 37°C and as mutational resistance occurred more frequently at such temperatures with three out of four species studied, perhaps ciprofloxacin may be more prone to therapeutic failure when used to treat skin infections caused by such bacteria. It may be relevant that the development of mutational ciprofloxacin resistance in patients occurs rarely with most bacteria but is significant with Staph. aureus and Pseudomonas aeruginosa, (Kresken and Weidmann 1988; Blumberg et al 1989) and both cause skin infections. It is also possible that mutational resistance may occur on fomites at room temperature.

Blumberg, H.M.,Rimland, D.,Wachsmuth, I.K. (1989) 29th ICAAC Abstract 7 page 102
Kresken, M., Weidemann, B. (1988) Antimicrob. Agents Chemother. 32:1285-1288
Smith, J.T. (1986) Scand J Inf Dis 49 (Suppl.):115-123