Studies on the Accumulation and Postantibiotic Effect of Fluoroquinolone Antimicrobials in Staphylococcus aureus

By

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

Department of Medical Microbiology

Faculty of Medicine

University of Manitoba

Winnipeg, Manitoba

ISBN 0-315-99104-6

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STUDIES ON THE ACCUMULATION AND POSTANTIBIOTIC EFFECT OF FLUOROQUINOLONE ANTIMICROBIALS IN *Staphylococcus aureus*

BY

ROSS J. DAVIDSON

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

DOCTOR OF PHILOSOPHY

To my wife Brenda for all her sacrifice, love and support

and

to my father, for encouraging me to pursue my dream.

Acknowledgment

I would like to express my sincere gratitude to my advisor, Dr. Daryl J. Hoban, for his guidance, support, and generosity. I thank my fellow graduate students, Dr. George Zhanel and James Karlowsky for their valuable input and for making the lab an enjoyable place to work and learn. Also thanks to fellow lab mates Lorraine Palatnick and Barb Weshnowesky. Thanks to Theresa Birkholz for all her valuable help. A special thank-you to Dr. Neil Simonsen and the boys of the "Worm Lab", Keith Fowke, Trevor Fudyk, and Blake Ball for their assistance.

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Abstract

The postantibiotic effect (PAE) is defined as the persistent suppression of bacterial growth following brief exposure to antimicrobial agents. Clinically, the PAE is important, given the implications of the PAE in influencing dosage regimens of antimicrobials. During the course of this thesis, the PAE was examined in both fluoroquinolone susceptible and resistant Staphylococcus aureus. Initial studies established the fluoroquinolone PAE to be a reproducible phenomenon in both methicillin susceptible (MSSA) and methicillin resistant (MRSA) S.aureus. The hypothesis that the PAE of fluoroquinolones in quinolone resistant S.aureus would be similar to their isogenic wild type counterparts was explored, however, several fluoroquinolone resistant S.aureus strains failed to produce a prolonged PAE when exposed to antimicrobial concentrations up to 10x their minimum inhibitory concentration (MIC). DNA sequence analysis of the gyrA, gyrB, and norA coding regions failed to detect mutations which might account for the observed loss of PAE.

Fluoroquinolone uptake into S.aureus cells was found to proceed via a non saturable energy independent passive diffusion process, however, studies with metabolic inhibitors established the presence of an energy dependent efflux system. Fluoroquinolone accumulation in the presence of these inhibitors was significantly increased. During the PAE phase, accumulation of fluoroquinolones were also significantly increased. Unlike actively growing cells, addition of metabolic inhibitors during the PAE phase did not significantly increase drug accumulation. This suggested that the norA efflux system is depressed during the PAE. Accumulation kinetic studies demonstrated that normal steady state levels of

fluoroquinolone accumulation did not return to pre-exposure kinetics for an average of 6 hours after the end of the PAE phase. This suggests that the duration of the PAE grossly underestimates the time required for bacterial cells to fully recover from the effects of antimicrobial exposure. Several fluoroquinolone resistant strains displayed reduced accumulation kinetics of fluoroquinolones confirming that permeability can also play a role in fluoroquinolone resistance. DNA sequence analysis detected two nucleotide substitutions in the norA promoter region of these strains. The mutations coincided with increased production or stability of norA transcripts as detected by Northern analysis and reduced intracellular accumulation of fluoroquinolones.

INTRODUCTION

1. Fluoroquinolones

a) History

The fluoroquinolones of the early 1980's, launched with the synthesis of norfloxacin, are not unlike other antimicrobial families, in that they are derived from chemical modifications of existing agents. The progenitor quinolone, nalidixic acid, now some 30 years old, was first described by Lesher et al in 1962 (Lesher et al,1962). Nalidixic acid itself is a derivative of the 1,8-naphthyridines; compounds obtained during chloroquine synthesis. Nalidixic acid, while active against a number of gram negative organisms, had little activity against Pseudomonas aeruginosa and gram positive organisms. The pharmacokinetic properties of nalidixic acid limited its utility in treating systemic infections although it did attain concentrations in urine sufficient to eradicate most gram negative organisms. Thus the primary clinical use of nalidixic acid was in the treatment of urinary tract infections (Siporin,1989). Soon after the introduction of nalidixic acid into clinical use, reports of toxicity, clinical failures, and the development of resistance were frequently documented. Nalidixic acid was soon replaced with other agents.

During the 1970's other quinolone agents were introduced including oxolinic acid, pipemidic acid and cinoxacin. Unfortunately these agents were only marginal improvements over nalidixic acid with oxolinic acid being approximately 4x more active than nalidixic acid and pipemidic acid having a significant (but not clinically relevant) improvement in activity against P.aeruginosa (Bryskier,1993).

The early 1980's saw the synthesis of norfloxacin, the first of the second generation quinolones. The addition of a 6 - fluorine atom and a 7 - piperazinyl ring gave rise to the agents now referred to as the fluoroquinolones, 4 - quinolones, carboxyquinolones, and the quinolone carboxylic acids. Norfloxacin had outstanding activity against the Enterobacteriaciae, having 50 - 100x more activity than nalidixic acid. More importantly, norfloxacin also had significant activity against P.aeruginosa and Staphylococcus aureus. Other second generation quinolones were soon to follow including ciprofloxacin, enoxacin, pefloxacin, and ofloxacin. From the time of its launch in the mid 1980's ciprofloxacin has been the "gold standard" by which all other potential fluoroquinolones have been measured. The attempt to develop fluoroquinolones with better oral availability, greater gram positive activity and activity against anaerobes has led to a plethora of third generation compounds including lomefloxacin, temafloxacin, tosufloxacin, sparfloxacin, and BAY y3118.

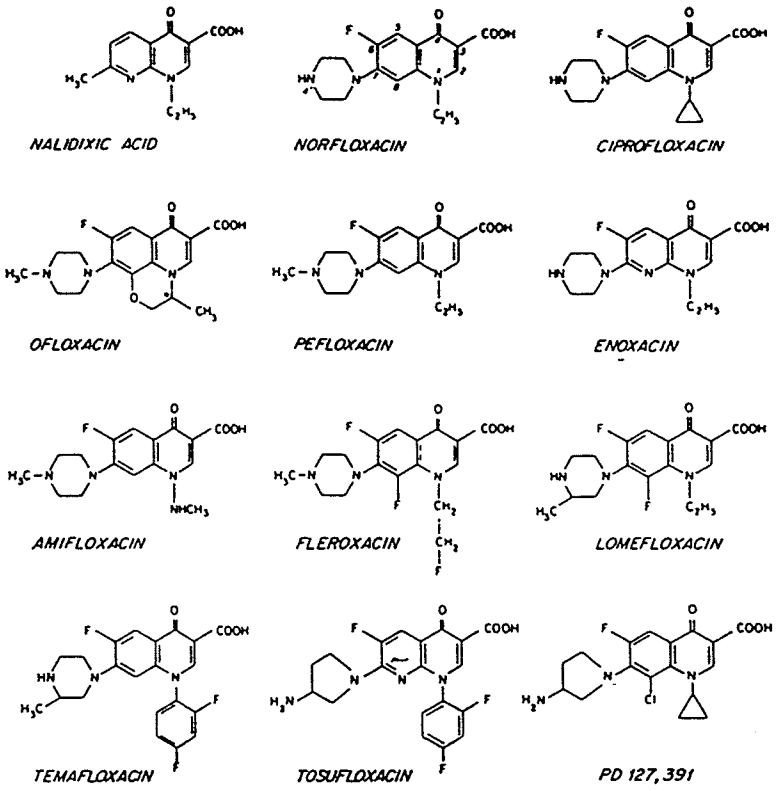
b) Structure

As stated previously, the basic fluoroquinolone structure is based on the much older quinolone analogs such as nalidixic acid and oxolinic acid. The two primary features or modifications that distinguish the two are the addition of a fluorine atom at position 6 and a piperazinyl or pyrrolidinyl substitution at position 7 of the quinolone nucleus (Wolfson and Hooper, 1989). Figure 1. depicts the structures of nalidixic acid and several of the second and third generation fluoroquinolones.

The fluoroquinolones are not large molecules. The typical molecular weight of these compounds is generally in the range of 300 - 500 daltons. Because of their

ionisable functional groups, the ionic and non-ionic states of the fluoroquinolone molecules depend on the pH of the surrounding environment. At neutral pH the majority of the fluoroquinolones are present in two forms; 90% zwitterionic form and 10% non-dissociated acid form (Bryskier,1989). These two characteristics are primarily responsible for the excellent cell penetration exhibited by most fluoroquinolones.

Figure 1. The structures of nalidixic acid and several second and third generation fluoroquinolones.

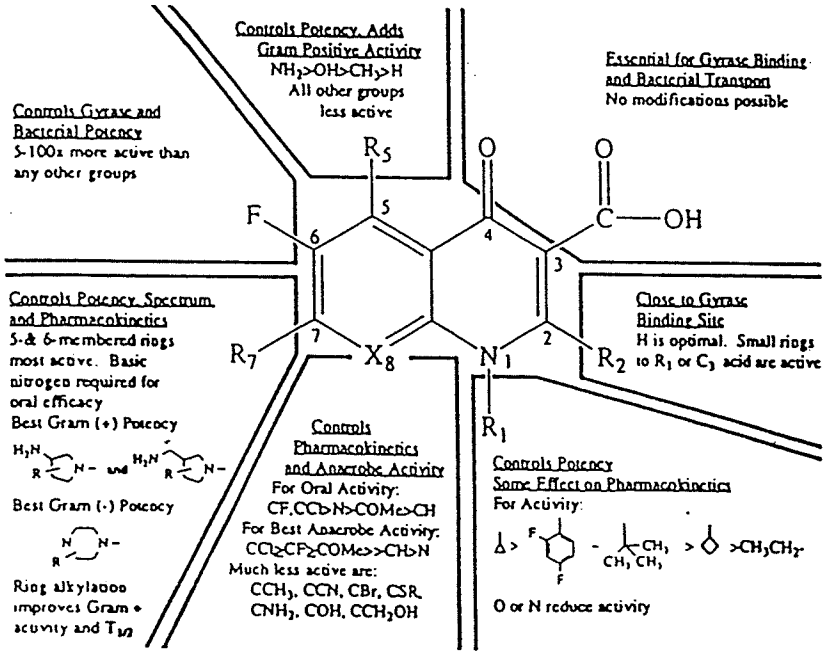


The structure activity relationships of the fluoroquinolones have and are being extensively studied. Modifications to existing structures have been shown to result in wider antibacterial spectrums, increased gram positive activity, greater oral availability,

and anaerobic activity. For example the replacement of the 7 - piperazinyl group in ciprofloxacin with a 7 - [3' - (ethylamino) methyl] - pyrrolidinyl group results in a 7.5 fold increase in activity against S.aureus and a 16 fold increase in activity against Streptococcus pyogenes. The addition of an amino group at position C-5 further augments these activities another 2 fold (Wolfson and Hooper,1989).

Figure 2. summarizes a number of the fluoroquinolone antibacterial structure activity relationships (Domagala,1994). Positions 2,3,4, and 6 of the fluoroquinolone molecule are extremely important for antibacterial potency as they all have roles mediating binding of the molecule to the DNA gyrase complex. The 3 - carboxylate and the 4 - carbonyl groups are absolutely essential in this regard. R-2 is generally an H atom and the fluorine atom alone can increase activity 100 fold. Very few modifications are made at these positions as most have been shown to decrease antibacterial activity.

Figure 2. Summary of quinolone antibacterial structure activity relationships.



Substitution at positions C-5 and C-7 have been shown to increase both gram positive activity as well as the entire antibacterial spectrum. Modifications at these positions can also affect serum half-life. Position X-8 primarily controls *in-vivo* efficacy. Substitution at this position with groups such as CF, CCl, or COMe can expand the antibacterial spectrum to include activity against anaerobes (Domagala,1994).

c) Antibacterial activity:

Unlike the first quinolone agents which were hampered by a narrow spectrum of activity limited to aerobic gram negative bacteria, the newer fluoroquinolones are broad spectrum bactericidal agents. These new agents not only demonstrate greater potency against common gram negative bacteria but also provide activity against P.aeruginosa and various gram positive organisms such as S.aureus (Eliopoulos and Eliopoulos,1993). In addition, the fluoroquinolones demonstrate good activity against fastidious gram negative bacteria such as Haemophilus influenzae and sexually transmitted organisms such as Chlamydia trachomatis (Wolfson and Hooper,1989; Danger et al,1988; Hoppe and Simon,1990). Activity against Rickettsia spp., Coxiella burnetti, and the malarial parasite Plasmodium falciparum have been reported (Divo et al,1988;Raoult et al,1987, Yeaman et al,1987). Table 1. lists the susceptibilities of many clinically relevant gram negative and gram positive organisms. The minimum bactericidal concentration (MBC) is generally within 2 dilutions of the MICs. When susceptible enteric organisms are exposed to fluoroquinolones at two to four times the MIC, a 3 $\log_{10}$ reduction in the viable count is usually observed within two hours. Often the bactericidal effects are seen within one hour of exposure (Eliopoulos and

Table 1. The *in-vitro* activities of selected fluoroquinolones.

	MIC_{90s} (µg / ml)						
Organism	Cfx♦	Nfx	Pfx	Lfx	Ofx	Tfx	Sfx
Gram negative							
Escherichia coli	0.06	0.12	0.12	0.2	0.12	0.12	0.06
Klebsiella pneumoniae	0.12	0.5	1.0	1.0	0.25	1.0	0.12
Enterobacter cloacae	0.12	0.5	0.5	0.5	0.25	0.5	0.25
Salmonella spp.	0.06	0.12	0.12	0.12	0.06	0.12	0.06
Pseudomonas aeruginosa	0.5	2.0	2.0	2.0	2.0	2.0	0.5
Haemophilus influenzae	0.008	---	---	0.06	0.06	0.06	0.06
Legionella pneumophila	0.12	---	---	0.06	0.06	0.12	0.06
Gram positive							
Staphylococcus aureus	0.5	2.0	0.5	2.0	0.5	0.25	0.12
Staphylococcus epidermidis	0.5	2.0	1.0	2.0	1.0	0.25	0.12
Streptococcus pneumonia	2.0	16.0	8.0	8.0	2.0	1.0	0.5
Enterococcus faecalis	2.0	8.0	8.0	8.0	4.0	2.0	1.0

Table 1. Continued

Organism	MIC_{90s} (µg / ml)						
	Cfx	Nfx	Pfx	Lfx	Ofx	Tfx	Sfx
Others							
Mycoplasma pneumoniae	2.0	---	4.0	4.0	1.0	2.0	0.1
Chlamydia trachomatis	2.0	16.0	2.0	2.0	1.0	0.25	0.06
Ureaplasma urealyticum	2.0	16.0	8.0	4.0	2.0	4.0	0.5
Mycobacterium tuberculosis	1.0	16.0	4.0	---	2.0	1.0	0.12

♦ Cfx - ciprofloxacin, Nfx - norfloxacin, Pfx - pefloxacin, Lfx - lomefloxacin, Ofx - ofloxacin, Tfx - temafloxacin, Sfx - sparfloxacin.

Eliopoulos,1993). The rate of killing in gram positive bacteria however has generally been slower than that of gram negative bacteria. A number of factors can reduce the activity of the fluoroquinolones. In vitro activity has been shown to be reduced under anaerobic conditions, acidic pH, excessively large inocula, and high concentrations of divalent cations. With limited options currently available for the treatment of many infections, interest in the fluoroquinolones as alternative chemotherapeutic agents has dramatically increased.

2) Mechanisms of action

a) Fluoroquinolone entry into the bacterial cell

Before any antimicrobial can exert its full antibacterial effects it must first gain access to its site of action or target site. The target site for most antimicrobial agents including the fluoroquinolones is found within the bacterial cell. Therefore uptake of antimicrobial agents into the bacterial cell can serve as an important factor in determining the activity and antibacterial spectrum of a given antimicrobial agent.

At the time this work began a certain degree of controversy existed in the literature regarding the exact mechanism of fluoroquinolone entry and accumulation in the bacterial cell (Bedard et al,1987; Chapman and Georgopapadakou,1988; Chapman and Georgopapadakou,1989; Cohen et al,1988; Diver,1989; Diver et al,1990; Kotera et al,1991; Piddock et al,1990). As well, the majority of investigators concentrated primarily on the uptake of fluoroquinolones in gram negative bacteria. This introduction will therefore limit itself to a brief review of this work, leaving the most recent investigations to be reviewed along with my findings in the discussion section.

Most of the earlier work concentrated on the uptake kinetics of fluoroquinolones in E.coli. In 1987, Bedard et al examined the uptake of ^{14}C-enoxacin in E.coli . Their results indicated that the uptake of enoxacin was not saturable even at concentrations as high as 150 μg/ml. Competition experiments using unlabelled ciprofloxacin at various ciprofloxacin:enoxacin ratios did not modify enoxacin uptake further supporting the unsaturable nature of uptake. Experiments performed at 4°C clearly demonstrated the rate of enoxacin uptake was decreased at this temperature. A number of metabolic inhibitors (sodium azide [NaN_3], carbonyl cyanide m-chlorophenylhydrazone [CCCP], 2,4-dinitrophenol [2,4-DNP], sodium arsenate, and sodium fluoride) were employed to determine their effect. None of the inhibitors had any effect on enoxacin uptake. Given the above results, the authors concluded that enoxacin uptake must be an energy independent passive diffusion process. The investigators then examined the role of the outer membrane and more specifically the role of several outer membrane proteins (Omp) in fluoroquinolone uptake. Accumulation of enoxacin was studied in E.coli mutants deficient in Omp F, Omp C, or both. Little difference in quinolone uptake was observed in Omp C deficient mutants, however Omp F deficient mutants had significantly decreased quinolone uptake as well as a corresponding two fold increase in enoxacin MICs. The uptake of enoxacin was also examined in Bacillus subtilis with similar findings to those reported for E.coli.

Consistent with the hypothesis that Omp F was responsible for fluoroquinolone permeation through the outer membrane of E.coli, Chapman and Georgopapadakou

reported that MICs of fleroxacin in Omp F deficient mutants were increased (Chapman and Georgopapadakou,1988). The role of divalent cations and in particular magnesium was examined in fleroxacin uptake. They discovered the uptake of fleroxacin was decreased and its MIC was increased in the presence of magnesium. Chapman and Georgopapadakou (1988), proposed that fluoroquinolones interact with the outer membrane as chelating agents. This hypothesis was based on the observation that outer membrane perturbations typical of those seen with gentamicin and EDTA were seen in quinolone treated cells. Aminoglycosides and EDTA are known to expose the lipid bilayer by displacing and chelating divalent cations respectively (Hancock,1984; Leive,1968). The afore mentioned quinolone induced alterations, in addition to the antibacterial action of fleroxacin, was abrogated or prevented by the addition of magnesium. Finally, the authors proposed that in addition to the porin pathway, quinolones may gain access into the cell via a non-porin "self promoted" pathway similar to that of aminoglycosides.

In 1988 while studying norfloxacin transport in E.coli, Cohen et al discovered an endogenous energy dependent efflux system. This was the second such efflux system described for antimicrobials, the first being described for the tetracyclines (McMurry et al,1983). The fluoroquinolone efflux system was, however, the first described specific for a fully synthetic antimicrobial. The genes encoding the tet efflux system are known to be plasmid mediated, however the fluoroquinolone system appears to be chromosomally controlled. The authors noted that norfloxacin rapidly diffused into energy depleted cells, but accumulation was significantly decreased in energized cells.

They further characterized the apparent efflux system using everted inner membrane vesicles. Results suggested that the efflux system was carrier mediated and was driven by proton motive force. Other hydrophilic but not hydrophobic quinolones competed with norfloxacin for transport. The authors concluded that decreased permeability caused by Omp F deficient mutants coupled with active efflux may permit the emergence of strains resistant to fluoroquinolones.

In 1990, Diver et al described a biphasic pattern of fluoroquinolone uptake in E.coli. Accumulation began with an initial rapid phase followed by steadily increasing accumulation over thirty minutes. They also reported that there was no direct relationship between quinolone accumulation and antibacterial activity. Consistent with other investigators, they described reduced uptake of fluoroquinolones at low temperature and acid pH. Studies performed in the presence of metabolic inhibitors (2,4-DNP, potassium cyanide [KCN], and sodium azide [NaN_3]) indicated that fluoroquinolone accumulation was reduced. This contradicted some earlier reports and suggested that uptake is partly dependent on cell metabolism and may proceed via an active transport mechanism.

b) Fluoroquinolone accumulation into S.aureus:

One of the first reports comparing the uptake of fluoroquinolones (^{14}C - lomefloxacin) in E.coli and S.aureus was published in 1990 by Piddock et al. The accumulation pattern between the two organisms were shown to be similar suggesting the mechanism of cell entry may be similar in both gram positive and gram negative cells. In both species saturation of the uptake system with lomefloxacin was not

observed.

Okuda et al (1991), examined the accumulation of ciprofloxacin and sparfloxacin in three strains of S.aureus, FDA209-P (fluoroquinolone susceptible), mutant 19 and 6171 (fluoroquinolone resistant). They observed that the fluoroquinolone susceptible isolate, FDA209-P, accumulated both ciprofloxacin and sparfloxacin to a higher level than did the fluoroquinolone resistant strains. The observed difference in quinolone accumulation between the susceptible and resistant strains was, however, abolished with the addition of CCCP. The authors state that these results suggest that energy dependent reduction systems exist in both the fluoroquinolone resistant strains.

Yoshida et al (1991), examined the accumulation of norfloxacin and sparfloxacin in both susceptible and resistant strains of S.aureus. Initial experiments with their susceptible strain (MS16008) demonstrated that accumulation increased in a linear fashion with increasing concentrations of drug and that accumulation was not saturable at concentrations as high as 80 μg/ml. They reported that binding of both quinolones appeared to be reversible as 80% and 90% of cell associated norfloxacin and sparfloxacin, respectively, were removed by two additional washings. Plateau levels of both agents was achieved within five minutes, however levels of sparfloxacin were approximately six-fold higher in this strain. The norfloxacin resistant, sparfloxacin susceptible strain, (MS16401), showed less accumulation of norfloxacin than in the susceptible strain, however, sparfloxacin levels were comparable. S.aureus strain NMS54, resistant to both agents, showed decreased accumulation of both agents. The addition of CCCP significantly increased the accumulation of both agents in all three

strains suggesting the presence of an energy dependent efflux system. The authors were also able to establish a relationship between the antibacterial activities of the two agents and the amount of quinolone accumulation in these strains.

Piddock and Zhu, 1991, examined the accumulation of sparfloxacin in members of the family Enterobacteriaceae, P.aeruginosa and S.aureus using three different methods. The first method, a fluorometric technique, was found to be unsuitable because the sparfloxacin molecule fluoresced poorly resulting in unreliable data. The second method, a vacuum filtration technique, was also found to be unsuitable because sparfloxacin bound to the filters resulting in an unacceptable background. A third technique based on the partitioning of cells in silicon oil, centrifugation, and scintillation counting was adopted.

The authors found that all strains tested with the exception of P.aeruginosa rapidly accumulated sparfloxacin, with high levels of the drug accumulated by the Staphylococci. They also demonstrated that the steady state levels of sparfloxacin were reduced threefold in the presence of the cation magnesium chloride (7M solution). Steady state levels of ciprofloxacin were not as high as sparfloxacin in both S.aureus and S.epidermidis. Accumulation experiments performed with fluoroquinolone resistant mutant S.aureus demonstrated no change in the accumulation of quinolones.

The authors concluded that sparfloxacin is accumulated to higher steady state levels than ciprofloxacin in Staphylococci and to higher overall levels than gram negative bacteria. The authors suggest that the inhibition of sparfloxacin accumulation

by magnesium ions may be the result of a sparfloxacin complex formed in the presence of these ions. This complex may be too bulky to diffuse through the cell envelope or that this agent uses a "self-promoted" pathway.

McCaffrey et al, 1992, examined the accumulation of numerous fluoroquinolones, (fleroxacin, pefloxacin, norfloxacin, difloxacin, A56620, ciprofloxacin, ofloxacin, and Ro 09-1168) in E.coli, P.aeruginosa, and S.aureus. Their results suggest that quinolones are taken up by a passive diffusion process, however, studies with metabolic inhibitors also suggest the presence of an endogenous energy dependent efflux system. The metabolic inhibitors CCCP and 2,4-DNP had a dramatic effect on the accumulation of quinolones in the gram negative species, increasing accumulation four fold, however, the same dramatic effect was not observed in S.aureus. Although the uptake assays were performed over a thirty minute period, maximal accumulation was achieved in approximately five minutes. McCaffrey et al, 1992, also observed a relationship between the relative hydrophobicity of the agents and the amount of fluoroquinolone accumulation. High quinolone hydrophobicity was associated with decreased uptake in the gram negative species but was associated with higher accumulation in S.aureus.

Bazile et al, 1992, examined the accumulation of eleven fluoroquinolone agents in E.coli, P.aeruginosa, and S.aureus. They observed a direct relationship between the hydrophobicity of the fluoroquinolone agents and the degree of accumulation in S.aureus. In addition their experiments demonstrated that while size of the quinolone agents was a limiting factor in gram negative species, this was not true in S.aureus.

The authors did not find any correlation between antibacterial activity and accumulation with the three microorganisms tested.

Furet et al, 1992, examined the uptake of pefloxacin into both intact S.aureus cells and protein free phosphatidylglycerol liposomes. Furet et al, 1992, confirmed that quinolones enter the bacterial cell via simple diffusion and also speculated that quinolone species with no net charge, (zwitterionic and uncharged species), have a predominant role in crossing the membrane. In addition their work suggested that negatively charged phospholipids facilitate quinolone diffusion across the membrane, while zwitterionic phospholipids are unlikely to be involved.

The most surprising result generated by this paper was the observation that CCCP could increase pefloxacin uptake in liposomes devoid of protein and energy production at both 30°and 4°C. Given these observations, the authors speculated that the CCCP effect in S.aureus may be the result of acidification of the internal bacterial pH with increased binding of the positive quinolone species to the inner leaflet of the cytoplasmic membrane. They also demonstrated that acidic outer pH optimized both the CCCP effect in whole cells and pefloxacin labeling of everted membrane vesicles. The authors do state however that their experiments were determined using a fluoroquinolone susceptible strain. They suggested that an endogenous active efflux is unlikely in quinolone susceptible strains but do not rule out its existence in resistant isolates.

The accumulation of four agents, rufloxacin, ofloxacin, fleroxacin and MF961, were studied in S.aureus, P.aeruginosa, and members of the family Enterobacteriaceae

by Piddock et al, 1993. The accumulation of all four agents was rapid with steady state concentrations achieved within five minutes. Higher steady state concentrations were achieved in S.aureus than in gram negative species with rufloxacin taken up at the highest concentrations. Interestingly, although rufloxacin was accumulated to the greatest degree, it was the least active agent in S.aureus suggesting that no correlation exists between uptake and antibacterial activity.

In 1993, Asuquo and Piddock published a study comparing the accumulation kinetics of 15 quinolone agents; nalidixic acid, eight mono-fluoroinated agents , three di-fluoroinated agents and three tri-fluoroinated agents in S.aureus, E.coli, and P.aeruginosa. The authors observed a biphasic pattern of accumulation for all agents studied with an initial rapid phase of uptake in the first ten seconds followed by a slow steady increase in quinolone accumulation over the next 50 minutes. The number of fluorine atoms on the quinolone nucleus did not appear to influence uptake of the agents as no correlation between the number of fluorine atoms and level of accumulation was observed. In addition, Asuquo and Piddock, 1993, were unable to correlate hydrophobicity and the degree of accumulation. Lastly, no correlation was found between the amount of quinolone accumulated and the antibacterial activity of the agents tested.

Denis and Moreau, 1993, examined the accumulation of sparfloxacin and three other fluoroquinolones of decreasing hydrophobicity, pefloxacin, ofloxacin, and ciprofloxacin in both fluoroquinolone susceptible and resistant clinical isolates of Enterobacteriaceae, P.aeruginosa, and S.aureus. These studies demonstrated the

importance of hydrophobicity on fluoroquinolone uptake. Sparfloxacin, the most hydrophobic agent studied, was accumulated in S.aureus to a higher concentration than the other quinolones. The opposite was seen in gram negative species with ciprofloxacin being accumulated the most and sparfloxacin the least. CCCP did not appear to have any effect on the accumulation of sparfloxacin in S.aureus in both susceptible and resistant cells.

In 1994, Marshall and Piddock examined the interaction of cations, quinolones and bacteria. While the majority of the work was performed using gram negative species, the authors do make some interesting observations with S.aureus. They observed that both Mg^{++} and Ca^{++} increased the MICs of quinolones in S.aureus. In addition the IC_{50}'s of the drugs were increased and correlated with the increase in the agents MICs. Steady state concentrations of quinolones were also reduced in the presence of cations. The authors end their paper with an interesting hypothesis. They speculate that there may be a direct antagonism by cations on the interaction between quinolones and DNA gyrase and suggest that studies be undertaken to test this hypothesis.

c) Topoisomerases, DNA gyrase and its interaction with fluoroquinolones:

Once the fluoroquinolones have penetrated the cell membrane and entered the bacterial cell they interact with their target site, the DNA-DNA gyrase complex. It is this interaction that is thought to be responsible for cell death. This section will review the functions of DNA gyrase as well as how fluoroquinolones interact with DNA, DNA gyrase and the DNA-DNA gyrase complex.

Deoxyribonucleic acid or DNA encodes genetic information, allows mutations

and recombination and serves as a template for semi-conservative replication and transcription (Hooper and Wolfson,1993; Watson et al,1987). The configuration of the DNA molecule, a linear double helical structure poses many problems. Firstly, the DNA exists in a highly condensed state within the bacterial cell. In a typical gram negative cell such as E.coli, the chromosome is a circular DNA molecule approximately 1,100 μm long. As the cell is typically only 1 to 2 μm long the DNA molecule is condensed approximately 1000 fold (Hooper and Wolfson,1993; Watson et al,1987; Kornberg and Baker,1991). Despite this, the DNA must still be able to replicate, segregate into daughter chromosomes, and allow transcription of individual genes (Hooper and Wolfson,1993).

With each turn of the DNA helix, which occurs on the average every 10.4 base pairs, two single strands are wrapped around each other. In a DNA molecule containing 4 million base pairs the strands are intertwined 400,000 times (Hooper and Wolfson,1993). This is the root of the second problem. The strands must unwind 400,000 times to allow semi-conservative replication.

Prokaryotes have a third unique problem in that negative supercoils are present in bacterial DNA. The slightly underwound state of bacterial DNA is thought to facilitate strand separation required for DNA replication and initiation of transcription (Hooper and Wolfson,1993; Kornberg and Baker,1991).The bacterial cell must expend energy to generate these as negative supercoils are energetically unfavorable.

Lastly, another problem arises during the transcription of certain genes by RNA polymerase. For constrained segments of DNA, the tracking of RNA polymerase along

the helical DNA template generates positive supercoils ahead of and negative supercoils behind the enzyme (Hooper and Wolfson,1993; Watson et al,1987; Kornberg and Baker,1991; Sutcliffe et al,1989).

A class of enzymes known as the topoisomerases have evolved in both prokaryotes and eukaryotes to solve many of the above problems. DNA topoisomerases are enzymes that control and modify the topological states of DNA (Wang,1985). There are two recognized types of topoisomerases; type I topoisomerases act by transiently breaking a DNA strand and passing another strand through the transient break. Type II topoisomerases act in a similar fashion but transiently break a pair of complementary strands and pass another double stranded segment through the transient break (Wang,1985). Bacteria contain 4 topoisomerase enzymes (I - IV). Topoisomerase I and III are classified as type I enzymes where as topoisomerase II and IV are classified as type II enzymes. Table 2. lists the topoisomerases identified to date in E.coli.

Table 2. Characterized topoisomerases in E.coli.

Topoisomerase	Type	Subunit	Genes
I	I	TopA	topA
II (DNA gyrase)	II	GyrA	gyrA
		GyrB	gyrB
III	I	TopB	topB
IV	II	ParC	parC
		ParE	parE

Topoisomerase I first isolated from E.coli in 1969 (Wang,1971) is a type I enzyme. It is a 110 Kda protein encoded for by the topA gene (Hooper and Wolfson,1993; Truckis et al,1981). Found in both prokaryotes and eukaryotes, it catalyzes the removal of negative supercoils from DNA in the absence of ATP (Wang,1971). In bacteria, topoisomerase I acts in concert with DNA gyrase to regulate the levels of negative supercoiling of intracellular DNA and is required for the transcription of certain operons (Drlica,1984; Reece and Maxwell,1991).

Topoisomerase III, another type I enzyme was first characterized in E.coli by Srivenugopal et al (Srivenugopal et al,1984). It is also able to remove negative supercoils in the absence of ATP.The enzyme is encoded for by the topB gene. Topoisomerase III does not appear to be an essential enzyme,however,the observation that topB deletion mutants display an increased frequency of spontaneous DNA deletions suggests that it may have a role in DNA recombination(Wang et al,1990).

Little is known about the function of topoisomerase IV *in vivo*. This enzyme, a type II topoisomerase, is encoded by the parC and parE genes (Kato et al,1990). *In vitro* , the purified enzyme has been shown to catalyze the ATP dependent relaxation of negative and positively supercoiled DNA and the unknotting of un-nicked duplex DNA. While its role is unclear, studies with conditional lethal mutants indicate that the parC and parE genes are essential.

Topoisomerase II or DNA gyrase, the intracellular target of fluoroquinolones, is a type II enzyme first isolated from E.coli by Gellert et al in 1976. DNA gyrase was unique in that unlike other topoisomerase enzymes it had the ability to catalyze, using

ATP, the introduction of negative superhelical turns into closed-circular double stranded DNA.

DNA gyrase has now been cloned from a variety of organisms including E.coli, K.pneumoniae, S.aureus, B.subtilis, C.jejuni, and most recently P.aeruginosa. The enzyme is encoded by two genes, gyrA and gyrB. In the majority of organisms, E.coli being an exception, gyrA and gyrB are found contiguously within the genome. In S.aureus, the recF, gyrB, and gyrA genes lie in the same open reading frame separated by only 9 and 36 base pairs sequentially. The absence of obvious termination signals for recF and gyrB and promoter functions for gyrA suggest that the three genes form an operon transcribed as a polycistronic transcript (Margerrison et al,1992). DNA gyrase is a tetramer composed of two A subunits coded for by gyrA and two B subunits coded for by gyrB. In S.aureus the A subunit has an approximate molecular weight of 99 Kda and the B subunit is approximately 73 Kda (Brockbank and Barth,1993). Thus the A_2B_2 holoenzyme has a predicted molecular weight of approximately 344 Kda. Both subunits appear to be required for the enzyme to function, although each subunit has a unique role. The A subunits form a transient double stranded break in DNA by linking to each complementary strand via Tyr-122 (Horowitz and Wang,1987). A second DNA duplex is then passed through the gyrase-DNA gate in a process coupled to ATP hydrolysis by the B subunits.

DNA gyrase is capable of performing a number of topological interconversions of DNA molecules (Table 3.). It is capable of the ATP dependent negative supercoiling of closed circular double stranded DNA, a feature unique to this enzyme. DNA gyrase

Table 3. The Reactions of DNA Gyrase:

	Subunits req.	ATP req.	Inhib by quinolones
Supercoiling	A/B	Yes	Yes
Relaxation	A/B	No	Yes
-ve supercoils*	A/B	Yes	Yes
+ve supercoils*	A/B	Yes	Yes
Catenation	A/B	Yes	Yes
Decatenation	A/B	Yes	Yes
Unknotting	A/B	Yes	Yes
DNA cleavage	A/B	No	No
ATPase	B	Yes	Yes

* template dependent

can relax negative supercoiled DNA in an ATP dependent manner and relax positively supercoiled DNA in an ATP independent fashion. Other observed functions include the formation and resolution of catenated DNA and the resolution of knotted DNA.

DNA gyrase is the primary target of the fluoroquinolones. The most compelling evidence in support of this is the existence of single point mutations in the gyrA gene which confer high level resistance to fluoroquinolones (Fasching et al,1991;Sreedharan et al,1991; Goseitz et al,1992). An important effect of quinolones on DNA gyrase occurs after site-specific cleavage of DNA by the enzyme. In this reaction, staggered single strand breaks on both strands are introduced four base pairs apart on a DNA duplex and the GyrA subunit is covalently attached to the 5' single stranded ends at

the cleavage site. Covalent linkage occurs at Tyr-122 of the GyrA subunit. All the DNA breaks are found within a 120-150 base pair region that binds to the enzyme. Quinolones are thought to stabilize the gyrase mediated DNA breaks leading to a rapid cessation of DNA synthesis. That is; quinolones are thought to trap the cut intermediate by stabilizing the enzyme-DNA complex.

It should be noted that although DNA gyrase is considered to be the primary target of fluoroquinolones, these agents do not bind specifically to the enzyme. Shen and Pernet found that quinolones did not bind to DNA gyrase but in fact bind to DNA (Shen and Pernet,1985). They also demonstrated that quinolones prefer to bind single stranded DNA and only bind double stranded DNA in a weak non specific manner. However fluoroquinolone binding to a relaxed double stranded DNA substrate can be induced by the addition of DNA gyrase. This finding lead Shen and colleagues to suggest that the binding of DNA gyrase to the DNA substrate created a site that allowed the drug to bind in a cooperative manner (Shen et al,1989). Further experimentation provided evidence to suggest that the bound enzyme induced a drug binding site on the relaxed DNA molecule and that the binding site was formed during the gate-opening step. The separated short, single stranded DNA segments form a simulated denatured DNA bubble that is an ideal site for the drug to bind (Shen et al,1989; Shen,1993). Their working model is depicted in figure 3.

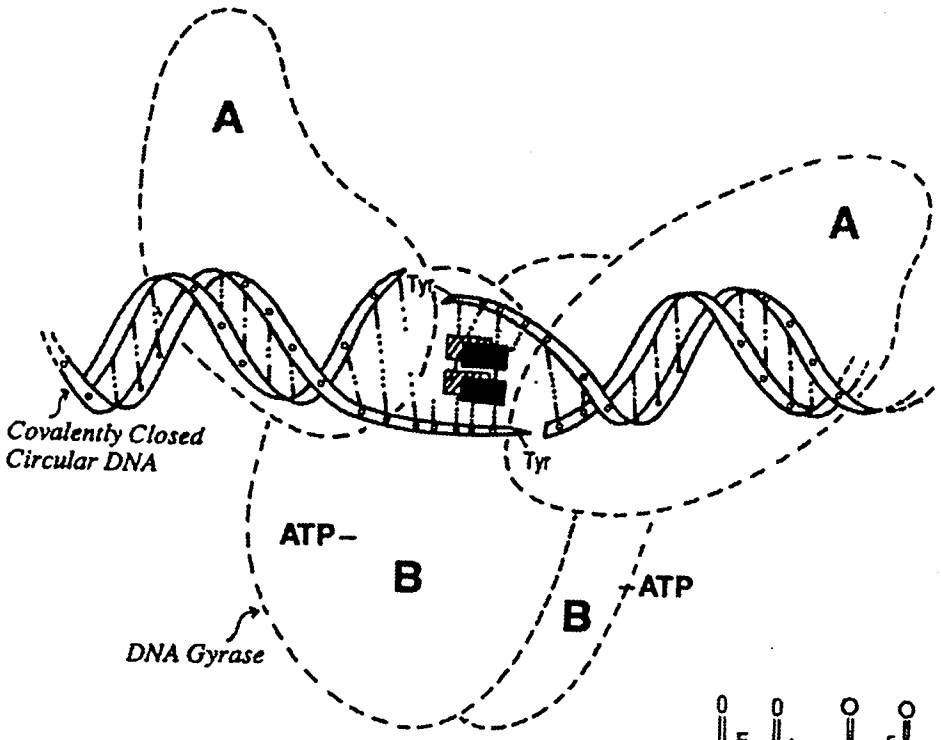
3. Fluoroquinolone Resistance

Since the advent of chemotherapeutics in the treatment of microbial infections, the development of resistance has always been a concern. Today, however, common

clinical pathogens are rapidly becoming resistant to many agents once considered first line therapy. The situation is becoming so precarious that some experts have predicted that we are entering a phase similar to the pre-antibiotic era for certain bacterial infections. Bacteria generally use one or more of three different techniques to acquire resistance to antimicrobial agents. They can limit access of the drug to its site of action via decreased permeability (or efflux), the drug can be modified or destroyed, or the target site can be modified.

The frequency of resistance to fluoroquinolone agents differs greatly among these agents. The older agents such as nalidixic acid had a selection frequency of approximately 10^{-7-8} while the newer agents such as ciprofloxacin and ofloxacin select resistant mutants with frequencies in the range of 10^{-9} to 10^{-11}. To date, no evidence exists to suggest that bacteria are capable of modifying or destroying fluoroquinolone agents but much data has been collected to provide evidence in support of decreased permeability and / or target site modification. Most of this discussion will focus on S.aureus, but it is important to first note the role of the outer membrane in gram negative bacteria. Uptake studies have clearly demonstrated that porin channels of gram negative bacteria play a crucial role in the transport of fluoroquinolone agents. Experiments have shown that porin deficient mutants accumulate less drug and display a corresponding decreased susceptibility to fluoroquinolone agents. Omp F appears to play the major role in gram negative permeability mutants.

Gram positive organisms do not have an outer membrane and as such rely on a different mechanism to limit access of the fluoroquinolone agents to their site of


A
A
Tyr
Tyr
Covalently Closed
Circular DNA
ATP–
B
B
–ATP
DNA Gyrase

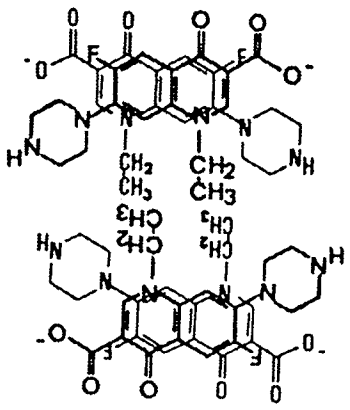


Figure 3. Proposed Quinolone-DNA Cooperative Binding Model for DNA Gyrase Inhibition. The filled and hatched boxes denote the quinolone molecules that self assemble to form a supermolecule inside the gyrase- induced DNA pocket. The binding pocket is believed to be induced during the intermediate gate opening step of the DNA supercoiling process (50).

action. Early studies examining the uptake of norfloxacin in E.coli demonstrated the existence of an endogenous active efflux pump (Cohen et al,1988). This putative pump, while first described in gram negative cells has also been shown to exist in S.aureus (Ubukata et al,1989;Yoshida et al,1990; Ohshita et al,1990; Kaatz et al,1993). The norA gene, responsible for this efflux pump, encodes a protein of approximately 43 Kda (Yoshida et al,1990). The deduced NorA polypeptide has 12 predicted hydrophobic membrane spanning regions and is partly homologous (26%) to the tet efflux protein. The NorA pump appears to be present in both susceptible and resistant organisms. A report by Ohshita et al described a mutation in the open reading frame of the norA apparently responsible for quinolone resistance (Ohshita et al,1990). This has not been confirmed by other workers. Recent reports involving the norA gene as well as my results will be handled in the discussion section of this thesis.

While the NorA efflux protein has been shown to contribute to low level fluoroquinolone resistance, high level resistance (MIC>8μg/ml) is acquired through modifications at the gyrase target (Fasching et al,1991; Sreedharan et al,1991; Goseitz et al,1992; Nakamura et al,1989; Sreedharan et al,1990; Piddock et al,1991). Documented changes to gyrA in both gram negative and gram positive organisms appear to be clustered in the amino terminal portion of the polypeptide near tyrosine 122. Table 4 describes common changes in gyrA associated with fluoroquinolone resistance at the DNA and amino acid level. The effects of these amino acid changes on the secondary and tertiary structures of the GyrA protein have not been fully elucidated, although it seems apparent that these changes somehow disrupt key

interactions involving the gyrase-quinolone complex. The most simple explanation would be that these changes reduce the affinity of the binding site for the quinolone molecules or in some cases block entry of the antibiotic to the binding site.

Table 4. Mutations in S.aureus gyrA associated with fluoroquinolone resistance.

Position	Codon	Amino Acid		Codon	Amino Acid	Type of Change*
84	TCA	Ser	change to	TTA	Leu	P to lg P
84	TCA	Ser	change to	GCA	Ala	P to NP
84/85	TCA/TCT	Ser/Ser	change to	TTA/CCT	Leu/Pro	P to NP
84	TCA	Ser	change to	TTT	Lys	P to +ve
88	GAA	Glu	change to	AAA	Phe	-ve to NP

* P = polar, NP = non-polar, lg = large, +ve = positive charge, -ve = negative charge

4. Postantibiotic Effect

a) History:

The postantibiotic effect or PAE is defined as the persistent suppression of bacterial regrowth following brief exposure to antimicrobial agents (McDonald et al,1977). The term post is to emphasize that the persistent suppression is due to previous exposure to antimicrobial agents. The observation that delayed regrowth of surviving bacteria following removal of an antibiotic was first described almost 50

years ago (Bigger,1944). Bigger noted the delayed development in turbidity after adding penicillinase to cultures of staphylococci and streptococci previously exposed to penicillin G. Other investigators also noted that cultures of staphylococci exposed to penicillin G for 5 to 30 minutes and then transferred to drug free medium did not resume normal growth for approximately 1 to 3 hours (Parker and Marsh,1946; Parker and Luse,1948). These investigators also noticed a time-concentration relationship in this effect. Increasing concentrations of drug and/or longer exposure times resulted in greater bacterial kill and a longer time for resumption of normal growth. Parker and Luse, 1948, reported that this effect was not due to the selective killing of dividing cells, leaving resting cells unaffected, because the effect was as apparent when there was no change in the viable cell count after penicillin treatment as when the population was moderately reduced (Parker and Luse,1948). Eagle and co-workers extended this work to include other gram positive bacteria and confirmed that this persistent effect exists both *in vitro* and *in vivo* (Eagle,1949; Eagle and Musselman,1949; Eagle et al,1950). Surprisingly, research in this area was not resurrected until just over a decade ago. The long void in the continuation of this work is somewhat disconcerting considering the implications of this area in the practice of chemotherapeutics. In the last decade however, studies on PAE have been expanded to include most clinically relevant gram positive and gram negative organisms as well as most antimicrobial agents.

b) Factors affecting the PAE:

The PAE appears to be a feature of virtually every antimicrobial examined and

has been documented with most common bacterial pathogens. Nevertheless, the type of organism, the class and concentration of antimicrobial agent, and the duration of exposure all influence the duration or presence of the PAE (Craig and Vogelman,1987). As stated above, the PAE has been evaluated in many common gram positive and gram negative pathogens but data regarding the PAE for organisms other than aerobic gram positive cocci and gram negative bacilli are limited. Experiments with anaerobic gram negative bacilli such as Bacteroides fragilis demonstrate no PAE with cefoxitin, but PAEs lasting several hours have been observed with clindamycin, metronidazole, and chloramphenicol (Craig and Gudmundsson,1986). Studies with antituberculosis agents indicate a PAE lasting several days when Mycobacterium tuberculosis is exposed to isoniazid (Beggs and Jenne,1969). Limited studies with antifungal agents show significant PAEs produced by amphotericin B and flucytosine with Candida spp. and one strain of Cryptococcus neoformans. In contrast, little or no PAE was observed with the imidazole agents ketoconazole and miconazole (Craig and Gudmundsson,1986). Tables 5 and 6 display the maximal *in vitro* PAEs achieved with several antimicrobial / microorganism combinations. The majority of antimicrobial agents used in clinical practice today have been evaluated for their PAE (tables 5 and 6). In general, inhibitors of protein and nucleic acid synthesis (including chloramphenicol, tetracycline, clindamycin, aminoglycosides, rifampin, and fluoroquinolones) induce prolonged PAEs (2 to 6 hours) against most gram positive cocci and gram negative bacilli within their spectrum of activity (Craig and Vogelman,1987). The apparent difference in the duration of the PAE between gram

positive and gram negative bacteria with aminoglycosides may be somewhat misleading. The rapid and complete bactericidal activity of these agents against gram positive cocci may not allow accurate quantitation of the true PAE whereas the slower killing of gram negative bacilli leaves sufficient cells to monitor regrowth. In contrast, cell wall-active agents (penicillins, cephalosporins, carbapenems, monobactams, and vancomycin) and trimethoprim induce PAEs of approximately 2 hours duration in gram positive cocci, but very short or no effects in gram negative bacilli (Craig and Vogelman,1987). Imipenem, a cell wall-active agent is unique in that it can produce significant PAEs with some strains of P.aeruginosa. In some instances gram negative bacilli exposed to penicillins and cephalosporins may produce what appears to be a negative PAE. That is; the culture exposed to antimicrobials appears to grow faster than the growth control. This phenomenon is most likely the result of filamentous forms, induced by exposure to β-lactam antimicrobials, dividing faster than control organisms soon after drug removal.

Table 5*: Maximal *in vitro* Postantibiotic Effect of several antimicrobial classes for gram positive cocci.

	PAE (hours)				
Antimicrobial	MSSA	MRSA	S.pneumoniae	E.faecalis	S.pyogenes
Aminoglycosides	0.5-1.5	0.5-1.5	-----	-----	-----
Cephalosporins	1.5-2.5	-----	2.5-3.5	-----	-----
Clindamycin	2.5-3.5	-----	>3.5	-----	>3.5
Macrolides	>3.5	-----	>3.5	-----	>3.5
Imipenem	2.5-3.5	2.5-3.5	-----	1.5-3.5	-----
Penicillins	1.5-3.5	-----	2.5-3.5	1.5-3.5	2.5-3.5
Fluoroquinolones	1.5-3.5	1.5-2.5	0.5-2.5	-----	-----
Tetracyclines	1.5-3.5	-----	1.5-2.5	-----	1.5-2.5
Vancomycin	2.5-3.5	1.5-2.5	-----	1.5-2.5	-----

* adapted from references 67, 69, 71

MSSA = methicillin susceptible S.aureus MRSA = methicillin resistant S.aureus

Table 6*: Maximal in vitro Postantibiotic Effect of several antimicrobial classes for gram negative bacilli.

	PAE (hours)					
Antimicrobial	E.coli	E.cloacae	P.aeruginosa	H.influenzae	K.pneumoniae	P.mirabilis
Aminoglycosides	2.5>3.5	2.5>3.5	2.5>3.5	-----	1.5-2.5	-----
Cephalosporins	<0.5-1.5	<0.5-1.5	<0.5	-----	<0.5-1.5	<0.5-1.5
Chloramphenicol	1.5-3.5	-----	-----	-----	2.5-3.5	0.5-1.5
Imipenem	1.5-2.5	1.5-2.5	1.5-3.5	-----	0.5-1.5	-----
Penicillins	<0.5-1.0	<0.5	<0.5	<0.5-1.5	<0.5-1.5	<0.5-1.5
Fluoroquinolones	1.5-3.5	-----	1.5-3.5	-----	-----	-----
Rifampin	>3.5	-----	2.5>3.5	-----	2.5>3.5	2.5-3.5

* adapted from references 67, 69, 71

c) Effects of concentration and duration of exposure:

Two of the most important factors that determine the duration of the PAE for any antimicrobial / organism combination are the concentration of antimicrobial and the length of exposure. In general, the persistent suppression of bacterial growth is only seen after bacteria have been exposed to drug concentrations that approach or exceed the MIC. When gram positive cocci are exposed to β-lactams, both the bactericidal activity and the duration of the PAE increase with increasing antimicrobial concentrations. There is a ceiling effect, however, that occurs between 5 and 10 times the MIC. At this point, maximal bactericidal activity and maximal PAE occur and increasing drug concentrations beyond this point do not increase either parameter (McDonald et al,1977; MacKenzie and Gould,1993). Gram negative bacilli exhibit little or no PAE with β-lactams (with the exception of imipenem) regardless of antimicrobial concentration (MacDonald et al,1977; MacKenzie and Gould,1993). Unlike the β-lactams, which show little concentration dependent bactericidal activity, the aminoglycosides display marked concentration dependent bactericidal activity. *In vitro* data document more extensive and rapid bacterial killing and continually increasing PAEs with increasing aminoglycoside concentration (Isaksson et al,1988). Isaksson (1988), has documented continually increasing PAEs with E.coli and P.aeruginosa using aminoglycoside concentrations of 1-64 μg/ml. Aminoglycosides are also one of the few antimicrobials that appear to induce a PAE even at concentrations below the MIC of the organism (Isaksson et al,1988). The bacteriostatic agents, erythromycin, tetracycline, and chloramphenicol induce PAEs in both gram positive

and gram negative organisms only at concentrations above the MIC. Maximal PAE is obtained at concentrations 5 to 10 times the MIC (MacDonald et al,1977; Bundtzen et al,1981). Rifampin does not appear to have a ceiling on its effect. Studies with S.aureus reveal that rifampin PAEs continue to increase even at concentrations as high as 200 times the MIC (Isaksson et al,1988).

Increasing the antimicrobials length of exposure has been shown to prolong the duration of the PAE. The length of exposure has also been shown to have a ceiling effect for many antimicrobials, i.e.; a maximal PAE is obtained regardless of the duration of antimicrobial exposure. A number of experiments by Craig and co-workers have demonstrated that the length of exposure and concentration of antimicrobials appear to have equal effects on the PAE (Fig. 4). Doubling the concentration has the same approximate effect as doubling the exposure time. Thus the "area under the concentration curve" (AUC - concentration x duration of exposure) appears to be the major determinant on the PAE duration for most antimicrobial / organism combinations (Craig and Gudmundsson,1986).

d) Quantitation of the PAE:

The PAE can be demonstrated both *in vitro* and *in vivo* by determining bacterial growth patterns after the antibiotic has been removed. Comparison of the growth kinetics of a treated organism (exposed to antimicrobial) with that of an untreated control enables us to assess the duration of the PAE (Craig and Vogelman,1987). Investigators performing *in vitro* experiments generally use rapid drug removal techniques such as repeated washing, dilution, or drug inactivation (Fig.5).

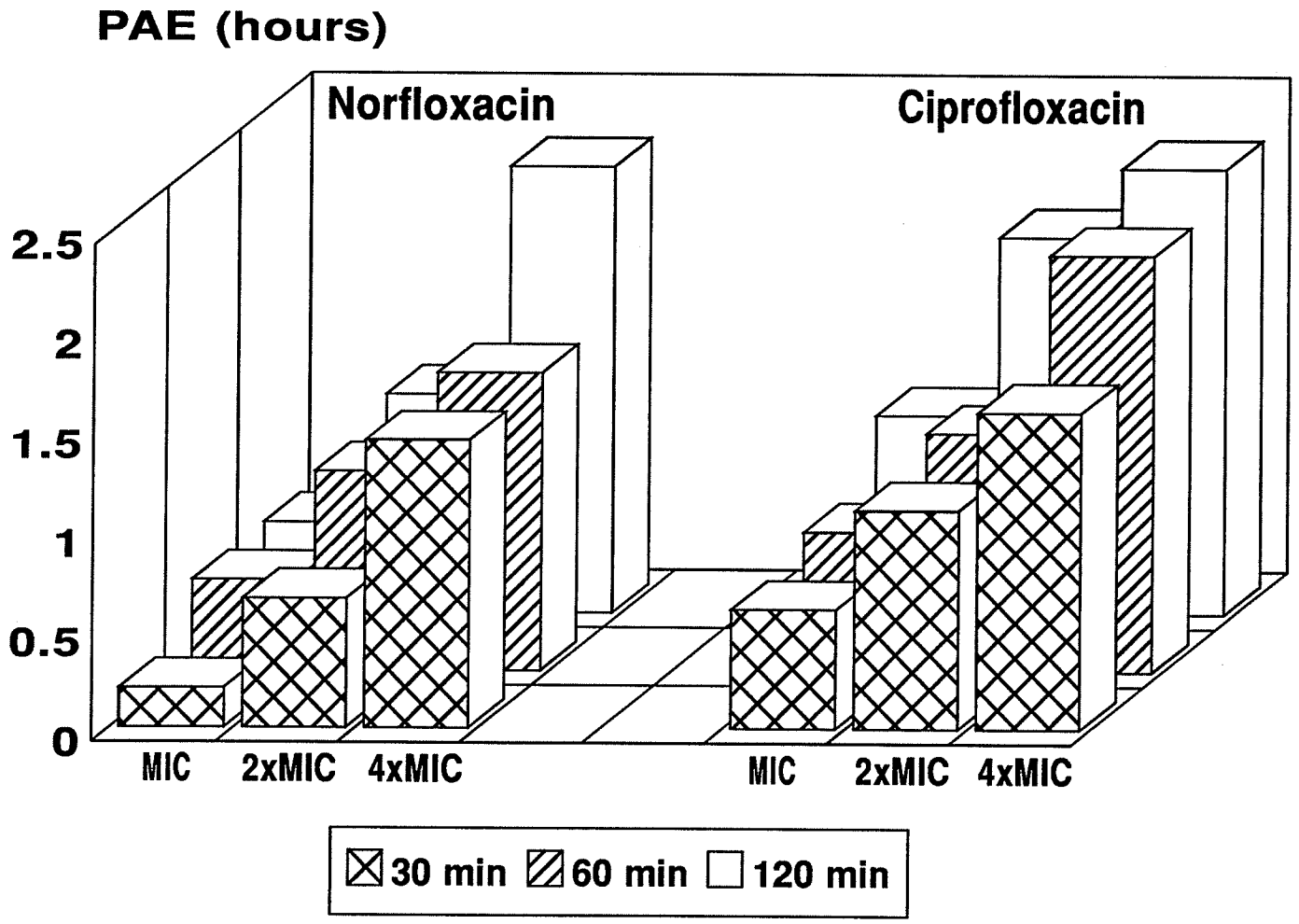
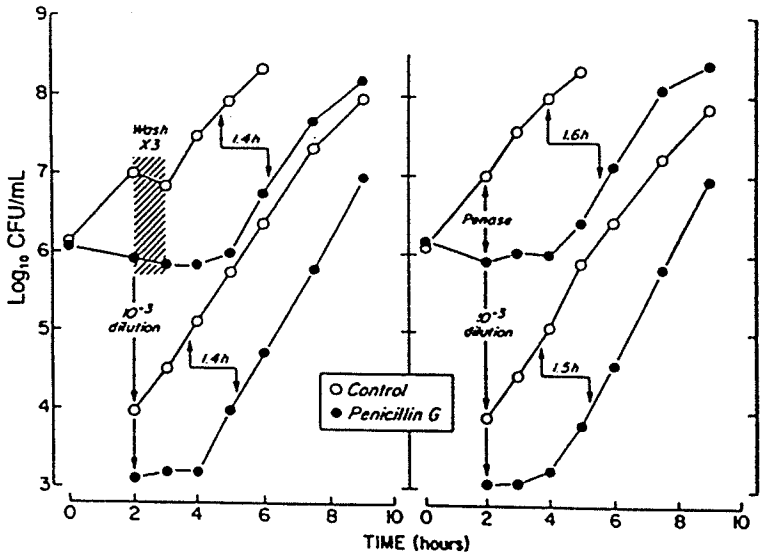

PAE (hours)
Norfloxacin
Ciprofloxacin
2.5
2
1.5
1
0.5
0
MIC
2xMIC
4xMIC
MIC
2xMIC
4xMIC
30 min
60 min
120 min

Figure 4. The effect of antimicrobial concentration and duration of exposure on the postantibiotic effect. Escherichia coli was exposed to either norfloxacin or ciprofloxacin at its MIC, 2xMIC, or 4xMIC. The organism was exposed to each concentration for 30, 60, or 120 minutes.

Figure 5. Growth kinetics of a typical PAE experiment.



The *in vitro* PAE of penicillin G (0.05 µg/ml) after a 2 hour exposure with S.aureus. Drug removal to inactive concentrations was performed by repeated washing, a 1:1000 dilution, or addition of penicillinase.

In vivo, four animal models exist to study the phenomenon; thigh infection in neutropenic mice, meningitis in rabbits, endocarditis in rats, and pneumonia in guinea pigs (Vogelman et al,1988; Tauber et al,1984; Ingerman et al,1986; Kapusnik et al,1985). In each of the preceding animal models, regrowth of bacteria is assessed after serum and tissue levels decrease to less than the MIC of the test agent against the infecting organism. Table 7 displays the *in vivo* PAE results for several antimicrobial / organism combinations. Regardless of the technique used to determine the PAE, the equation PAE = T - C is used to quantify the phenomenon. T represents the time required for the bacterial count (cfu/ml) in the test culture to increase one $\log_{10}$ above the count observed immediately after drug removal and C represents the time required for an untreated control culture to increase one log10 above the count observed

Table 7♦. In vivo PAE results for several antimicrobial - organism combinations.

	PAE (hours)			
Antimicrobial	Staphylococci	Streptococci	Enterobacteriaceae	Pseudomonas
Penicillins	2-6	<0.5	<0.5-2	<0.5
Cephalosporins	2-6	-----	<0.5-2	<0.5
Imipenem	2-4	-----	0.5-2	2-4
Vancomycin	4-6	-----	-----	-----
Aminoglycosides	2-4	-----	4>6	4>6
Fluoroquinolones	2-4	-----	2-6	2-6
Tetracyclines	4-6	4-6	2-4	-----
Macrolides	4>6	4>6	-----	-----

♦neutropenic mouse thigh model

immediately after drug removal (MacDonald et al,1977; Craig and Gudmundsson,1986). To calculate the PAE *in vivo* a similar equation is used. Using the neutropenic mouse thigh model as an example the equation PAE = T - C - M is employed (Vogelman et al,1988). M represents the time serum levels exceed the MIC; T is the time required for the number of cfu in the thighs of treated mice to increase one $\log_{10}$ above the count at time M and C is the time required for the number of cfu in the thighs of untreated controls to increase one $\log_{10}$ above the viable counts at zero time. It is important to note that the above equations require cells to increase by one $\log_{10}$ cfu before normal growth kinetics are considered to be "normal". To understand the importance of this one must first consider the cultures that are being examined. A bacterial culture is composed of a large population of individual cells. Early studies have demonstrated quite clearly that individual cells within the bacterial population can vary tremendously in their growth characteristics. Therefore, by requiring an overall increase in cfu by one $\log_{10}$ the variability among individual cells is reduced to acceptable levels and the growth characteristics of the population as a whole is more accurately reflected.

e) Mechanism of the PAE:

The precise mechanism by which antimicrobials induce PAEs are as yet not understood. The observed differences in PAEs of various antimicrobial / organism combinations suggest that multiple mechanisms are involved (Craig and Gudmundsson,1986; Zhanel et al,1991; MacKenzie and Gould,1993). It does not appear to be the result of a population shift in the test cultures to slower growing

variants as the growth curves of test and control cultures are parallel in the post PAE phase. The two most commonly proposed mechanisms are drug induced nonlethal damage at the cellular site of action and/or the limited persistence of the drug at its binding site (Craig and Vogelman,1987; Craig and Gudmundsson,1987; Zhanel et al,1991; MacKenzie and Gould,1993). For agents such as the macrolides, tetracycline, and chloramphenicol, which reversibly bind to specific subunits of susceptible bacterial ribosomes it has been suggested that the PAE represents the time required for the antibiotics to diffuse from the ribosome. An argument against this hypothesis stems from experiments by Gerber and Craig (Gerber and Craig,1981). They demonstrated that the PAE of erythromycin with S.pneumoniae was not lost during a 24 hour period at 4°C, during which time the drug would have been expected to diffuse from the ribosomes.

The aminoglycosides are a class of agents that exhibit their highly lethal effects by irreversibly binding to ribosome subunits. It has been suggested that the prolonged PAE induced by these agents could represent the binding of sublethal amounts of drug with subsequent disruption of protein synthesis. Therefore a possible result of this may be the depletion of functional proteins required by the cell for its intermediary metabolism and unhampered growth. The PAE might represent a period of resynthesis of these proteins (Craig and Gudmundsson,1987).

β-lactam antimicrobials show a marked difference in their effect on gram positive and gram negative bacteria. A prolonged PAE is observed in gram positive organisms, however, little or no PAE is observed in gram negative organisms. In both organisms,

β-lactams are known to bind to multiple penicillin binding proteins (PBP), some of which are required for cell wall synthesis. Craig and Gudmundsson (Craig and Gudmundsson, 1987) have postulated that the PAEs of β-lactam agents represent the time required for the organism to synthesize new enzymes. It has also been shown that the covalently bound penicillin-enzyme complex can break down regenerating active enzyme molecules (Tomasz,1979). Thus the PAE could represent the time required for the restoration of pre-existing PBPs. A recent paper by Yan et al (1994), has shown the new synthesis hypothesis to be the most likely scenario. They demonstrated that the PAE induced by penicillin in S.pyogenes is the result of irreversible binding to and the subsequent resynthesis of PBP 1-3.

Fuursted has speculated that the PAE induced by fluoroquinolones may represent the time required for these agents to dissociate from their binding site and diffuse from the cell (Fuursted,1987). This explanation seems reasonable, however, one cannot discount a multifactorial mechanism.

f) Clinical implications of the PAE:

Clinically the PAE has very important implications, its major impact being on the frequency of antimicrobial administration. At present, most antimicrobial regimens are designed to ensure serum and tissue levels of drug remain above the MIC for most of the dosing interval. While this has proved to be relatively successful for most cases, it may not be the optimal dosing regimen for drug efficacy, toxicity and cost. Studies have already shown that once daily aminoglycoside therapy is as efficacious as bid (twice a day) or tid (three times a day) dosing and the likelihood of toxicity is reduced

(Gilbert,1991). Demonstration of a prolonged PAE should allow for increased periods of time between dosing. Regrowth of the infecting organisms would be suppressed by the PAE allowing serum and tissue levels of the antimicrobial agent to fall below the MIC without any loss in drug efficacy.

5. Staphylococcus aureus:

S.aureus resides in the family Micrococcaceae. Its genus name "Staphylococcus" is derived from the Greek term grape-like cocci. This name is appropriate because the cellular arrangement of these organisms resembles a cluster of grapes.

The Staphylococci are small gram positive cocci 0.5 to 1.5 μm in diameter, non motile, and facultatively anaerobic. They are catalase positive, salt tolerant, and grow well at temperatures ranging from 18° to 40°C (Kaplan and Tenenbaum,1982). S.aureus is differentiated from other Staphylococcal species by its ability to produce coagulase.

Some consider S.aureus to be one of the most versatile pathogens in existence today (Brumfitt and Hamilton-Miller,1989). It produces at least five cytolytic toxins (α, β, δ, γ, and leucocidin) as well as exfoliative toxin (Staphylococcal scalded skin syndrome) and toxic shock syndrome toxin-1. S.aureus also produces five serologically distinct enterotoxins (A - E) that are resistant to hydrolysis by gastric and jejunel enzymes and are thermostable. As a result S.aureus is commonly associated with cases of food-borne poisonings.

Treatment of Staphylococcal infections is becoming increasingly problematic. The term "antibiotic resistant" and "S.aureus" is rapidly becoming synonymous. The

advent of penicillin G in the early 1940's temporarily solved the problem of Staphylococcal infections, but the constant use of this agent caused the selection of resistant (β-lactamase producing) strains. By 1948 the prevalence of resistant strains had seriously reduced the value of penicillin G (Barber and Rozwadowska-Dowzenko,1948). By the end of the 1950's S.aureus had acquired resistance to virtually all available systemic antimicrobials including erythromycin, streptomycin, and the tetracyclines. The semi-synthetic penicillins, (methicillin, oxacillin etc.) produced in the early 1960's seemed hopeful as they were not inactivated by β-lactamases. Resistance to these agents were soon detected however. The methicillin resistant S.aureus (MRSA) strains of the 1960's and 1970's, while resistant to most penicillins, were adequately covered by numerous other agents. The late 1980's and early 1990's saw the arrival of new highly resistant MRSA strains. So multiply resistant are some of these strains that vancomycin is the only option left available for many staphylococcal infections. Add to this the fact that vancomycin resistant Enterococci have now been isolated. The genes vanA and vanB, responsible for vancomycin resistance reside on highly mobile transposons. How long will it be before MRSA acquires vancomycin resistance? With this information in mind it is clear that research dedicated to finding new antimicrobial agents is an absolute priority. The information gleaned from studying pharmacodynamic parameters of existing agents and the resistance mechanisms directed against them should help us in the search for new agents.

The studies in this thesis were undertaken in part to expand our knowledge of

fluoroquinolone pharmacodynamics in gram positive bacteria. While the PAE of many antimicrobials and the accumulation of these agents has been well studied in gram negative organisms, examination of the same in gram positive organisms has not been as well defined. It is only with the study of existing agents can we hope to better understand their mechanisms of action and apply this knowledge to the rational design of new and improved chemotherapeutic agents.

Methods and Materials

1. Bacterial strains

The following methicillin susceptible strains of S.aureus were obtained from the clinical microbiology laboratory, Health Sciences Center; F651, F629, F495, F447, F988. The methicillin resistant S.aureus strains F192, F201, and F321 were also obtained from the clinical microbiology laboratory, Health Sciences Center. S.aureus strains ATCC #25923, #29213 (MSSA), and #33592 (MRSA) were obtained from the American Type Culture Collection, Rockville, Maryland. The methicillin resistant-fluoroquinolone resistant S.aureus strains #603, #609, #614, and #617 were obtained from Dr. Donald E. Low, Mount Sinai Hospital, Toronto, Ontario. Escherichia coli DH5α was obtained from Dr. J. Neil Simonsen, Dept. of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba.

2. Antimicrobials

The following antimicrobials were used throughout the course of this thesis; **Fluoroquinolones:** ciprofloxacin, pefloxacin, norfloxacin, lomefloxacin, and sparfloxacin. **β-lactams:** cloxacillin , cephalexin, and ampicillin. **Aminoglycosides:** gentamicin. Choramphenicol was also used in the large scale plasmid preparations. Ciprofloxacin was obtained from Miles Pharmaceuticals, Rexdale, Ontario, Canada; pefloxacin and sparfloxacin were obtained from Rhone Poulenc Rorer, Montreal, Quebec, Canada; norfloxacin was obtained from Merck Frosst, Rahway, N.J., USA;

lomefloxacin was obtained from Searle, Chicago, Illinois,USA; cloxacillin was obtained from Bristol-Myers, Syracuse, N.Y., USA.; cephalexin was obtained from Sigma chemical company, St. Louis, Mo, USA., ampicillin was obtained from Sigma Chemical Company, St. Louis, MO, USA., and gentamicin was obtained from Schering Corp. Ltd., Pointe Claire, Quebec Canada. Chloramphenicol was obtained from Parke-Davis, Morris Plains, N.J. USA.

3. Radiolabelled antimicrobials

All ^{14}C-antimicrobials were obtained from their respective manufacturers. ^{14}C-pefloxacin and ^{14}C-sparfloxacin were obtained from Rhone Poulenc Rorer, Montreal, Quebec; ^{14}C-norfloxacin was obtained from Merck Frosst, Rahway, N.J.; and ^{14}C-lomefloxacin was obtained from Searle, Chicago, Illinois. Specific activities of the radiolabelled compounds were as follows: ^{14}C-pefloxacin 50.8μCi/mg, ^{14}C-sparfloxacin 31μCi/mg, ^{14}C-norfloxacin 46.5μCi/mg, and ^{14}C-lomefloxacin 152μ Ci/mg.

4. Susceptibility testing

Initial minimum inhibitory concentrations (MICs) were performed using a macrotube procedure described by the National Committee for Clinical Microbiology Standards. Serial doubling dilution's of antimicrobials were prepared in 1ml cation supplemented Mueller-Hinton broth (MHB) (Difco Laboratories, Detroit, MI.) (final concentration Ca^{+2} 50mg/l, Mg^{+2} 25mg/l). The bacterial inoculum was prepared from

an exponential phase culture and adjusted to yield a 0.5 MacFarland turbidity standard (1×10^8 cfu/ml). Tubes were incubated at 37°C for 16-18 hours and the MIC determined as the lowest concentration of antimicrobial that inhibited bacterial growth.

MICs determined by the above macrotube method were repeated using a more accurate arithmetic series of antimicrobial dilution's (0.02 mg/l increments). A microtitre method as described by the National Committee for Clinical Microbiology Standards was employed. Microtitre plates were inoculated with a MIC-2000 automatic inoculator (Dynatech Laboratories Inc., Alexandria, Virginia, USA) resulting in a final inoculum of approximately 5×10^5 cfu/ml. MICs were defined as the lowest concentration of antimicrobial that inhibited bacterial growth after 18 hours incubation at 37°C.

5. Selection of Fluoroquinolone resistant mutants.

Brain Heart Infusion (BHI) (Oxoid-Unipath Ltd., Basingstoke, Hampshire) agar plates were prepared with fluoroquinolone added to a final concentration equal to the MIC of the organism to be sub-cultured. The organism was plated onto the BHI agar and incubated at 37°C for 24-48 hours. After incubation colonies were picked from each plate and sub-cultured onto fresh fluoroquinolone containing BHI agar plates and allowed to incubate a further 24-48 hours at 37°C. This process was repeated until a) good growth was achieved and b) colony morphology resembled a single clone. Once good growth was achieved with only a single colony type apparent, organisms were sub-cultured on BHI agar containing twice the concentration of fluoroquinolone as the

original plates (Fig.6). The above process was repeated increasing the fluoroquinolone concentration with each subsequent sub-culture until MICs could no longer be increased. The stability of the resistant isolates was evaluated by sub-culturing the organisms on drug free medium and assessing their MICs.

6. Determination of the Postantibiotic Effect

A) preparation of bacterial culture

1-2 colonies from a 24 hour old blood agar plate were used to inoculate 50ml of MHB and the culture incubated overnight for 16-18 hours at 37°C. 5ml of the overnight culture was transferred to 45ml of fresh MHB and allowed to incubate at 37 °C for several hours until the optical density at λ580nm approached 0.3. All optical densities were measured with a Spectronic 1201 spectrophotometer, (Milton Roy Company, Rochester, N.Y., USA). 1ml of the resulting mid-log phase culture was then transferred to: a) a growth control containing 9ml of MHB and b) a test culture containing 8.9ml of MHB and 100µl of the test antimicrobial stock solution. Antimicrobial stock solutions were made to an initial concentration of 100x the test concentration. The final concentration of organisms in both the growth control and the test culture should be approximately 10^6-10^7 cfu/ml. A small aliquot from each tube was immediately removed to verify the inoculum by viable colony counts and the tubes transferred to a 37°C water bath for 2 hours.

B) removal of the antimicrobials

Two different methods were used to remove or reduce the antimicrobial

- Resistant strains were selected for by serially passing organisms on BHI agar containing increasing concentrations of fluoroquinolones

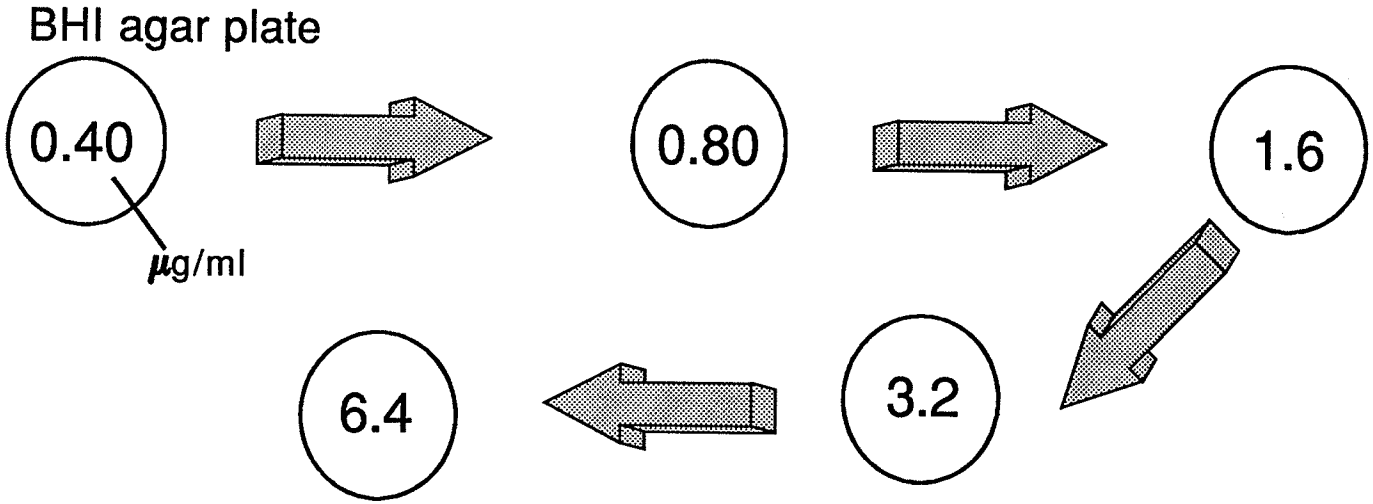


Figure 6. Diagramatic representation of the technique used to select for fluoroquinolone resistant isolates. Fluoroquinolone susceptible clinical isolates were plated on BHI agar containing a fluoroquinolone at the organisms MIC. After repeated subculture at this concentration, the organism was subcultured on BHI agar containing twice the previous drug concentration. This was repeated until the organisms would no longer grow.

concentrations to levels that no longer had any effect on the bacterial cells.

1. Dilution Method:

100µl of both the test culture and the growth control were transferred to 9.9ml of fresh MHB resulting in a 1:100 dilution. The diluted cultures were incubated in a 37°C waterbath for several hours until marked turbidity was observed (Fig.7).

2. Washing Method:

The test culture and growth control were centrifuged at 4000 xg for 5 minutes and the pellet resuspended in phosphate buffered saline (PBS) (appendix A). Cultures were recentrifuged and the pellets resuspended in 10ml fresh MHB. The resuspended cultures were allowed to incubate in a 37°C waterbath until marked turbidity was observed.

C: residual antimicrobial control

A residual antimicrobial control (RAC) was developed for use in the test system to ensure levels of diluted antimicrobials were no longer having any significant effect on the growth rate of the test organisms. The RAC is designed to contain the same concentration of drug found in the diluted antimicrobial test tube. 100µl of the antimicrobial stock solution was added to 9.9ml of MHB. A further 100µl aliquot was transferred from this new antimicrobial dilution to the RAC tube which consists of 9.8ml of fresh MHB and a 100µl aliquot from the growth control. The RAC tube was incubated and treated identically as the diluted test and growth control tubes.

D: viable colony counting

After antimicrobial dilution / removal, 100µl aliquots were removed from the test

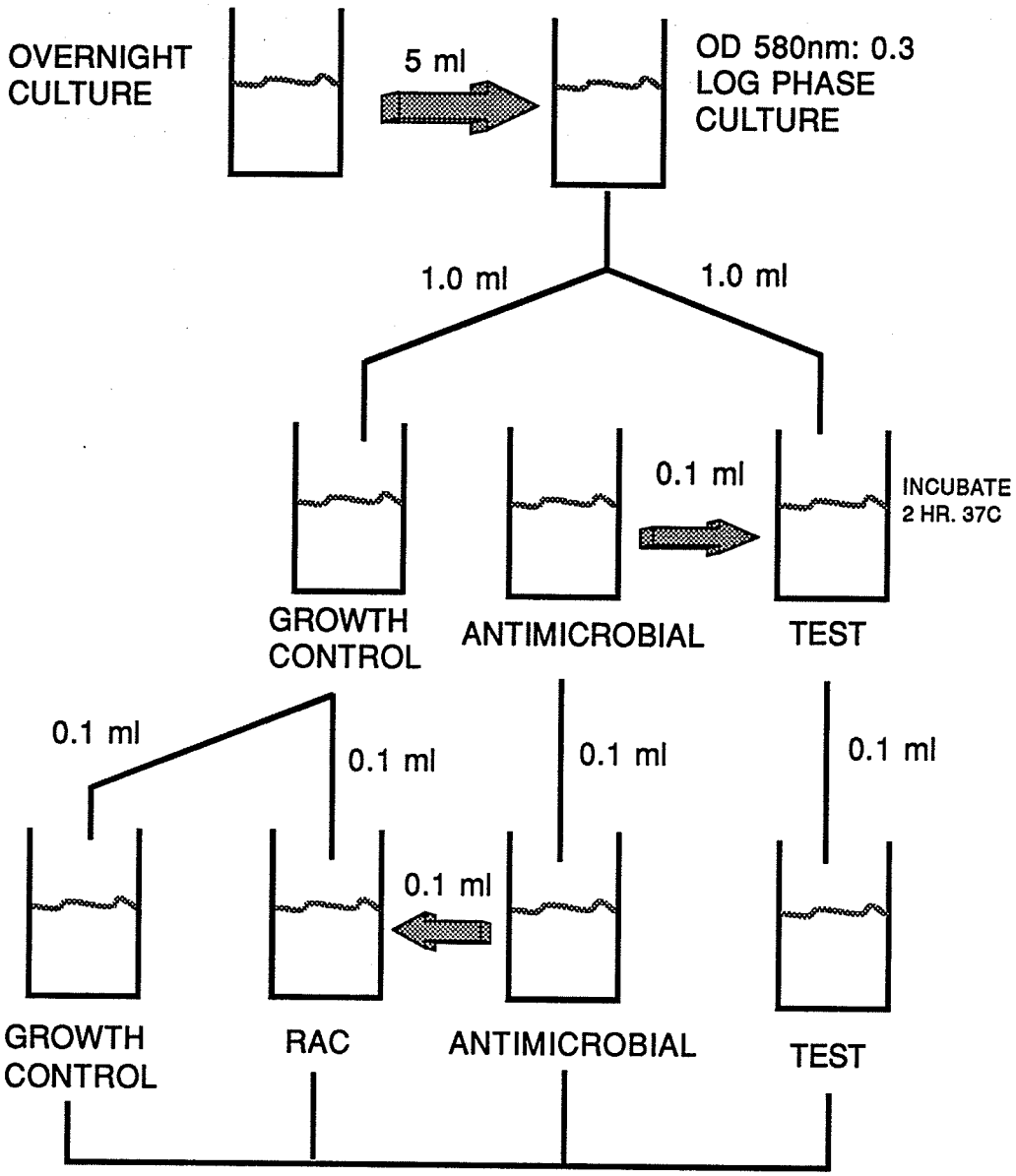

OVERNIGHT CULTURE
5 ml
OD 580nm: 0.3 LOG PHASE CULTURE
1.0 ml
1.0 ml
0.1 ml
INCUBATE 2 HR. 37C
GROWTH CONTROL
ANTIMICROBIAL
TEST
0.1 ml
0.1 ml
0.1 ml
0.1 ml
0.1 ml
GROWTH CONTROL
RAC
ANTIMICROBIAL
TEST
REGROWTH EVALUATED EVERY 30 MINUTES

Figure 7. Diagrammatic representation of the dilution technique used to determine the PAE.

culture, growth control, and RAC tubes every 0.5 hours until marked turbidity is observed. The aliquots were serially diluted in PBS and plated on sheep blood agar plates. Plates were incubated for 18 hours at 37°C and manually counted. All plates containing less than 25 colonies or greater than 250 colonies were rejected.

E: calculation of the PAE

The following formula was used to calculate the PAE:

$$\mathbf{PAE = T - C}$$

where T represents the time required for the organisms in the test culture to increase one $\log_{10}$ above the viable colony count immediately after dilution

and

where C represents the time required for the organisms in the control culture to increase one $\log_{10}$ above the viable colony count immediately after dilution.

7. Fluoroquinolone accumulation in exponential phase S.aureus cells

A modified method of Diver et al, 1990, was employed for the determination of fluoroquinolone accumulation in S.aureus. This method results in exponential phase cultures containing approximately 10^8 cfu/ml.

One to two colonies from a 24 hour old blood agar plate were used to inoculate 50ml of Iso-Sensitest broth (Oxoid-Unipath, Basingstoke, Hampshire) and the culture incubated overnight for 16-18 hours at 37°C. Five hundred microlitres of the overnight culture was transferred to 50ml of fresh Iso-Sensitest broth and allowed to incubate at 37°C for 2.5 hours in a shaking water bath. A 10ml aliquot was removed and

centrifuged at 4000 x g for 10 minutes, washed with PBS and the resulting pellet resuspended in 1ml of PBS. A 50µl aliquot was removed for determination of total cell protein and the PBS-pellet resuspended in 9ml of fresh Iso-Sensitest broth. The culture was allowed to equilibrate for 10 minutes at 37°C after which time a 50µl aliquot was removed for determination of the viable cell count. The reaction was initiated by addition of ^{14}C-fluoroquinolone. At timed intervals, a 0.5ml aliquot was removed and immediately diluted in 20ml PBS at 7°C. The sample was filtered through a 0.45µm pore, 25mm diameter nylon membrane filter (Micron Separations Inc., Westbora, M.A.) using a vacuum filtration manifold (Millipore Corp., Bedford, M.A.). Just prior to filtration, filters were presoaked in PBS. Filters were washed with 20ml PBS , removed from the manifold, dried at 60°C for 1 hour and placed in 10ml Cytoscint scintillation cocktail (ICN, Costa Mesa, CA.) for counting in a LKB Rackbeta 1217 counter. Cell associated radioactivity was determined after correction for non-specific binding of the radiolabel to filters in the absence of bacterial cells. Total cell protein was determined by the method of Lowry et al (1951).

8. Treatment with metabolic inhibitors

The effect of several metabolic inhibitors on the uptake of ^{14}C-fluoroquinolones was examined. The inhibitors 2,4-dinitrophenol (DNP), carbonyl cyanide m-chlorophenyl-hydrazine (CCCP), sodium azide (NaN_3), and potassium cyanide (KCN) were added to final concentrations of 2mM, 50µM, 5mM, and 2mM respectively. All inhibitors were added 10 minutes after the addition of the radiolabeled

fluoroquinolone. The inhibitors were determined not to be bactericidal over the course of the assay as determined by viable colony counts in the absence of antibiotics.

9. Fluoroquinolone accumulation in PAE phase cells

In order to ensure sufficient numbers of viable cells were available for uptake experiments, the uptake assay as described above was modified. In addition, to ensure organisms were in the PAE phase during the time frame of the uptake assays, PAE determinations were simultaneously performed with all uptake experiments.

One to two colonies from a 24 hour old blood agar plate were used to inoculate 50ml of Iso-Sensitest broth. The culture was incubated overnight for 16-18 hours at 37°C. Five hundred microlitres of the overnight culture was transferred to 50ml of fresh Iso-Sensitest broth and allowed to incubate at 37°C for several hours in a shaking water bath. Ten millilitres of this culture was transferred to 90ml Iso-Sensitest broth containing antimicrobial at a given multiple of the MIC and incubated for 2 hours at 37°C. Antimicrobials were removed by centrifugation at 4000 x g, 10 minutes, washed twice with PBS and the pellet resuspended in fresh Iso-Sensitest broth. To determine the PAE, cultures were allowed to incubate at 37°C for several hours until marked turbidity was observed. Aliquots were removed every 0.5 hours to assess regrowth. The PAE was calculated as described earlier.

For uptake experiments the pellet (after washing to remove antimicrobials) was resuspended in 1 ml PBS and a 50µl aliquot removed for determination of total cell protein. The pellet-PBS was resuspended in 9ml fresh Iso-Sensitest broth and adjusted

to approximately 10^8 cfu/ml. Cell densities were adjusted with McFarland standards and confirmed by viable colony counts. Cultures were allowed to equilibrate 10 minutes at 37°C prior to the start of the experiment after which a 50µl aliquot was removed for determination of the viable cell count. After addition of the ^{14}C-fluoroquinolone 0.5ml aliquots were removed at timed intervals and immediately diluted in 20ml PBS at 7°C. The sample was filtered through a 0.45µm pore, 25mm diameter nylon membrane filter (Micron Separations Inc., Westbora, M.A.) using a vacuum filtration manifold (Millipore Corp., Bedford, M.A.). Just prior to filtration, filters were presoaked in PBS. Filters were washed with 20ml PBS , removed from the manifold, dried at 60°C for 1 hour and placed in 10ml Cytoscint scintillation cocktail (ICN, Costa Mesa, CA.) for counting in a LKB Rackbeta 1217 counter. Cell associated radioactivity was determined after correction for non-specific binding of the radiolabel to filters in the absence of bacterial cells. Total cell protein was determined by the method of Lowry et al (1951).

10. Protein determination using the Lowry Method.

Fifty microlitres of the S.aureus pellet-PBS was added to 150µl of 1.0N NaOH in a 5ml test tube. The tube was heated to just under boiling for 10 minutes after which 1ml of reagent D (appendix A) was added, mixed and allowed to stand for 10 minutes at room temperature. 100µl of reagent E (appendix A) was added and mixed very rapidly. After 30 minutes incubation at room temperature the sample was read at λ750nm using a Spectronic 1201 spectrophotometer. The amount of protein was

calculated from a previously determined standard curve.

11. Isolation of Genomic DNA from S.aureus

One to two colonies from a 24 hour old blood agar plate were used to inoculate 250ml of BHI broth. The culture was incubated shaking overnight for 18-24 hours at 37°C. Bacteria were harvested from the overnight broth culture by centrifuging for 15 minutes at 5000 x g. The pellet was resuspended in 20ml of T20E1Na100 buffer (20mM Tris-HCl pH 7.5, 1mM EDTA, 100mM NaCl) to which 100-150 μg of lysostaphin was added. The resuspended pellet was incubated for 30 minutes in a 37°C water bath. 1ml of Triton X-100 (20% v/v) and 20μg of proteinase K was then added and the suspension allowed to incubate for 30 minutes in a 37°C water bath. After incubation, 20g of cesium chloride (CsCl) and 0.8ml of ethidium bromide (10mg/ml) were added. The mixture was centrifuged to equilibrium in a fixed angle rotor (Ti70) at 175,000 x g for 24 hours. The chromosomal band was collected through the side of the centrifuge tube with a sterile 16 gauge needle. The chromosomal band was transferred to a clean test tube and sterile water added to bring the volume to 5ml. To remove the EtBr the DNA solution was extracted with an equal volume of isoamyl alcohol and this procedure repeated until the solution remained clear. The isoamyl alcohol was removed and the solution decanted into sterile pre-silanized corex tubes. The DNA was precipitated with two volumes cold absolute ethyl alcohol and recovered by spooling onto a clean sterile glass rod. The spooled DNA was washed twice in 70% cold ethyl alcohol, air dried, and resuspended gently in 500 to 1000μl of

T10E1 buffer (10mM Tris-HCl pH 8.0, 1mM EDTA).

12. Quantitation of genomic DNA:

Genomic DNA was evaluated spectrophotometrically to determine both the quantity of isolated DNA and to determine its purity. A 1:50 dilution of genomic DNA was made in T10E1 and the optical density measured at λ260nm and λ280nm. Purity was determined by the λ260nm / λ280nm ratio. The quantity of genomic DNA was determined with the following formula:

$$[DNA] = \Sigma_{260} \times OD_{260} \times \text{dilution}$$

where the dilution was 1:50 and the $\Sigma 260 = 50$

13. Preparation of Southern blots:

1 μg of genomic DNA was incubated at 37°C overnight with several units of one or more restriction enzymes. The digested DNA was electrophoresed in a 1% EtBr containing agarose gel for several hours at 100V. After electrophoresis the gel was exposed to UV light.(>1Kb-15 minutes, < 1Kb-5 minutes). Alternatively the gel could be soaked in 2N HCl for 15 minutes. The treated gel was then soaked for 20 minutes (x3) in a 0.5N NaOH / 1.5M NaCl solution and rinsed briefly in distilled water. The gel was then neutalized in a 0.5M TRIS pH 7.5 / 1.5M NaCl solution for 20 minutes (x2) and rinsed briefly in distilled water. The transfer was set up in 10x SSC (appendix A) using the following technique. A pool of 10x SSC was poured into a glass dish and a glass plate placed across the dish. Two strips of 3MM paper were placed across the

plate and into the pool of SSC on each side. The strips of paper were then soaked with SSC. Three pieces of 3MM paper were cut to the size of the gel, soaked with SSC and placed on top of the plate / 3MM paper. Air bubbles were removed. The treated gel was placed bottom side up in the center of the 3MM paper. Genescreen membrane was cut to fit gel, soaked with 10x SSC and placed on top of the gel, as always smoothing out bubbles. The membrane was surrounded with even edged pieces of parafilm, draping over soaked 3MM paper. Three more trimmed strips of 3MM paper were placed on top of the membrane, soaked with SSC and a large stack of paper towels placed on top. The paper towels were compressed with a heavy weight on top for 16-72 hours. With membrane still moist from transfer, UV crosslinking was performed for three minutes, the membrane soaked in 2x SSC to reduce salt content, and the membrane baked at 80°C for 2 hours in vacuum oven. The baked membrane was sealed in a plastic bag until required.

14. End labeling oligonucleotides with γ^{32}P:

All oligonucleotides used in the course of this thesis were synthesized at the DNA synthesis laboratory, University of Calgary, Calgary, Alberta.

Oligonucleotides were diluted to a concentration of 100ng / µl in T10E1 prior to labelling. The following reactants; oligonucleotide 1µl, 10x PNK buffer (appendix A) 2µl, sterile water 11µl, γ^{32}P-ATP (tricine)(~50µCi) 5µl, and T4 polynucleotide kinase (10U) 1µl were added together and incubated for 1 hour at 37°C. Eighty microlitres of T10E1 was then added and the total 100µl volume placed in a G-25 Sephadex

column and centrifuged at 1500 xg for 2 minutes. The purified oligonucleotide was collected and stored in a 1.5 ml ependorf tube at -20°C.

15. Preparation of G-25 Sephadex column

To a tuberculin syringe add enough silanized glass wool to create a plug. Fill the syringe with a sephadex solution and centifuge 1500 xg for 2 minutes and repeat. The sephadex should fill the syringe to the 0.8ml mark.

16. Southern hybridization:

To a previously prepared Southern blot, 10 to 15ml of hybridization buffer (appendix A) was added in a plastic bag. All air was removed, the bag sealed with a heat sealer and placed into a second plastic bag. Again, all air was removed and the bag placed in a shaking waterbath at 50°C* for 0.5 hours. The old buffer was decanted and replaced with 10 to 15ml of fresh buffer and approximately 50µl γ^{32}P• oligonucleotide (~$2x10^6$cpm) was introduced. All air was removed, the bag heat sealed and double bagged as before. The membrane was incubated overnight in a shaking waterbath at 50°C. The genescreen was carefully removed and placed in a plastic container where it was washed with 2x SSC / 0.1% SDS for 1 hour at 50°C and repeated. The washed genescreen was wrapped in saranwrap, taped to a cardboard support and placed with an intensifying screen in an autoradiograph cassette. In a darkroom, x-ray film was placed between the genescreen and the intensifying screen and the film exposed overnight at -80°C. The film was developed the next day.

* Incubation temperature is dependent on the melting temperature of the oligonucleotides. Melting temperature was determined according to the following formula:

$$Tm = 69.3 + 0.41\ (G + C\%) - 650 / L$$

where L = length of oligonucleotide

17. Polymerase chain reaction:

A: reaction protocols:

The polymerase chain reaction (PCR) was used to amplify the genes gyrA, gyrB, norA, and the norA promoter region directly from S.aureus genomic DNA. The following reaction mixture, sufficient for 6 reactions and 1 control was prepared fresh just prior to the start of each PCR experiment.

H_2O	164µl
10x reaction buffer (appendix A)	70µl
1.25 mM dNTPs (dATP, dCTP, dGTP, dTTP)	112µl
100µM 5' oligonucleotide	8µl
100µM 3' oligonucleotide	8µl
Taq polymerase	3.5µl

To 50µl of the above reaction mixture, a 50µl solution of genomic DNA consisting of 1ng total DNA was added and mixed. This 100µl volume was overlayed with 50µl sterile mineral oil and placed in a Perkin Elmer 4800 series thermocycler (Perkin-Elmer Corporation, Norwalk, CT) A 3 temperature cycling protocol was used in all

experiments. Each experiment was performed for 30 complete cycles with the following parameters:

Denaturing step	94°C	60 seconds
Annealing step	50°C*	60 seconds
Extension step	72°C	2- 3 minutes**

* The annealing temperature is dependent on the Tm of the oligonucleotide.

**2 minutes were chosen for amplifying the coding region of gyrB and norA, 3 minutes were chosen for gyrA. 45 seconds were used to amplify the norA promoter region.

Note: The Perkin-Elmer 4800 series thermocycler was replaced with a Perkin-Elmer GeneAmp PCR 9600 system during these studies. The time frames for each of the 3 reaction steps were reduced by half due to the increased efficiency of the 9600 series system. The addition of sterile mineral oil was not required with the 9600 series.

After completion of 30 cycles a final 10 minute extension step was performed at 72°C. 20µl (20%) of the completed PCR reactions were removed and loaded onto a 1% ethidium bromide containing agarose gel to assess products. The remaining 80µl was extracted with 25:24:1 phenol:choroform:isoamyl alcohol followed by a final 24:1 chloroform:isoamyl alcohol extraction. The PCR products were then precipitated with a 1/10 volume of 3M sodium acetate and 2 volumes of cold absolute ethyl alcohol. The precipitated products were resuspended in 100µl T10E5-SDS (Tris-HCl 10mM pH 7.5, 5mM EDTA, 0.5% SDS) containing 50µg/ml proteinase K. The products were allowed to incubate for 30 minutes in a 37°C water bath followed by a 10 minute

incubation at 68°C. The products were again extracted with 25:24:1 phenol:choroform:isoamyl alcohol and precipitated with 3M sodium acetate and cold absolute ethyl alcohol. If the PCR products were destined to be inserted into a cloning vector they were digested overnight with the appropriate restriction endonuclease.

B: recovery of PCR products by agarose gel purification and electroelution:

PCR products were run on a 1% ethidium bromide containing agarose gel, visualized under UV light and the corresponding bands cut out as gel slices using a scalpel. The electroelution device (IBI Inc., New Haven, CT.) was filled with 0.5 x TBE buffer (appendix A). Collection tubes were filled with 80µl of a 3M sodium acetate-bromophenol blue solution. Gel slices were placed in the wells and the reaction was run at 90 volts until DNA was no longer visible under UV light. The sodium acetate-bromophenol blue-DNA solution was collected, precipitated with cold absolute ethyl alcohol, washed with cold 70% ethyl alcohol and resuspended in 10- 50 µl T10E1. 1µl of the final product was accessed on a 1% ethidium bromide containing agarose gel to determine final quantity of product.

18. Preparation of pBluescribe plasmid vector:

Approximately 5µg of plasmid DNA was digested with the desired restriction endonuclease overnight at 37°C. The plasmid was then extracted with 25:24:1 phenol:chloroform:isoamyl alcohol, reextracted with 24:1 chloroform:isoamyl alcohol, and precipitated with a 1/10 volume of 3M sodium acetate and 2 volumes cold absolute ethyl alcohol. The precipitate was resuspended in 10µl T10E1. The 10µl

volume was divided into two 5µl volumes; 5µl to serve as a control and 5µl to be phosphatased with calf intestinal phosphatase in the following reaction: Digested plasmid 5µl, 10x buffer 2µl, calf intestinal phosphatase (1 unit) 1µl, and 12µl of sterile water. The reaction was incubated at 37°C for 30 minutes, extracted with 25:24:1 phenol:chloroform:isoamyl alcohol, reextracted with 24:1 chloroform:isoamyl alcohol, and precipitated with a 1/10 volume of 3M sodium acetate and 2 volumes of cold absolute ethyl alcohol. The precipitate was washed with 70% cold ethyl alcohol and resuspended in 10µl T10E1. A 1µl aliquot of both cut and uncut plasmid was run on a 1% ethidium bromide containing agarose gel to ensure efficient cutting of the plasmid.

19: Ligation of PCR products into Bluescribe vector:

A: control reactions:

The following controls were performed prior to each experiment:

	C1	C2	C3	C4
	vector cut, not phosphatased	vector cut and phosphatased	vector cut, not phosphatased	vector cut and phosphatased
plasmid	50ng	50ng	50ng	50ng
5x ligase salts	4µl	4µl	4µl	4µl
10mM ATP	0.5µ	0.5µl	0.5µl	0.5µl
H_2O	15.5µl	15.5µl	15µl	15µl
T4 DNA ligase	----	----	0.5µl	0.5µl

The control reactions were incubated for 24 hours at 16°C and then precipitated with 5µg of yeast tRNA and 2 volumes cold absolute ethyl alcohol. The precipitate was resuspended in 10µl T10E1Na100 (Tris-HCl 10mM pH 7.5, EDTA 1mM, NaCl 100mM).

B: ligation reactions:

The ligation reactions were performed in the same manner as the controls using only the cut and phosphatased vector. The PCR products were added to the reaction mixture in vector / PCR product molar ratios of 1:1 and 1:3.

	Length	1:1	1:3
Plasmid vector	~ 3 Kb	50ng	50ng
gyrA	~ 2.6 Kb	43ng	130ng
gyrB	~ 1.8 Kb	30ng	90ng
norA	~ 1.2 Kb	20ng	60ng

The ligation reactions were incubated and precipitated as above.

20. Preparation of competent DH5α E.coli:

One to two colonies of DH5α E.coli were inoculated into 50ml of SOC broth (appendix A) and incubated overnight for 16-18 hours in a 37°C shaking waterbath. One millilitre of the overnight DH5α E.coli culture was added to 100ml of SOC broth and incubated 2.5 hours in a 37°C shaking waterbath until the optical density measured at λ600nm approached 0.46. The organisms were placed on ice for 5 minutes and centrifuged at 4000 x g for 10 minutes. The supernatant was removed

and the pellets resuspended in 33ml of FSB (appendix A). The FSB-E.coli solution was split into 2 tubes with 16.5ml of solution per tube. Tubes were then incubated on ice for 15 minutes and centrifuged 10 minutes at 4000 x g. The supernatant was removed and the pellet gently resuspended in 8ml FSB (4ml / tube). 280µl of DMSO was added (to 3.5%) and the tubes placed on ice for 5 minutes. An additional 280µl of DMSO was added (to 7.0%) and the tubes placed on ice for 5 minutes. 0.6ml aliquots were flash frozen and stored at -80°C until required.

21. Transformation of competent DH5α E.coli:

20µl aliquots of competent cells (**previously thawed on ice**) were added to a **prechilled** 1.5ml Eppendorf tube. One microlitre of precipitated ligation (resuspended in T10E1Na100) was then added to prechilled cells (maximum DNA 5ng) and incubated on ice for 30 minutes. The cells were heat shocked by placing them in a waterbath at 42°C for 45 - 60 seconds and then placed on ice. Eighty microlitres of SOC broth was added and the cells allowed to incubate for 60 minutes at 37°C. Ten and 90µl were plated on selective media (eg. ampicillin, tetracycline, etc.) and incubated overnight for 16-18 hours at 37°C.

22. Boiled plasmid preparations:

This is a quick screening procedure used to identify clones successfully transformed with the cloning vector.

A putative clone was inoculated in 5ml of BHI or LB broth containing the appropriate selection pressure and incubated at 37°C in a shaking waterbath for 16 to

18 hours. Fifteen hundred microlitres of the overnight culture was centrifuged in an Eppendorf tube for 1 minute, the supernatant removed and the pellet resuspended in 350µl of Boiled Prep Buffer (BPB) (appendix A). Twenty five µl of **fresh** lysozyme solution (10mg/ml in BPB) was added and the tube vortexed briefly. The tube was incubated at room temperature for 1 minute and placed in a 100°C water or sand bath for an additional 60 seconds. The sample was microfuged for 10-15 minutes at room temperature and the resultant insoluble slimy pellet from the bottom of the tube removed with a sterile toothpick. The sample was precipitated by adding 200µl of a 7.5M ammonium acetate solution and 600µl of isopropanol. The tube was vortexed and incubated for 10 to15 minutes at room temperature. After incubation the tube was microfuged for 10 to15 minutes at room temperature, the supernatant removed and the pellet allowed to air dry. The pellet was resuspended in 20-50µl of a T10E1/RNaseA/spermidine buffer (Tris-HCl 10mM pH 7.5, RNase A 10µg/ml, and 2mM spermidine). and a portion digested with restriction endonucleases as necessary, usually 5-10µl. An aliquot of the digested preparation was electrophoresed on a 1% ethidium bromide containing agarose gel with digested and undigested plasmid controls to assess results.

23. Large scale preparation of plasmid DNA:

A. plasmid amplification:

From a single bacterial colony harboring the desired plasmid, 5ml of LB or BHI broth containing an appropriate selection pressure was inoculated and allowed to

incubate for 18 to 24 hours at 37°C. One millilitre of this culture was used to inoculate 500ml of BHI broth containing an appropriate selection pressure and was incubated at 37°C with vigorous shaking until the culture achieved an $OD_{\lambda_{600}}$ reading of 0.6 to 0.8. Chloramphenicol was added to a final concentration of 25µg/ml and the culture incubated overnight at 37°C in a shaking waterbath.

B: harvesting and lysis of bacteria:

The 500ml broth culture was divided into two aliquots and centrifuged for 20 minutes at 4000 xg. The pellet from each tube was resuspended in 5ml of solution I (appendix A) containing lysozyme (5mg/ml). Solutions were transferred to polyallomer tubes and allowed to stand at room temperature for 5 minutes. Ten millilitres of solution II (appendix A) were added to each tube, shaken and stored on ice for 10 minutes. Seven and one half miliilitres of solution III (appendix A) was then added to each tube, shaken and stored on ice for another 10 minutes. The tubes were centrifuged for 30 minutes at 4000 xg, 4°C and the supernatant from each tube transferred to another polyallomer tube. Point six volumes of isopropyl alcohol was added and the tubes allowed to stand for 15 minutes at room temperature.The precipitate was collected by centrifugation at 12,000 xg for 30 minutes, room temperature, and the pellet from each tube resuspended in 10ml T10E1. The contents of each tube were combined to make 20ml.

C: isolation of plasmid DNA

To the 20ml suspension, 21.0g of CsCl, 1250µl of EtBr, and 625µl of Sarkosyl (10% solution) were added. The suspension was sealed in a 30ml ultracentrifuge tube

and centifuged for 18 to 24 hours at 175,000 xg. The plasmid band (bottom) was extracted with a 16 gauge needle and the collected DNA made to a 5ml volume with sterile water. Isoamyl alcohol (v/v) was added, gently mixed and the organic layer decanted. This procedure was repeated until both phases remained clear. The plasmid DNA was precipitated with 2 volumes of cold absolute ethyl alcohol and placed at -20 °C for 30 minutes. DNA was recovered by centrifugation at 8000 x g for 30 minutes, 4°C and the pellet resuspended in 5ml water, 1/10 vol NaOAc (3M), and 2 volumes cold absolute ethyl alcohol. Tubes were stored overnight at -20°C, centrifuged at 8000 xg for 15 minutes, air dried, and the pellet redissolved in 1ml T10E1.

24: Double stranded dideoxy DNA sequencing (USB-Sequenase method)

A: denaturing supercoiled plasmid:

Note: Three to four µg of plasmid DNA should be adequate for overnight exposure of ^{35}S-labeled sequence. Additional plasmid may lead to increased background and non-specific bands in sequencing lanes.

To 3-4µg of plasmid DNA, 2µl of **freshly** prepared 2N NaOH was added and the volume adjusted to 20µl with sterile water. This was incubated for 5 minutes at room temperature after which 2µl of 2M ammonium acetate, pH 4.5, and 50µl ice-cold absolute ethyl alcohol were added and the DNA frozen on dry ice (-80°C, 20 minutes). The denatured DNA was microfuged for 10 minutes at 4°C, and washed with 70% ethyl alcohol. The DNA was refrozen, microfuged, and air dried.

B: annealing of sequencing primer and sequenase reactions:

The denatured plasmid DNA was dissolved in a 10µl solution consisting of: 1pmole of sequencing primer, 2µl sequencing buffer (Sequenase kit) and sterile water to 10µl. The mixture was heated at 65°C, for 15 minutes; then room temperature for an additional 15 minutes. While primer was annealing, 2.5µl of each termination mixture (G, A, T, C) was added to four tubes per reaction and placed on ice.

C: labeling reactions:

To the annealed DNA, 1µl of 0.1M DTT, 2µl of dilute labeling mix, and 2µl of dilute Sequenase enzyme were added, mixed and allowed to incubate at room temperature for 5 minutes.

D: termination reactions:

Three and one half microlitres of the labeling reaction was transferred to each of the dNTP termination tubes and mixed. The reaction was allowed to proceed for 5 minutes at 37°C.

E: stop reaction:

Four microlitres of stop solution was added.

F: preparation of samples prior to loading sequencing gel:

Samples were heat denatured for 3 minutes at 95°C and immediately placed on ice. Two and one half microlitres of each sample was loaded onto a 4% or 8% polyacrylamide sequencing gel.

G: polyacrylamide sequencing gels:

Each sequencing reaction was run on a 4% double loaded (acrylamide:bis-

acrylamide 19:1) polyacrylamide gel and an 8% (acrylamide:bis-acrylamide 19:1) polyacrylamide wedge gel. The gel was fixed in a solution containing 7.5% glacial acetic acid and 7.5% methanol for 30 minutes. The gel was rinsed with distilled water, dried on Whatman filter paper using a Biorad gel drier for one hour and exposed to x-ray film for a period of up to seven days and the film developed.

25. Exo$^-$ Pfu sequencing (Stratagene).

Three µl of the appropriate ddNTP were added to each of four termination tubes and placed on ice. For each DNA template, the following reaction components were added, gently mixed and placed on ice; DNA template 200 fmol, sequencing primer 1 pmol, 10x sequencing buffer 4µl, radioactive label 10µCi (^{35}S), Exo$^-$ Pfu DNA polymerase 1µl, sterile water to 26µl, and DMSO 4µl. The final volume should be 30µl.

Note: It is essential to add DMSO last to avoid precipitation and to thoroughly mix all the components of the reaction mixture.

Seven µl of the reaction mixture were transferred into each of the four termination tubes and mixed thoroughly. The reactions were overlayed with mineral oil and the reactions cycled through an appropriate temperature profile.

Initial denaturation	95°C	5 minutes
Link to 30 cycles of:		
Denaturation	95°C	seconds
Annealing	60°C*	seconds
Extension	72°C	seconds

* Annealing temperature is dependent on the Tm of the oligonucleotide

Five µl of stop dye solution was added and mixed. Prior to loading samples on sequencing gel, the samples were heat denatured for 2-5 minutes at >85°C. Two to four µl of the samples were loaded onto sequencing gels previously described.

26. RNA Isolation from S.aureus.

Note: All water and solutions used in this procedure were first treated with diethylpyrocarbonate (DEPC) to inhibit RNase activity.

A 10ml BHI broth culture of S.aureus was grown at 37°C to mid-log phase. The cells were harvested by centrifugation at 12,000 xg for 10 minutes and the pellet resuspended in 0.5ml lysis buffer (Appendix A). The resuspended cells were then transferred to microcentrifuge tubes and frozen on dry ice. The samples were thawed and sonicated three times for 10 seconds with a microtip sonicator. The cell suspension should clear indicating cell lysis. After sonification, the lysed cells were incubated for 60 minutes in a 37°C water bath. The samples were extracted twice with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol and once with an equal volume of 24:1 chloroform:isoamyl alcohol. To 400µl of the aqueous phase, 15µl of a 5M NaCl solution was added and the microcentrifuge tube filled with ice-cold absolute ethanol. The tube was mixed and incubated overnight at -20°C. The precipitated RNA was microfuged for 15 minutes at 4°C, the pellet rinsed with 500µl of ice-cold 70% ethanol and air dried. The pellet was redissolved in 95µl of DNase digestion buffer (Appendix A), 4µl of a 2.5mg/ml solution of RNase-free DNase I added and the solution incubated for 60 minutes at 37°C. The redissolved pellet was then re-

extracted with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol. The aqueous layer was removed and 100µl of T10E1 buffer was added to the remaining organic layer and mixed. This was microfuged for 5 minutes at room temperature and the two aqueous layers pooled. A 24:1 chloroform:isoamyl alcohol extraction was performed after which 10µl of 5M NaCl and 600µl of absolute ethanol was added and mixed. The tube was incubated overnight at -20°C and the precipitate collected by microfuging 15 to 30 minutes at 4°C. The pellet was rinsed with 70% ethanol, air dried, and redissolved in 100µl of DEPC treated water. Purity and quantity of RNA was determined spectrophotometrically.

27. Northern blot analysis of RNA:

A: Agarose/Formaldehyde gel electrophoresis:

Twenty µg of total cellular RNA (measured spectrophotometrically) was loaded onto a 2.2M formaldehyde containing 1% agarose gel and electrophoresed in 1x MOPS buffer. Duplicate samples were loaded on one side of the gel and were visualized with EtBr after electrophoresis. Samples on the other side of the gel were transferred to a nylon filter.

B: Transfer of RNA to membrane:

The unstained portion of the gel was rinsed several times in sterile DEPC treated distilled water to remove the formaldehyde. The gel was soaked in 10 volumes of 0.05M NaOH / 1.5M NaCl for 30 minutes and then replaced with 10 volumes of 0.5M Tris-HCl (pH 7.4) / 1.5M NaCl. After soaking for 20 minutes the solution was

replaced with 10 volumes of 10x SSC and allowed to soak for 45 minutes. The remaining transfer procedure was previously described in section 13; Southern blotting.

C: Hybridization analysis:

The nylon membrane was soaked in 6x SSC, placed in a hybridization tube and 10ml of RNA hybridization solution added (appendix A). The membrane was incubated at 42°C for 45 minutes. A 100 bp Ssp I DNA fragment, internal to the norA coding region was used to probe the membrane. Prior to use the DNA fragment was labeled with ^{32}P using a random primer kit from Gibco. The probe was denatured at 100°C for 10 minutes and introduced into the hybridization tube. The membrane was allowed to incubate overnight at 42°C. The hybridization solution was removed after incubation and replaced with an equal volume of 2x SSC / 0.1% SDS. This solution was incubated at room temperature for 5 minutes, fresh solution added and the membrane incubated an additional 5 minutes. Two additional 5 minute incubations were performed with 0.2x SSC / 0.1% SDS. The membrane was then removed from the tube, placed in an autoradiograph cassette and exposed to x-ray film at -70° for several days before developing.

Results

1. Antimicrobial Susceptibility:

Minimum inhibitory concentrations (MICs) of all clinical isolates and ATCC strains used throughout this study are listed in table 8. With the exception of strains #603, #609, #614, and #617, all isolates were susceptible to all fluoroquinolones tested, (fluoroquinolone resistant breakpoint: 2.0μg/ml). MICs of cloxacillin, cephalexin, and gentamicin were only determined for those strains used in the serum PAE experiments. In addition MICs to lomefloxacin and sparfloxacin were determined late in the study and only selected strains were tested. MICs of ciprofloxacin in strains #603, #609, #614, and #617 were determined using only a doubling dilution technique.

2. Generation and stability of resistant mutants:

Selection of fluoroquinolone resistant S.aureus mutants was done to provide resistant isogenic mutants for PAE experiments. Our goal was to compare the PAE in these and their parental strains to determine if any difference in the PAE existed between resistant and susceptible isolates. The maximum MICs attained by serial passage of susceptible strains on antimicrobial containing BHI agar are listed in tables 9 and 10. All strains acquired low levels of resistance very rapidly, but development of high level resistance slowed as the MIC approached high levels. On the first serial passage at any new antimicrobial concentration, all strains exhibited a pleotrophic colony morphology. Upon repeated sub-culture a single colony type was evident. Morphologically, resistant mutants appeared similar to their susceptible parental

Table 8. Minimum inhibitory concentrations of clinical isolates and ATCC strains.

MICs (μg / ml)

Strain	Cfx♦	Pfx	Nfx	Lome	Spx	Clox	Ceph	Gent
F447	0.36	0.30	1.0	----	----	----	----	----
F495	0.34	0.30	0.76	----	----	----	----	----
F629	0.32	0.46	0.66	----	----	----	----	----
F651	0.34	0.25	0.68	0.40	0.12	0.25	2.0	0.50
F988	0.25	0.32	0.45	0.32	0.12	0.25	2.0	0.50
ATCC 25923	0.36	0.46	0.72	0.40	0.12	----	----	----
ATCC 29213	0.32	0.50	0.82	----	----	----	----	----
F192*	0.36	0.40	1.0	0.38	0.25	0.25	2.0	0.50
F201*	0.50	0.44	0.88	----	----	----	----	----
F321*	0.50	0.48	1.0	----	----	----	----	----
#603*	1024	----	----	----	----	----	----	----
#609*	1024	----	----	----	----	----	----	----
#614*	512	----	----	----	----	----	----	----
#617*	256	----	----	----	----	----	----	----
ATCC 33592*	0.50	0.50	1.0	----	----	----	----	----

* Methicillin resistant strain

♦cfx = ciprofloxacin, pfx = pefloxacin, nfx = norfloxacin, lome = lomefloxacin, spx = sparfloxacin, clox = cloxacillin, ceph = cephalexin, gent = gentamicin

strains, however a reduced generation time was observed in many of the strains. Most of the resistant mutants appeared to be quite stable as few reversions to wild type MICs were observed after repeated subculture on antimicrobial free agar.

Table 9. Maximum MICs of laboratory derived resistant mutants.

MICs (μg / ml)

Strain	Cfx		Pfx		Nfx	
	wt*	res	wt	res	wt	res
F651	0.34	44.0	0.40	51.0	0.62	128
F495	0.34	44.0	0.30	51.0	0.76	128
F629	0.32	44.0	0.46	51.0	0.66	128
F988	0.25	44.0	0.34	51.0	0.78	128
F192	0.36	44.0	0.40	51.0	1.0	128
ATCC 25923	0.44	36.0	0.50	44.0	0.88	128

* wt = wild type, res = resistant mutant

3. Postantibiotic Effect:

a) Comparison of PAEs in Methicillin susceptible (MSSA) and Methicillin resistant (MRSA) S.aureus.

The PAEs in several MSSA and MRSA strains were evaluated using ciprofloxacin, pefloxacin, and norfloxacin to determine if any significant difference in

the duration of the PAE could be detected between MS and MR S.aureus strains. Three MS and three MR S.aureus strains were examined. PAEs were evaluated at 1x,

Table 10. Strain numbers of resistant S.aureus isolates F651 and F192

Strain	MIC(μg/ml)	Strain	MIC(μg/ml)
F651-wt	0.35	F192-wt	0.35
R1	0.7	R8	0.7
R2	1.4	R9	1.4
R3	2.8	R10	2.8
R4	5.6	R11	5.6
R5	11	R12	11
R6	22	R13	22
R7	44	R14	44

4x and 10x MIC using a two hour exposure (fig.8). Results of this initial study are found in table 11 and all data presented are the resultant means of three independent experiments. No significant difference in the duration of the PAE was observed between any of the MS or MR S.aureus strains evaluated ($p>0.05$). This observation was true at all levels of the MIC tested.

Table 11. The PAEs in MSSA and MRSA of three fluoroquinolones.

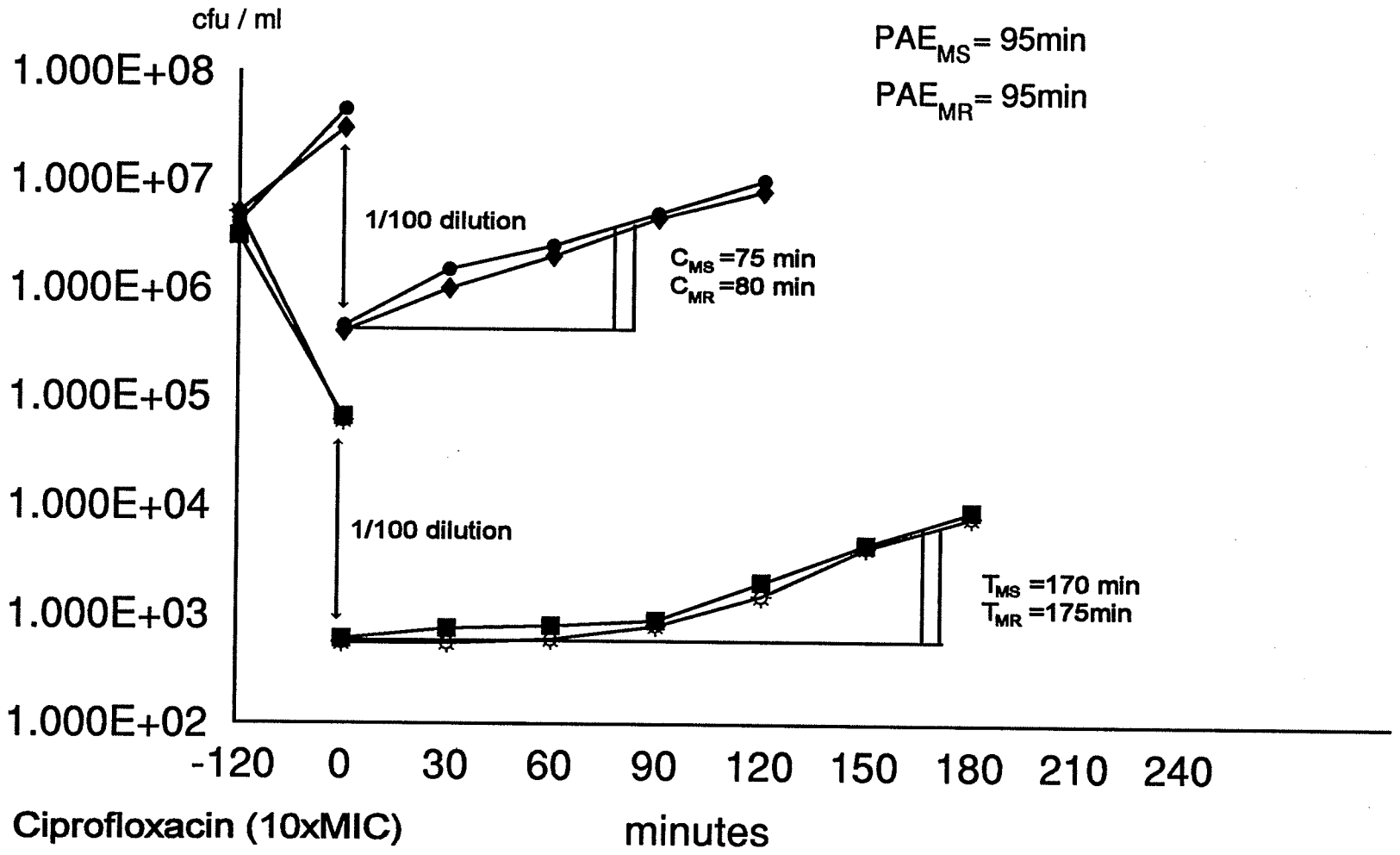
PAEs (minutes)

	Ciprofloxacin			Pefloxacin			Norfloxacin		
Strain	1x MIC	4x MIC	10x MIC	1x MIC	4x MIC	10x MIC	1x MIC	4x MIC	10x MIC
F447	4.8	55	92	4.9	54	88	5.4	49	81
F629	5.2	66	108	4.8	60	92	4.2	51	87
F988	4.4	72	88	5.2	68	86	5.1	43	79
F192*	6.7	76	114	4.3	69	94	6.3	57	86
F201*	4.0	61	93	5.8	56	86	4.0	50	81
F321*	5.1	60	91	5.0	51	88	4.9	48	82

* Methicillin resistant strain

b) Reproducibility of the Postantibiotic Effect:

Two S.aureus strains (F495 and F192) were chosen for all experiments to determine the reproducibility of the PAE. Both strains were exposed for 2 hours at three concentrations of each fluoroquinolone, 1x, 4x, and 10x MIC. Ciprofloxacin, norfloxacin, and pefloxacin were chosen as the test quinolones. All experiments were repeated 7 times. The individual results as well as the mean and standard deviation of these experiments are found in table 12. At 1x MIC, no significant difference was observed between any of the fluoroquinolones tested ($p>0.05$). At both 4x and 10x MIC, the PAE of ciprofloxacin was significantly longer than that of norfloxacin or pefloxacin ($p<0.05$).


cfu / ml
1.000E+08
1.000E+07
1.000E+06
1.000E+05
1.000E+04
1.000E+03
1.000E+02
PAE$_{MS}$= 95min
PAE$_{MR}$= 95min
1/100 dilution
C$_{MS}$ =75 min
C$_{MR}$ =80 min
1/100 dilution
T$_{MS}$ =170 min
T$_{MR}$ =175min
-120 0 30 60 90 120 150 180 210 240
Ciprofloxacin (10xMIC)
minutes

82

Figure 8. A graphical time course representation of the PAE in methicillin susceptible (MSSA) and methicillin resistant (MRSA) S.aureus. A MSSA (F651) and a MRSA (F192) were exposed to ciprofloxacin at 10xMIC for 2 hours. The antimicrobial was removed with a 1:100 dilution. MS growth control ●, MR growth control ◆, MS test culture ■, MR test culture ✡

Table 12. Reproducibility of the PAE with fluoroquinolones in S.aureus

	PAE (minutes)								
	Ciprofloxacin			Pefloxacin			Norfloxacin		
Trial #	1x MIC	4x MIC	10x MIC	1x MIC	4x MIC	10x MIC	1x MIC	4x MIC	10x MIC
1	5.5	58	93	5.0	36	86.5	6.0	44	78
2	-8.8	52	92	4.0	40.5	84	2.0	46	72
3	8.0	59	95	8.0	36.5	87	9.0	30	77
4	13.0	61	85	-7.0	44	88	-8.5	42.5	75
5	6.5	51	93	6.0	37.5	81	7.0	45	77
6	5.0	59	91	16	35	90.5	7.5	45	78
7	4.0	48	92	2.0	27	85.5	5.0	40	76
Mean	4.8	55.4	91.6	4.9	36.6	86.1	4.0	41.8	76.1
SD	6.4	5.0	3.2	6.9	5.3	3.0	5.9	5.6	2.1

c) The effect of fluoroquinolone hydrophobicity on the PAE in S.aureus

Four fluoroquinolones, sparfloxacin, pefloxacin, norfloxacin, and lomefloxacin were selected to examine the effect of hydrophobicity on the PAE. All PAE determinations were performed by exposing a logarithmic phase culture to the test agent for two hours at 4x and 10x MIC. The relative hydrophobicity of the fluoroquinolones was determined by their partition coefficients in n-octanol - 0.1M phosphate buffer (table 13). Of the agents examined, sparfloxacin was the most hydrophobic followed by pefloxacin > lomefloxacin > and norfloxacin.
All PAE calculations were based on three independent experiments. PAE results are displayed in table 13. A direct relationship between the duration of the PAE and the hydrophobicity of the fluoroquinolones was observed ($p<0.05$). Sparfloxacin produced the longest PAE followed by pefloxacin, lomefloxacin, and norfloxacin. A similar relationship was observed with respect to the agents MICs. As the hydrophobicity of the tested agent increased, the MIC decreased.

d) PAE in Human Serum:

While the PAE has been demonstrated *in vivo* using animal models, practical considerations limit the majority of investigations to more conventional *in vitro* laboratory models. In an attempt to bridge the gap between *in vivo* studies and bench level experiments, we examined the PAE in vitro using human serum as a replacement for MHB. Human serum was obtained from healthy adult volunteers with no history of antimicrobial therapy in the previous two months and from the Manitoba Red Cross.

Table 13. The hydrophobicity profiles and PAEs (minutes) of Sparfloxacin, Pefloxacin, Lomefloxacin, and Norfloxacin.

		F988			F651		
Fluoroquinolone	Partition coefficient*	MIC µg/ml	PAE 4x MIC	PAE 10x MIC	MIC µg/ml	PAE 4x MIC	PAE 10x MIC
Sparfloxacin	0.97	0.12	75	115	0.12	70	120
Pefloxacin	0.36	0.32	70	100	0.25	60	100
Lomefloxacin	0.12	0.32	60	90	0.40	60	90
Norfloxacin	0.07	0.45	55	85	0.68	55	80

* partition coefficient in n-octanol - 0.1M phosphate buffer, pH 7.2

Table 14 displays results of MICs determined in MHB or human serum. Note that while MICs of the fluoroquinolones were determined using arithmetic dilutions, MICs of gentamicin, cloxacillin, and cephalexin were calculated from a series of doubling dilutions. MICs of ciprofloxacin and norfloxacin increased less than two fold in serum whereas a four fold increase was noted for pefloxacin. Serum had little effect on the MIC of cephalexin, however, a sixteen fold increase in the MIC was noted for cloxacillin due to its high protein binding. A slight decrease in the MIC of gentamicin in serum was observed.

Table 14: The MICs of Antimicrobial Agents in MHB and Human Serum

MIC (μg/ml)

Strain	Cipro		Norflox		Peflox		Gent		Clox		Ceph	
	M	S	M	S	M	S	M	S	M	S	M	S
F651	0.34	0.50	0.62	0.85	0.40	1.3	0.50	0.12	0.25	4.0	2.0	3.0
F988	0.25	0.45	0.45	0.80	0.38	1.6	0.50	0.25	0.25	4.0	2.0	3.0
#29213	0.32	0.70	0.82	1.1	0.50	3.6	---	---	---	---	---	---

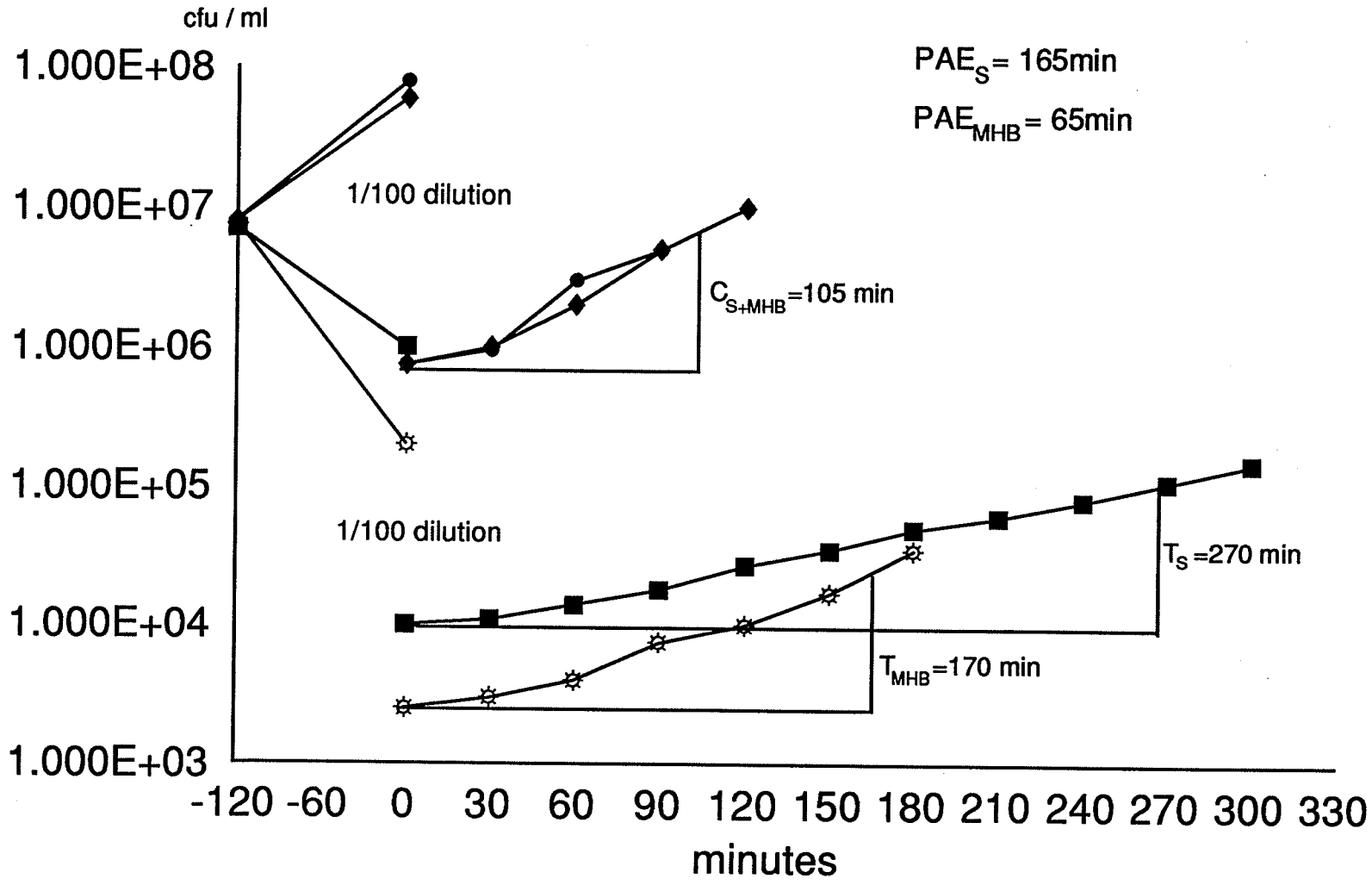
Growth kinetics of organisms in MHB and human serum were remarkably similar. In the initial two hour incubation period, growth controls in both MHB or serum grew at the same rate and achieved the same final cell densities. In addition, both growth controls were repeatedly observed to increase one log cfu/ml 80-90 minutes after dilution (fig.9).

Human serum was found to have a profound effect on the duration of the PAE. In the majority of experiments the PAE in serum was double ($p<0.05$) that of the corresponding experiments in MHB. (figs.10 and 11). Of interest is the observation that while the PAE appeared to be enhanced in human serum the bactericidal activity was reduced approximately 1 log (fig.9). Altering the pH of serum from 7.8-7.9 to 7.0-7.1 to reflect the pH of MHB had no effect on the PAE. Heat treating the serum at 56°C for 30 minutes significantly lowered the PAE of cells exposed to 10x MIC ciprofloxacin (2 hours) from 3.2 to 2.4 hours, a 25% decrease ($p<0.05$).

To confirm that this effect was not limited to the fluoroquinolones, similar experiments were repeated with gentamicin, and two β-lactam antimicrobials; cloxacillin and cephalexin. Both gentamicin and cephalexin had significantly longer PAEs in serum than in MHB with increases of 2 and 2.5 fold, respectively. The cloxacillin PAE in serum was reduced from 1.2 hours in MHB to 0.35 hours. When the high protein binding of cloxacillin was corrected for and the bacteria exposed to a higher concentration of antimicrobial, the PAE in serum was increased to 2.3 hours.

e) PAE in Fluoroquinolone Resistant S.aureus.

All previous PAE experiments had been conducted using fluoroquinolone susceptible S.aureus. In fact to the best of our knowledge, "susceptible strains" have been exclusively examined by other investigators. We therefore devised a series of experiments that would compare the PAE in fluoroquinolone resistant mutants and


cfu / ml
1.000E+08
1.000E+07
1.000E+06
1.000E+05
1.000E+04
1.000E+03
PAE$_S$= 165min
PAE$_{MHB}$= 65min
1/100 dilution
C$_{S+MHB}$=105 min
1/100 dilution
T$_S$ =270 min
T$_{MHB}$=170 min
-120 -60 0 30 60 90 120 150 180 210 240 270 300 330
minutes

68

Figure 9. A time course representation of the PAE in MHB and human serum. S.aureus (F651) was exposed to ciprofloxacin at 4x MIC for 2 hours in MHB or human serum. Antimicrobial was removed by a 1:100 dilution into fresh MHB or serum. Serum growth control ●, MHB growth control ◆, serum test culture ■, MHB test culture ✡

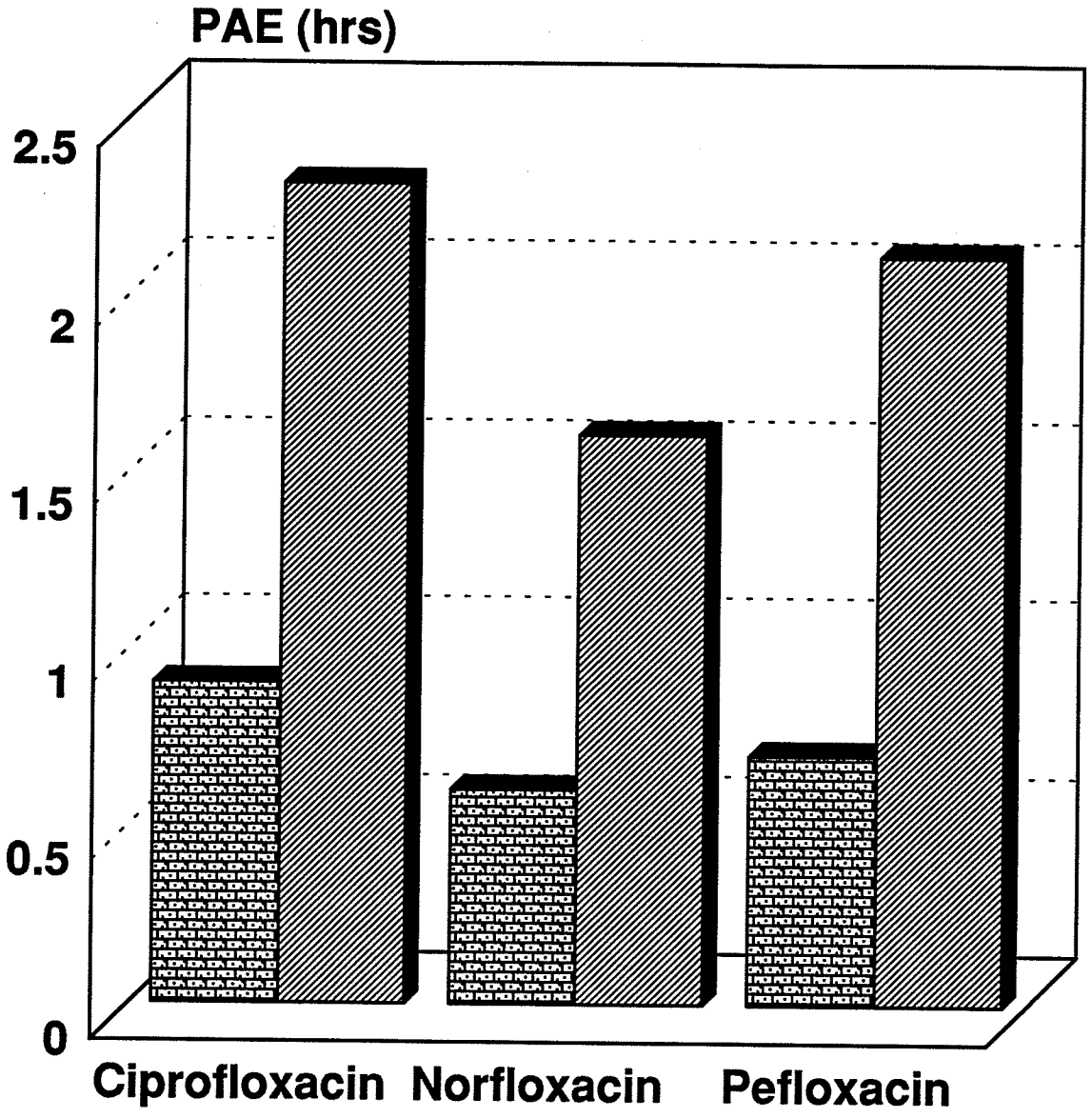

PAE (hrs)
2.5
2
1.5
1
0.5
0
Ciprofloxacin
Norfloxacin
Pefloxacin

Figure 10. Results of PAE experiments performed in MHB or human serum at 4x MIC. S.aureus (F651) was exposed to ciprofloxacin, norfloxacin, or pefloxacin at 4x MIC for 2 hours. Experiments were performed in either MHB ▩ or human serum ▩.

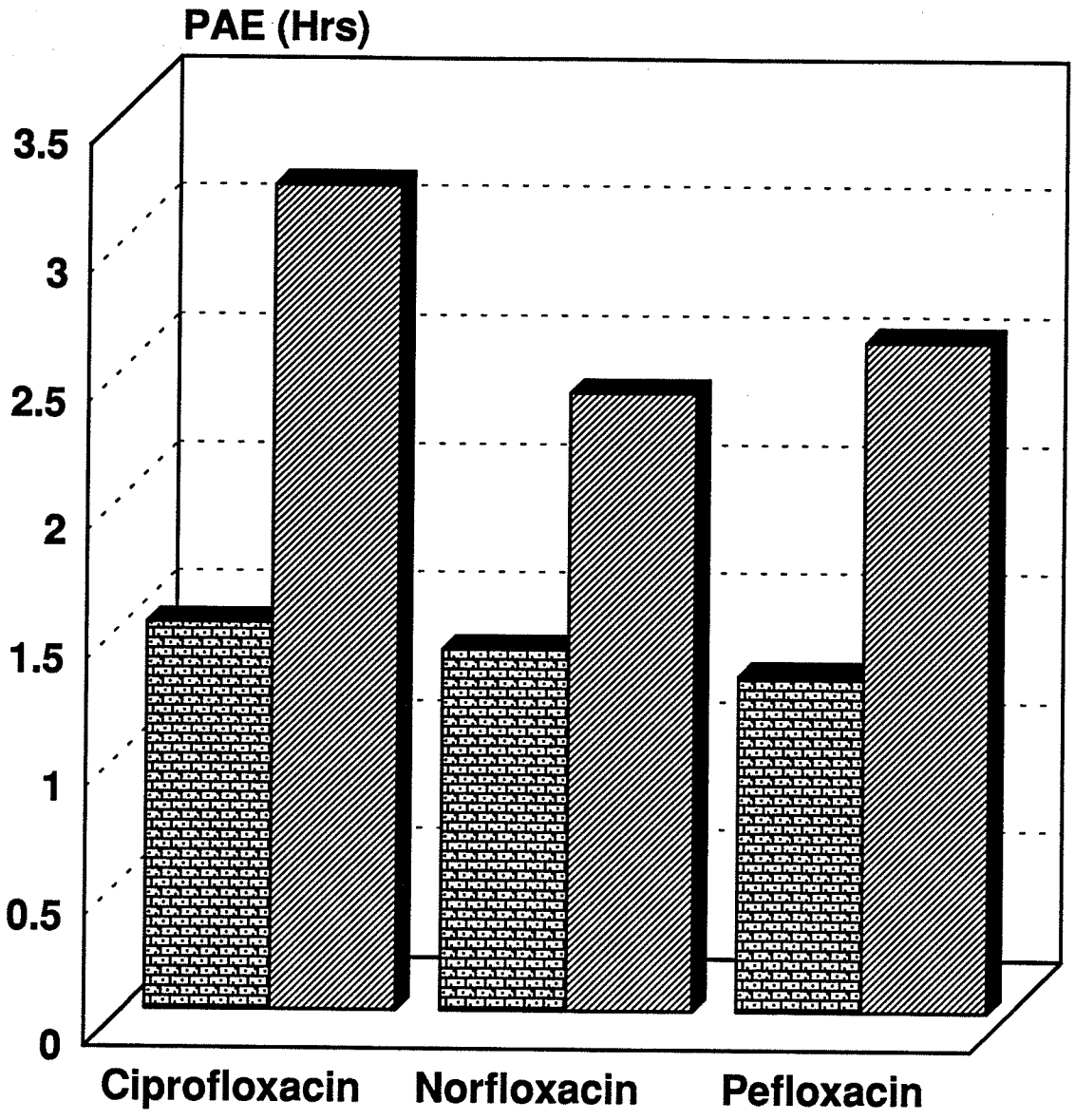

PAE (Hrs)
3.5
3
2.5
2
1.5
1
0.5
0
Ciprofloxacin
Norfloxacin
Pefloxacin

Figure 11. Results of PAE experiments performed in MHB or human serum at 10x MIC. S.aureus (F651) was exposed to ciprofloxacin, norfloxacin, or pefloxacin at 10x MIC for 2 hours. Experiments were performed in either MHB ▩ or human serum ▩.

their parental wild type S.aureus strains. Resistant mutants chosen for study are described in section 2 and their MICs detailed in tables 9 and 10.

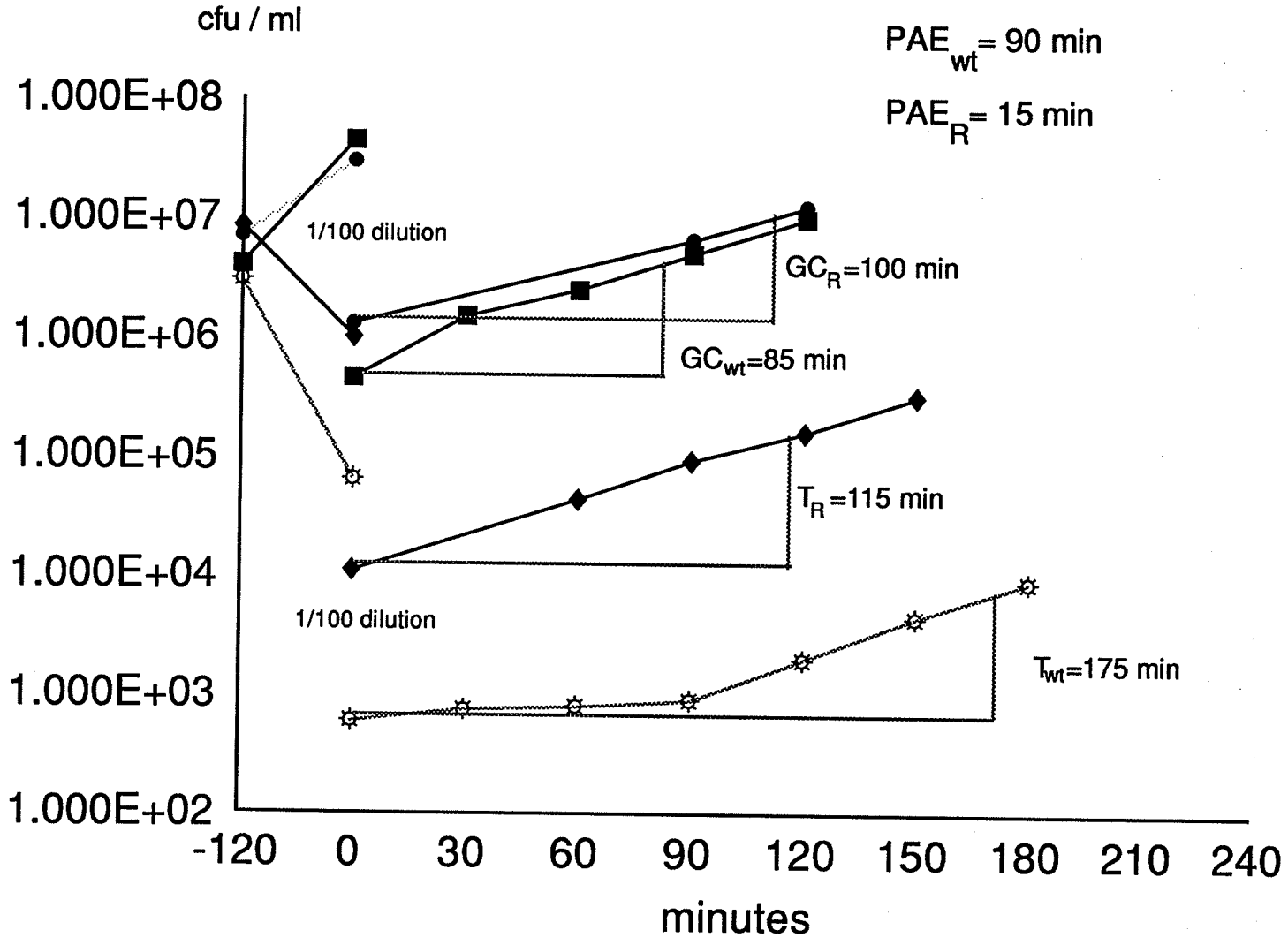
Initial experiments compared the PAEs in five fluoroquinolone resistant mutants and their isogenic wild type counterparts. Resistant mutants had ciprofloxacin MICs of 22 μg/ml compared with 0.25-0.36 μg/ml for the wild type (susceptible) strains. We had hypothesized that the PAE in the resistant mutants would be similar to the PAE seen in wild type strains assuming each was exposed to the same multiple of the MIC. The resistant organisms would be exposed to a higher concentration of antimicrobial owing to their elevated MICs.

Table 15 details the results of our initial experiments. Wild type strains, when exposed to ciprofloxacin or pefloxacin at 4x or 10x MIC, produced a PAE of approximately 50 to 60 and 90 to 100 minutes, respectively. Two of the resistant strains when exposed to ciprofloxacin or pefloxacin at 4x or 10x MIC had little or no PAE. A time course graph of the initial experiments is illustrated in figure 12. Unlike the wild type strains, resistant strains exposed to ciprofloxacin or pefloxacin resumed logarithmic growth similar to or parallel with their growth controls.

To determine if loss or abrogation of the PAE could be detected in other unrelated fluoroquinolone resistant S.aureus we examined the PAE of ciprofloxacin in high level fluoroquinolone resistant S.aureus (fig.13) obtained from Toronto. These strains were clinical isolates and had ciprofloxacin MICs ranging from 256 to 1024 μg/ml. As we had originally predicted these strains had PAEs of approximately 50 to 70 minutes at 4x MIC and PAEs of approximately 130 to 150 minutes at 10x MIC.

Table 15. The PAE of ciprofloxacin in isogenic fluoroquinolone susceptible and resistant S.aureus.

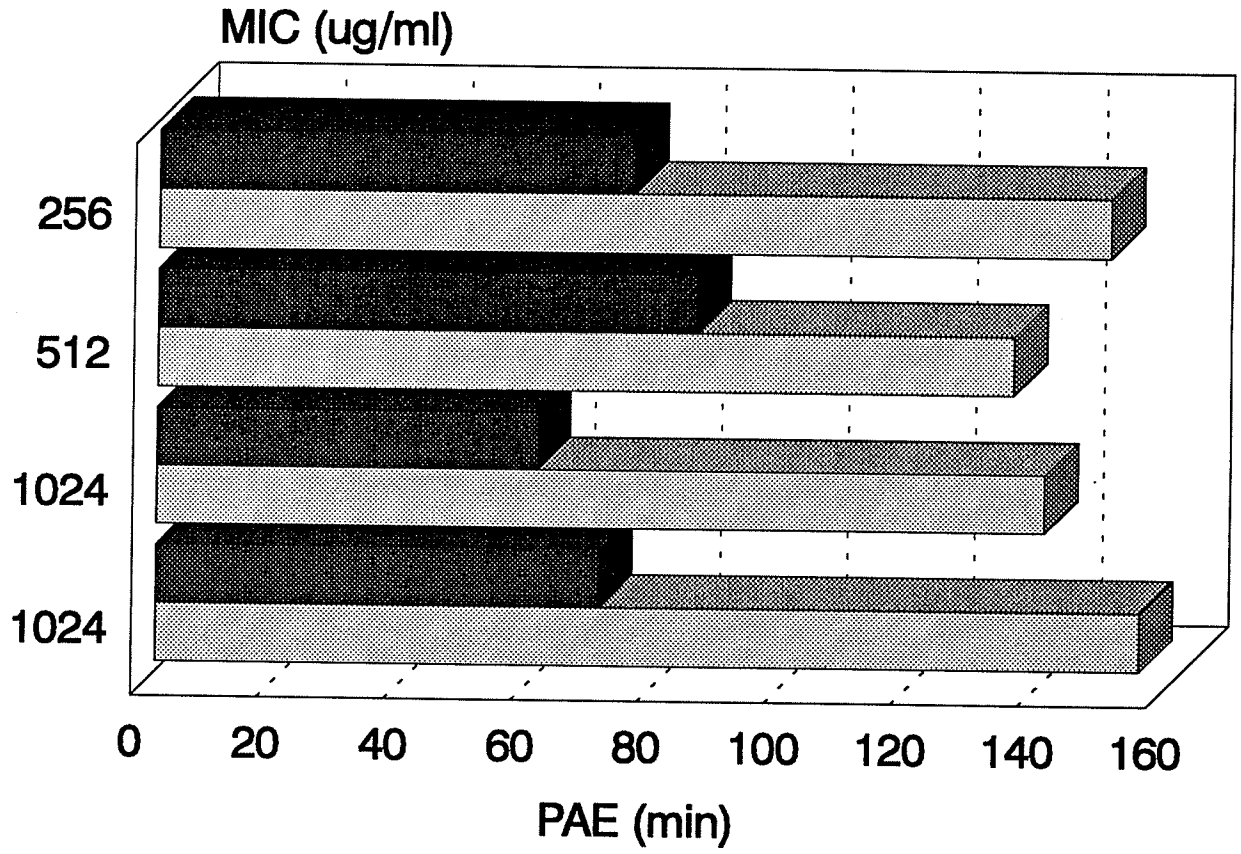
		PAE (minutes)			
S.aureus strain	MIC (μg/ml) ciprofloxacin	PAE of ciprofloxacin		PAE of pefloxacin	
		4x MIC	10x MIC	4x MIC	10x MIC
F192	0.36	60	100	50	90
F192 R-17	22.0	0-15	0-15	0-15	0-15
F495	0.34	60	100	60	90
F495-R	22.0	60	90	50	90
F629	0.32	70	110	60	95
F629-R	22.0	70	100	55	90
F651	0.34	60	100	60	90
F651 R-6	22.0	0-15	0-15	0-15	0-15
F988	0.25	75	90	70	90
F988-R	22.0	65	90	60	95


cfu / ml
PAE wt = 90 min
PAE R = 15 min
1.000E+08
1.000E+07
1.000E+06
1.000E+05
1.000E+04
1.000E+03
1.000E+02
1/100 dilution
GC R =100 min
GC wt =85 min
T R =115 min
1/100 dilution
T wt =175 min
-120
0
30
60
90
120
150
180
210
240
minutes

97

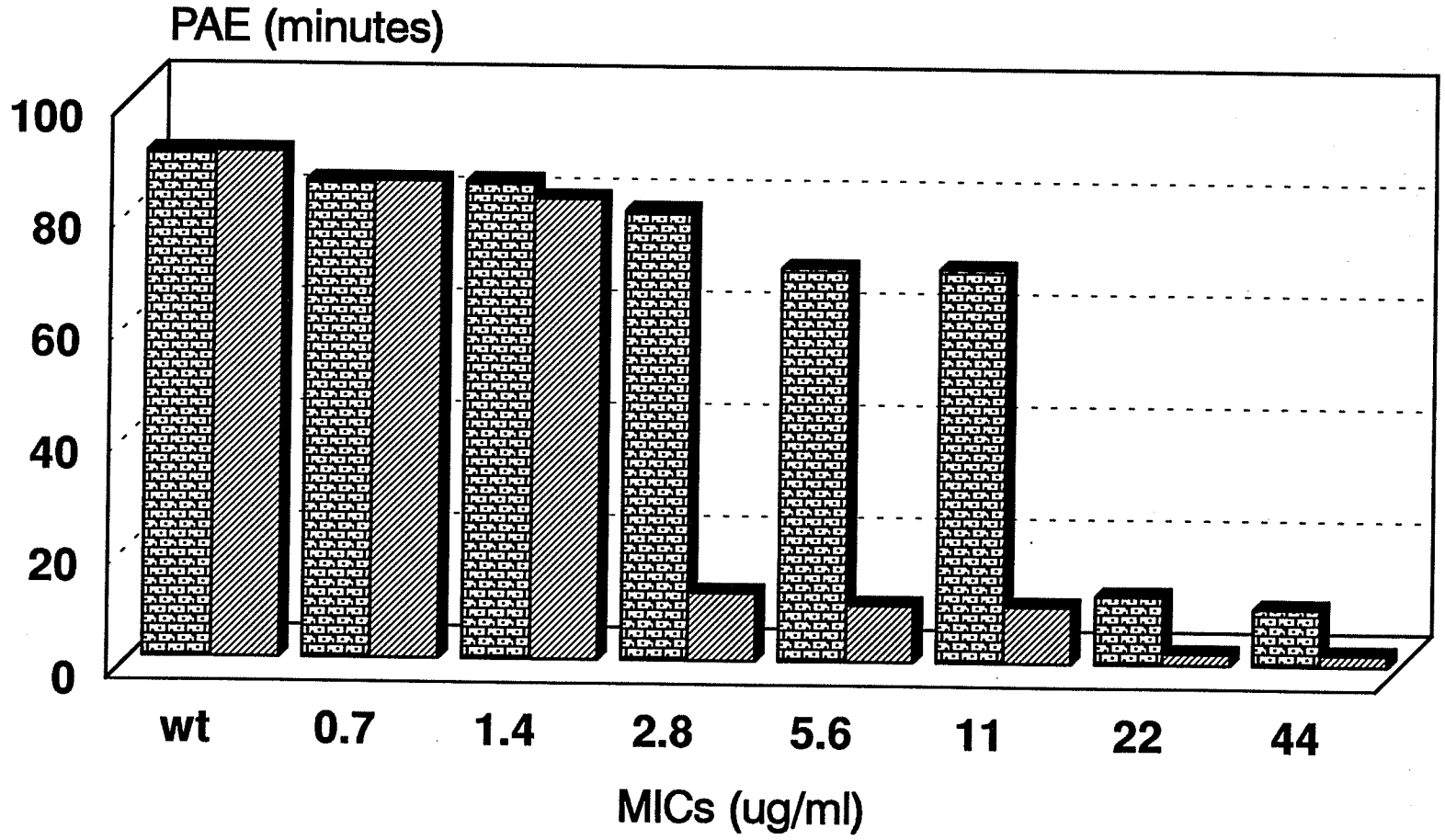
Figure 12. A time course representation of PAE experiments in isogenic wild type and resistant S.aureus. Isogenic S.aureus strains (F651 MIC = 0.35μg/ml) and (F651 R-6 MIC = 22.0μg/ml) were exposed to 10x MIC ciprofloxacin for 2 hours. Antimicrobials were removed with a 1:100 dilution. F651 growth control ■, F651 test culture ✡, F651 R-6 growth control ●, F651 R-6 test culture ◆.

Given these results we repeated the PAE experiments with our resistant mutants. It should be noted that as resistant mutants were selected in the laboratory they were stocked at each level of resistance. We therefore had a collection of isogenic strains with a wide spectrum of MIC phenotypes ranging from wild type to highly resistant. A series of PAE experiments were performed with wild type strains and repeated with their isogenic resistant counterparts at each level of ciprofloxacin resistance. Results of these experiments are detailed in figure 14. Wild type strains F651 and F192, when exposed to ciprofloxacin at 10x MIC, had PAEs of approximately 90 to 100 minutes. When repeated with isogenic strains having ciprofloxacin MICs double that of the wild type strains the duration of the PAEs were not significantly reduced ($p>0.05$). Similarly with isogenic strains having ciprofloxacin MICs of 1.4 μg/ml,(R2 and R9), only a slight reduction in the duration of the PAE was observed but was not found to be statistically significant ($p>0.05$). When experiments were conducted with isogenic strains having MICs of 2.8 μg/ml, (R3 and R10), a significant difference in the duration of the PAEs was observed. Strain F192 R-10 only produced a PAE of approximately 15 minutes. The duration of the PAE in strain F651 R-3 was not significantly different than its wild type strain ($p>0.05$), however, the 15 minute PAE observed in strain F192 R-10 was significantly different from both its wild type parental strain as well as F651 R-3 ($p<0.05$). Experiments performed with strains having ciprofloxacin MICs of 5.6 and 11.0 μg/ml, (R4/5 and R11/12), had similar results. Ciprofloxacin continued to produce a PAE of only 10 to 15 minutes in strain F192-R whereas the PAE in strain F651-R was still prolonged. Although the PAE in



100

Figure 13. Results of PAE experiments performed in high level fluoroquinolone resistant clinical isolates of S.aureus. Four clinical isolates of S.aureus with MICs to ciprofloxacin ranging from 256 to 1024 μg/ml were exposed to ciprofloxacin at 4x and 10x MIC for 2 hours. Antimicrobials were removed by a 1:100 dilution. 4x MIC, ■ 10x MIC ▒.



102

Figure 14. Abrogation of the PAE with isogenic high level fluoroquinolone resistant S.aureus. Wild type, low level and high level fluoroquinolone resistant isogenic strains of S.aureus were exposed to ciprofloxacin at 10x MIC for 2 hours. Antimicrobials were removed with a 1:100 dilution. F651, F651-R ■; F192, F192-R ■

strain F651-R was still quite apparent, the 20 minute reduction in the duration of the PAE at these levels was considered to be statistically different from its wild type parental strain($p<0.05$). When strains, (F651 R6/7 and F192 R-13/14), having ciprofloxacin MICs of 22.0 and 44.0 μg/ml were exposed to 10x MIC for 2 hours, both strains displayed little or no PAE.

f) Loss of PAE

From the results of the above experiments it appeared that development of fluoroquinolone resistance was necessary, but not sufficient to cause abrogation of the PAE. Given the apparent link or association between fluoroquinolone resistance and the loss of PAE, we examined the three known genetic loci known to confer fluoroquinolone resistance; gyrA, gyrB, and norA.

Genomic DNA from susceptible and resistant (Res PAE^+ and Res PAE^-) strains was digested with several restriction enzymes, electrophoresed, and transferred to nitrocellulose filters. The filters were subsequently probed with oligonucleotides internal to the open reading frame (ORF) of gyrA, gyrB, and norA. The resulting restriction fragment length polymorphism (RFLP) patterns did not indicate any change on gross examination between wild type and resistant PAE^+ or PAE^- strains (fig.15 and 16). gyrA amplified from high level fluoroquinolone resistant mutants and incubated with the restriction enzyme Hinf I did however exhibit the loss of a Hinf I site (fig. 17).

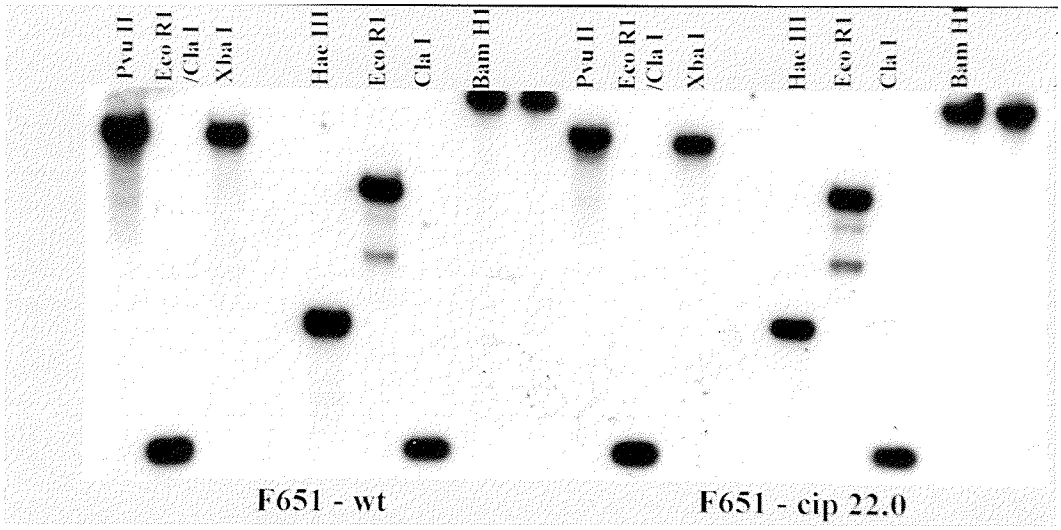
As the RFLP data was inconclusive we undertook the task of DNA sequencing

the entire ORF of gyrA, gyrB, and norA from our three types of S.aureus strains (wt PAE^+, Res PAE^+, Res PAE^-). The amplified gene products were sequenced directly via cycle sequencing and using a sequenase protocol after sub-cloning into bluescribe vectors. Both DNA strands from each gene were sequenced and any observed base change confirmed by sequencing several clones. Tables 16, 17, and 18 detail the DNA sequence from norA, gyrB, and gyrA, respectively.

No sequence changes were detected in the ORF of norA or gyrB. DNA sequences in both wild type and resistant strains were identical. In addition our DNA sequences from both norA and gyrB matched previously published DNA sequences from S.aureus (Brockbank,1993; Margerrison,1992; Ubukata,1989; Yoshida et al,1990). We did uncover two base changes in gyrA (table 19). The first base change was in fact a discrepancy between our DNA sequence and published sequences. Of interest is the fact that this change was only detected in S.aureus isolates (wt and resistant) collected from the clinical laboratory at the Health Sciences Centre, Winnipeg. This discrepancy was not detected in isolates collected from other sources. The base change occurred at nucleotide position 75 where a thymine residue was replaced with a cytosine residue. This results in an amino acid change from tyrosine to cysteine.

The second substitution, a cytosine to thymine transition was detected only in those strains exhibiting high level fluoroquinolone resistance (table 19). The transition resulted in an amino acid change from serine to leucine. The mutation was detected in both high level fluoroquinolone resistant isolates as well as laboratory mutants having

Figure 15. Southern hybridization analysis of chromosomal DNA isolated from fluoroquinolone susceptible and resistant S.aureus mutants. The blot was hybridized to a 20 mer ^{32}P-oligonucleotide internal to the gyrA ORF.



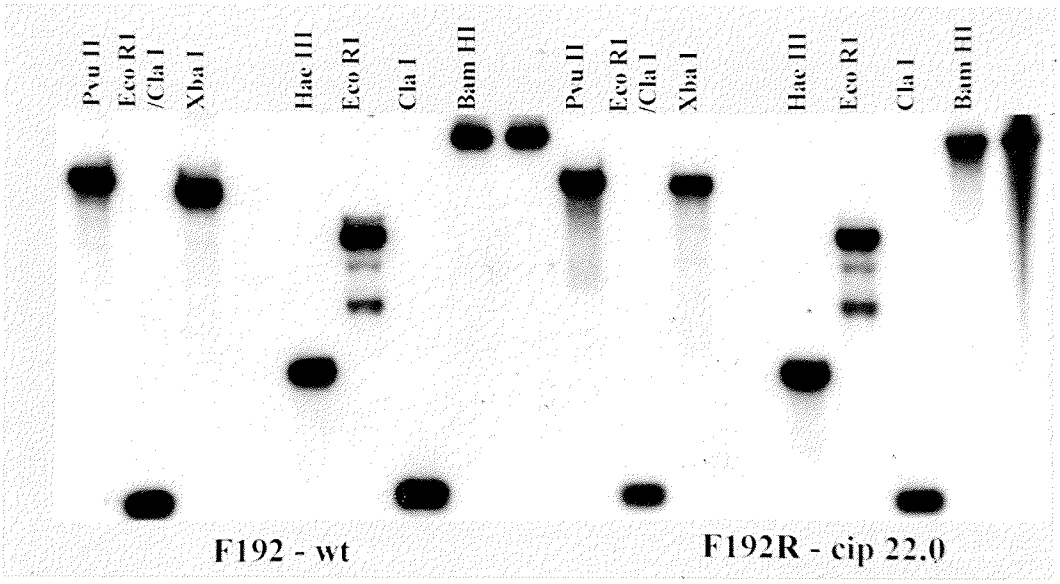
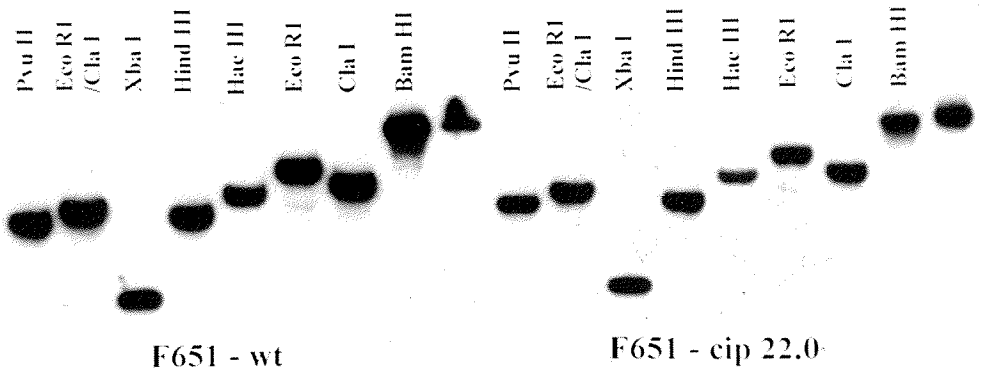


Figure 16. Southern hybridization analysis of chromosomal DNA isolated from fluoroquinolone susceptible and resistant S.aureus mutants. The blot was hybridized to a 20 mer ^{32}P-oligonucleotide internal to the norA ORF.



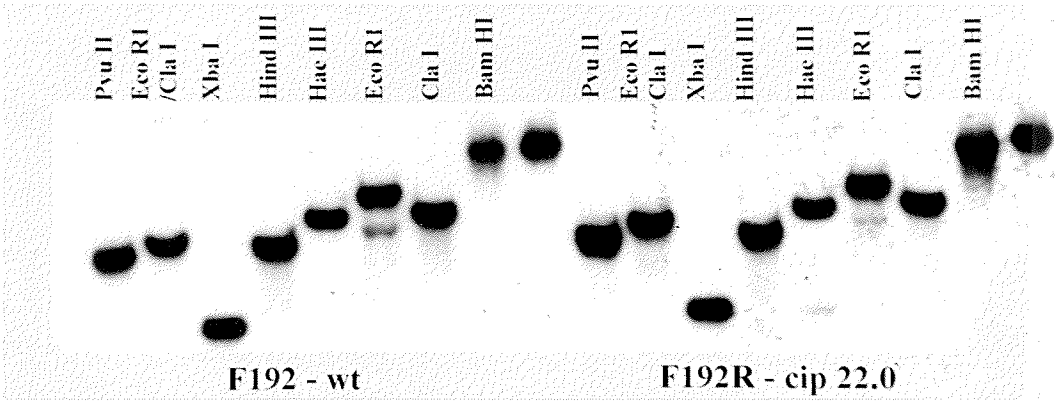


Figure 17. Southern hybridization analysis of a 2.4 Kb gyrA PCR fragment amplified from fluoroquinolone susceptible and resistant S.aureus. The PCR fragments were digested overnight with Hinf I. The blot was hybridized to a 20 mer ^{32}P-oligonicleotide internal to the gyrA ORF.

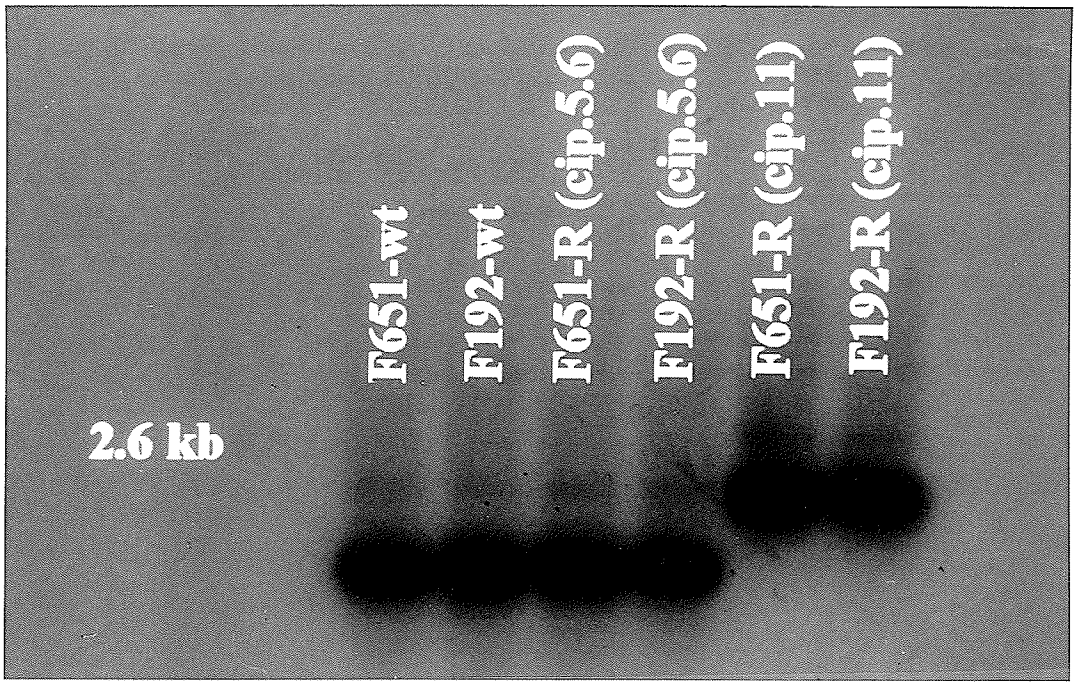
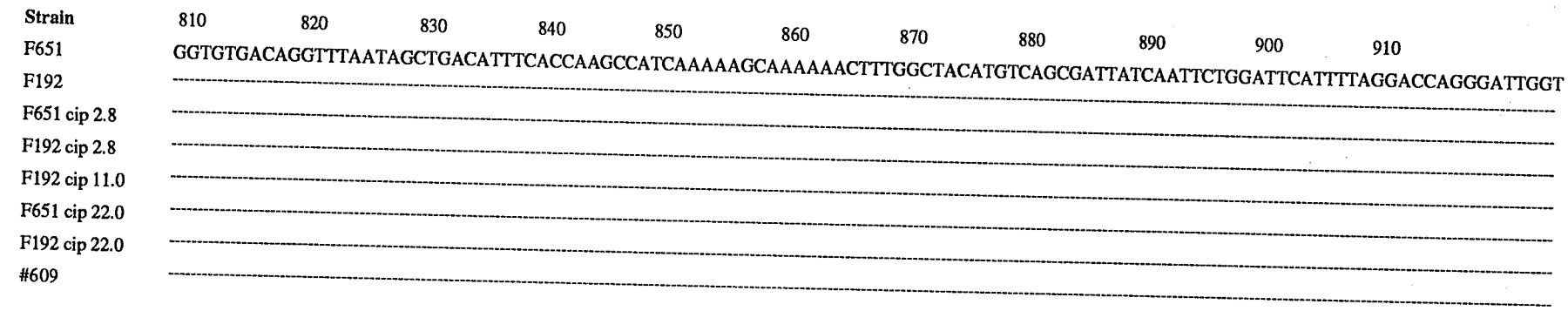


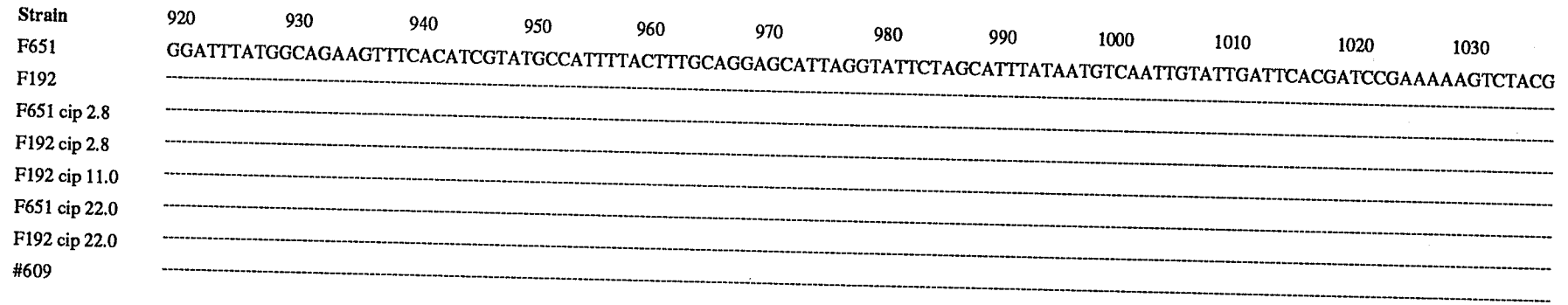
Table 16. The DNA sequence of a 1170 bp DNA fragment corresponding to the coding region of the norA gene. The ATG start codon is in boldtype.

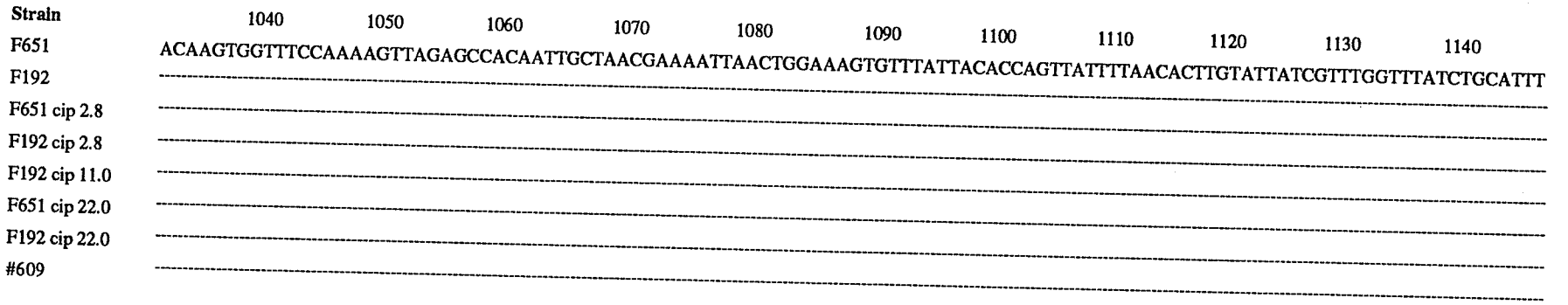
Strain	480	490	500	510	520	530	540	550	560	570	580
F651	CATATGAATAAACAGATTTTTGTCTTATATTTTAATATTTTCTTGATTTTTTTAGGTATCGGTTTAGTAATACCAGTCTTGCCTGTTTATTTAAAAGATTTGGGATTAACT										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

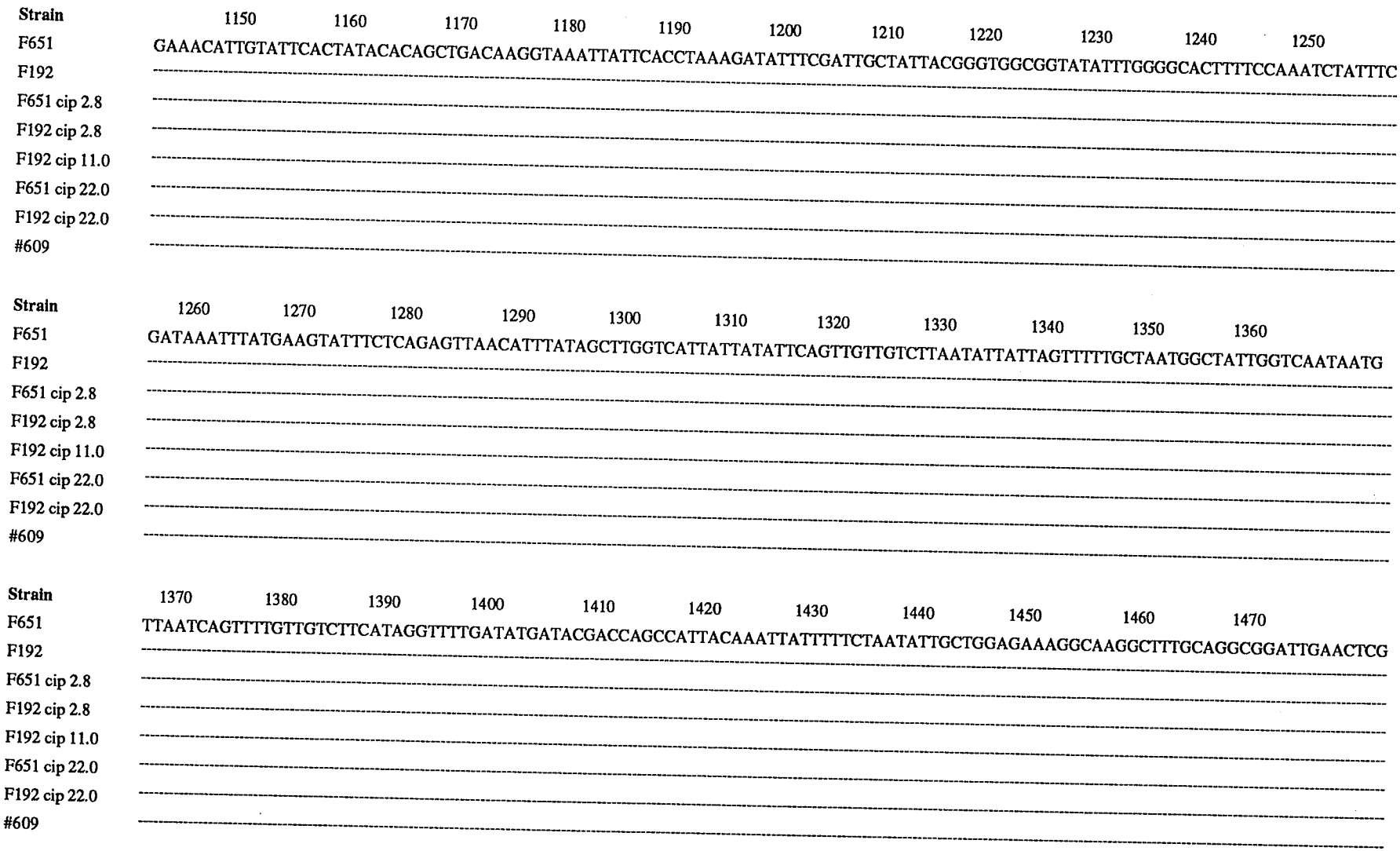
Strain	590	600	610	620	630	640	650	660	670	680	690
F651	GGTAGTGATTTAGGATTACTAGTTGCTGCTTTTGCGTTATCTCAAATGATTATATCGCCGTTTGGTGGTACGCTAGCTGACAAATTAGGGAAGAAATTAATTATATGTATA										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	700	710	720	730	740	750	760	770	780	790	800
F651	GGATTAATTTTGTTTTCAGTGTCAGAATTTATGTTTGCAGTTGGCCACAATTTTTCGGTATTGATGTTATCGAGAGTGATTGGTGGTATGAGTGCTGGTATGGTAATGCCT										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										


Strain
810 820 830 840 850 860 870 880 890 900 910
F651 GGTGTGACAGGTTTAATAGCTGACATTTCACCAAGCCATCAAAAAGCAAAAAACTTTGGCTACATGTCAGCGATTATCAATTCTGGATTCATTTTAGGACCAGGGATTGGT
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0
F651 cip 22.0
F192 cip 22.0
#609


Strain
920 930 940 950 960 970 980 990 1000 1010 1020 1030
F651 GGATTTATGGCAGAAGTTTCACATCGTATGCCATTTTACTTTGCAGGAGCATTAGGTATTCTAGCATTTATAATGTCAATTGTATTGATTCACGATCCGAAAAAGTCTACG
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0
F651 cip 22.0
F192 cip 22.0
#609


Strain
1040 1050 1060 1070 1080 1090 1100 1110 1120 1130 1140
F651 ACAAGTGGTTTCCAAAAGTTAGAGCCACAATTGCTAACGAAAATTAACTGGAAAGTGTTTATTACACCAGTTATTTTAACACTTGTATTATCGTTTGGTTTATCTGCATTT
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0
F651 cip 22.0
F192 cip 22.0
#609


Strain
1150 1160 1170 1180 1190 1200 1210 1220 1230 1240 1250
F651 GAAACATTGTATTCACTATACACAGCTGACAAGGTAAATTATTCACCTAAAGATATTTCGATTGCTATTACGGGTGGCGGTATATTTGGGGCACTTTTCCAAATCTATTTC
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0
F651 cip 22.0
F192 cip 22.0
#609
Strain
1260 1270 1280 1290 1300 1310 1320 1330 1340 1350 1360
F651 GATAAATTTATGAAGTATTTCTCAGAGTTAACATTTATAGCTTGGTCATTATTATATTCAGTTGTTGTCTTAATATTATTAGTTTTTGCTAATGGCTATTGGTCAATAATG
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0
F651 cip 22.0
F192 cip 22.0
#609
Strain
1370 1380 1390 1400 1410 1420 1430 1440 1450 1460 1470
F651 TTAATCAGTTTTGTTGTCTTCATAGGTTTTGATATGATACGACCAGCCATTACAAATTATTTTTCTAATATTGCTGGAGAAAGGCAAGGCTTTGCAGGCGGATTGAACTCG
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0
F651 cip 22.0
F192 cip 22.0
#609

Strain	1480	1490	1500	1510	1520	1530	1540	1550	1560	1570	1580	1590
F651	ACATTCACTAGTATGGGTAATTTCATAGGTCCTTTAATCGCAGGTGCGTTATTTGATGTACACATTGAAGCACCAATTTATATGGCTATAGGTGTTTCATTAGCAGGTGTT											
F192	----------											
F651 cip 2.8	----------											
F192 cip 2.8	----------											
F192 cip 11.0	----------											
F651 cip 22.0	----------											
F192 cip 22.0	----------											
#609	----------											

Strain	1600	1610	1620	1630	1640	1650
F651	ATTGTTTTAATTGAAAAGCAACATAGAGCAAAATTGAAAGAACAAAATATGTAGCATAAG					
F192	----------					
F651 cip 2.8	----------					
F192 cip 2.8	----------					
F192 cip 11.0	----------					
F651 cip 22.0	----------					
F192 cip 22.0	----------					
#609	----------					

Table 17. The DNA sequence of a 1920 bp DNA fragment corresponding to the coding region of the gyrB gene. The ATG start codon is in boldtype.

Strain	10	20	30	40	50	60	70	80	90	100	110
F651	ATGGTGACTGCATTGTCAGATGTAAACAACACGGATAATTATGGTGCTGGGCAAATACAAGTATTAGAAGGTTTAGAAGCAGTACGTAAAAGACCAGGTATGTATATAGGA
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F192 cip 11.0	----------
F651 cip 22.0	----------
F192 cip 22.0	----------
#609	----------

Strain	120	130	140	150	160	170	180	190	200	210	220
F651	TCGACTCAGAGAGAGTTGCACATTAGTGTGGAAATTGTCGATAATAGTATCGATGAAGCATTAGCTGGTTATGCAAATAAAATTGAAGTTGTTATTGAAAAAGATAACTGG
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F192 cip 11.0	----------
F651 cip 22.0	----------
F192 cip 22.0	----------
#609	----------

Strain	230	240	250	260	270	280	290	300	310	320	330
F651	ATTAAAGTAACGGATAACGGACGTGGTATCCCAGTTGATATTCAAGAAAAAATGGGACGTCCAGCTGTCGAAGTTATTTTAACTGTTTTACATGCTGGTGGTAAATTCGGC
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F192 cip 11.0	----------
F651 cip 22.0	----------
F192 cip 22.0	----------
#609	----------

Strain	340	350	360	370	380	390	400	410	420	430	440
F651	GGTGGCGGATACAAAGTATCTGGTGGTTTACATGGTGTTGGTTCATCAGTTGTAAACGCATTGTCACAAGACTTAGAAGTATATGTACACAGAAATGAGACTATATATCAT										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	450	460	470	480	490	500	510	520	530	540	550
F651	CAAGCATATAAAAAAGGTGTACCTCAATTTGACTTAAAAGAAGTTGGCACAACTGATAAGACAGGTACTGTCATTCGTTTTAAAGCAGATGGAGAAATCTTCACAGAGACA										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	560	570	580	590	600	610	620	630	640	650	660
F651	ACTGTATACAACTATGAAACATTACAGCAGCGTATTAGAGAGCTTGCTTTCTTAAACAAAGGAATTCAAATCACATTAAGAGATGAACGTGATGAAGAAAACGTTAGAGAA										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	670	680	690	700	710	720	730	740	750	760	770
F651	GACTCCTATCACTATGAGGGCGGTATTAAATCGTACGTTGAGTTATTGAACGAAAATAAAGAACCTATTCATGATGAGCCAATTTATATTCATCAATCTAAAGATGATATT										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

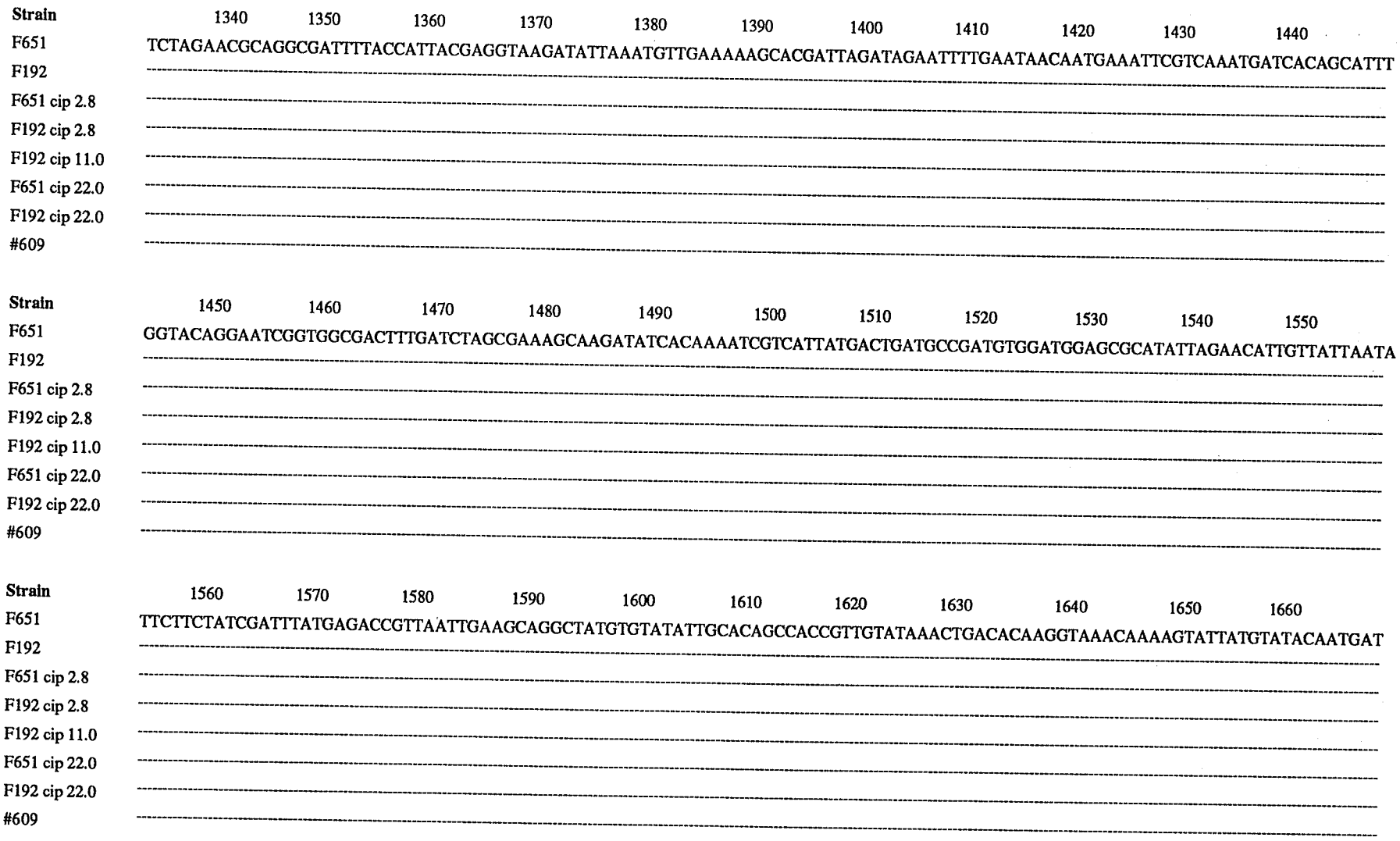
Strain	780	790	800	810	820	830	840	850	860	870	880
F651	GAAGTAGAAATTGCGATTCAATATAACTCAGGATATGCCACAAATCTTTTAACTTACGCAAATAACATTCATACGTACGAAGGTGGTACGCATGAAGACGGATTCAAACGT										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	890	900	910	920	930	940	950	960	970	980	990
F651	GCATTAACGCGTGTCTTAAATAGTTATGGTTTAAGTAGCAGATATGAAGAAGAAAAGATAGCTTCTGGTGAAGATACACGAGAAGGTATGACAGCAATTATATCTATCAAA										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	1000	1010	1020	1030	1040	1050	1060	1070	1080	1090	1100	1110
F651	CATGGTGATCCTCAATTCGAAGGTCAAACGAAGACAAAATTAGGTAATTCTGAAGTGCGTCAAGTTGTAGATAAATTATTCTCAGAGCACTTTGAACGATTTTTATATGAA											
F192	----------											
F651 cip 2.8	----------											
F192 cip 2.8	----------											
F192 cip 11.0	----------											
F651 cip 22.0	----------											
F192 cip 22.0	----------											
#609	----------											

Strain	1120	1130	1140	1150	1160	1170	1180	1190	1200	1210	1220
F651	AATCCACAAGTCGCACGTACAGTGGTTGAAAAAGGTATTATGGCGGCACGTGCACGTGTTGCTGCCAAAAAAGCGCGTGAAGTAACACGTCGTAAATCAGCGTTAGATGTA										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	1230	1240	1250	1260	1270	1280	1290	1300	1310	1320	1330
F651	GCAAGTCTTCCAGGTAAATTAGCCGATTGCTCTAGTCAAAGTCCTGAAGAATGTGAGATTTTCTTAGTCGAAGGGGACTCTGCCGGAGGGTCTACAAAATCTGGTCGTGAC										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

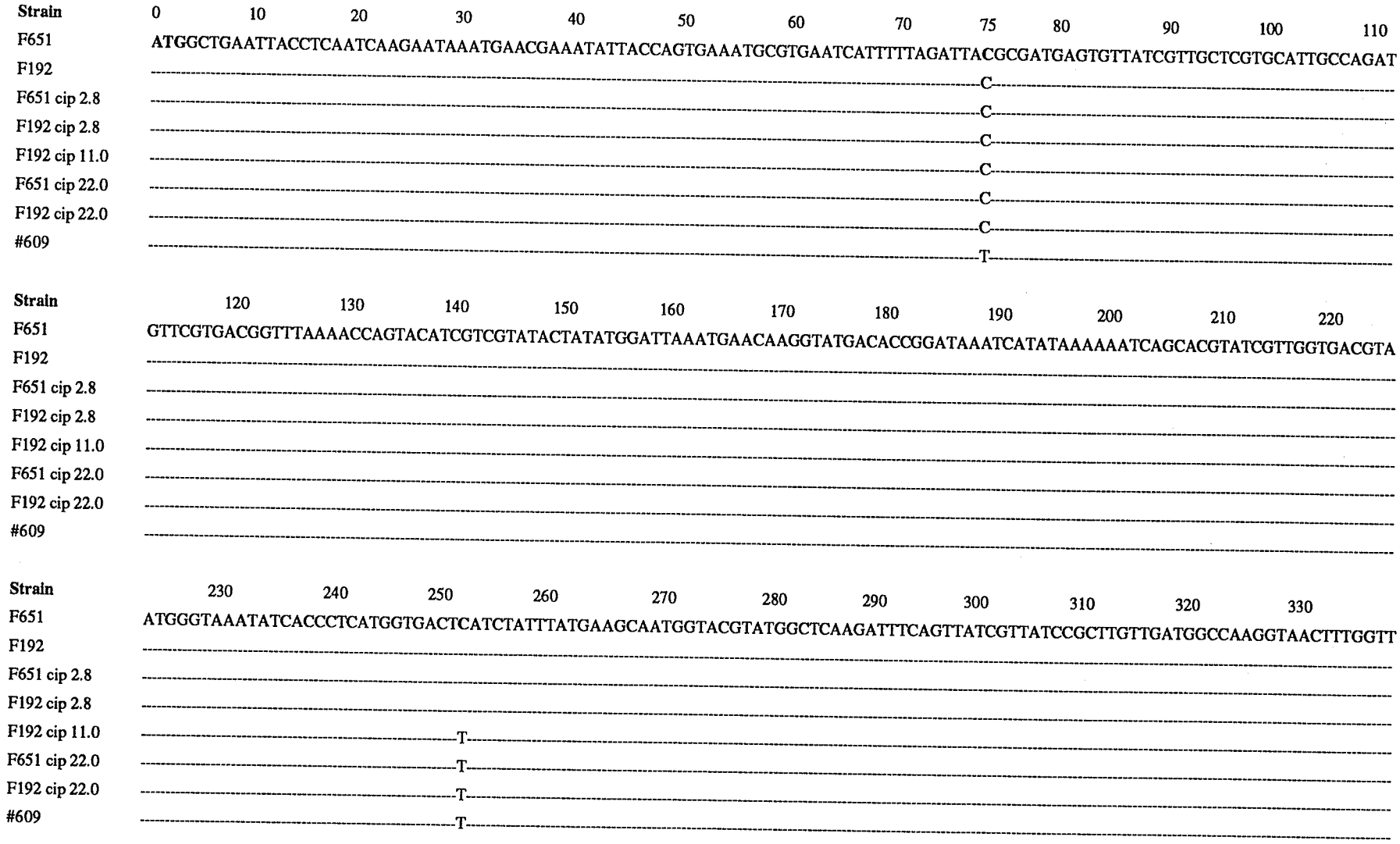

Strain
1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
F651 TCTAGAACGCAGGCGATTTTACCATTACGAGGTAAGATATTAAATGTTGAAAAAGCACGATTAGATAGAATTTTGAATAACAATGAAATTCGTCAAATGATCACAGCATTT
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0
F651 cip 22.0
F192 cip 22.0
#609
Strain
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550
F651 GGTACAGGAATCGGTGGCGACTTTGATCTAGCGAAAGCAAGATATCACAAAATCGTCATTATGACTGATGCCGATGTGGATGGAGCGCATATTAGAACATTGTTATTAATA
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0
F651 cip 22.0
F192 cip 22.0
#609
Strain
1560 1570 1580 1590 1600 1610 1620 1630 1640 1650 1660
F651 TTCTTCTATCGATTTATGAGACCGTTAATTGAAGCAGGCTATGTGTATATTGCACAGCCACCGTTGTATAAACTGACACAAGGTAAACAAAAGTATTATGTATACAATGAT
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0
F651 cip 22.0
F192 cip 22.0
#609

Strain	1670	1680	1690	1700	1710	1720	1730	1740	1750	1760	1770
F651	AGGGAACTTGATAAACTTAAATCTGAATTGAATCCAACACCAAAATGGTCTATTGCGCTATACAAAGGTCTTGGAGAAATGAATGCAGATCAATTATGGGAAACAACAATG										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	1780	1790	1800	1810	1820	1830	1840	1850	1860	1870	1880
F651	AACCCTGAGCACCGCGCTCTTTTACAAGTAAAACTTGAAGATGCGATTGAAGCGGACCAAACATTTGAAATGTTAATGGGTGACGTTGTAGAAAACCGTAGACAATTTATA										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	1890	1900	1910	1920
F651	GAAGATAATGCAGTTTATGCAAACTTAGACTTCTAAGCGC			
F192	----------			
F651 cip 2.8	----------			
F192 cip 2.8	----------			
F192 cip 11.0	----------			
F651 cip 22.0	----------			
F192 cip 22.0	----------			
#609	----------			

Table 18. The DNA sequence of a 2672 bp DNA fragment corresponding to the coding region of the gyrA gene. The ATG start codon is in boldtype.


Strain
0 10 20 30 40 50 60 70 75 80 90 100 110
F651 ATGGCTGAATTACCTCAATCAAGAATAAATGAACGAAATATTACCAGTGAAATGCGTGAATCATTTTTAGATTACGCGATGAGTGTTATCGTTGCTCGTGCATTGCCAGAT
F192
F651 cip 2.8 C
F192 cip 2.8 C
F192 cip 11.0 C
F651 cip 22.0 C
F192 cip 22.0 C
#609 T
Strain
120 130 140 150 160 170 180 190 200 210 220
F651 GTTCGTGACGGTTTAAAACCAGTACATCGTCGTATACTATATGGATTAAATGAACAAGGTATGACACCGGATAAATCATATAAAAAATCAGCACGTATCGTTGGTGACGTA
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0
F651 cip 22.0
F192 cip 22.0
#609
Strain
230 240 250 260 270 280 290 300 310 320 330
F651 ATGGGTAAATATCACCCTCATGGTGACTCATCTATTTATGAAGCAATGGTACGTATGGCTCAAGATTTCAGTTATCGTTATCCGCTTGTTGATGGCCAAGGTAACTTTGGTT
F192
F651 cip 2.8
F192 cip 2.8
F192 cip 11.0 T
F651 cip 22.0 T
F192 cip 22.0 T
#609 T

Strain	340	350	360	370	380	390	400	410	420	430	440
F651	CAATGGATGGAGATGGCGCAGCAGCAATGCGTTATACTGAAGCGCGTATGACTAAAATCACACTTGAACTGTTACGTGATATTAATAAAGATACAATAGATTTTATCGA
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F192 cip 11.0	----------
F651 cip 22.0	----------
F192 cip 22.0	----------
#609	----------

Strain	450	460	470	480	490	500	510	520	530	540	550
F651	TAACTATGATGGTAATGAAAGAGAGCCGTCAGTCTTACCTGCTCGATTCCCTAACTTGTTAGCCAATGGTGCATCAGGTATCGCGGTAGGTATGGCAACGAATATTCCACC
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F192 cip 11.0	----------
F651 cip 22.0	----------
F192 cip 22.0	----------
#609	----------

Strain	560	570	580	590	600	610	620	630	640	650	660
F651	ACATAACTTAACAGAATTAATCAATGGTGTACTTAGCTTAAGTAAGAACCCTGATATTTCAATTGCTGAGTTAATGGAGGATATTGAAGGTCCTGATTTCCCAACTGCTGG
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F192 cip 11.0	----------
F651 cip 22.0	----------
F192 cip 22.0	----------
#609	----------

Strain	670	680	690	700	710	720	730	740	750	760	770
F651	ACTTATTTTAGGTAAGAGTGGTATTAGACGTGCATATGAAACAGGTCGTGGTTCAATTCAAATGCGTTCTCGTGCAGTTATTGAAGAACGTGGAGGCGGACGTCAACGTAT										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	780	790	800	810	820	830	840	850	860	870	880
F651	TGTTGTCACTGAAATTCCTTTCCAAGTGAATAAGGCTCGTATGATTGAAAAAATTGCAGAGCTCGTTCGTGACAAGAAAATTGACGGTATCACTGATTTACGTGATGAAAC										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	890	900	910	920	930	940	950	960	970	980	990
F651	AAGTTTACGTACTGGTGTGCGTGTCGTTATTGATGTGCGTAAGGATGCAAATGCTAGTGTCATTTTAAATAACTTATACAAACAAACACCTCTTCAAACATCATTTGGTGT										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	1000	1010	1020	1030	1040	1050	1060	1070	1080	1090	1100	1110
F651	GAATATGATTGCACTTGTAAATGGTAGACCGAAGCTTATTAATTTAAAAGAAGCGTTGGTACATTATTTAGAGCATCAAAAGACAGTTGTTAGAAGACGTACGCAATACAA											
F192	----------											
F651 cip 2.8	----------											
F192 cip 2.8	----------											
F192 cip 11.0	----------											
F651 cip 22.0	----------											
F192 cip 22.0	----------											
#609	----------											

Strain	1120	1130	1140	1150	1160	1170	1180	1190	1200	1210	1220
F651	CTTACGTAAAGCTAAAGATCGTGCCCACATTTTAGAAGGATTACGTATCGCACTTGACCATATCGATGAAATTATTTCAACGATTCGTGAGTCAGATACAGATAAAGTTGC										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	1230	1240	1250	1260	1270	1280	1290	1300	1310	1320	1330
F651	AATGGAAAGCTTGCAACAACGCTTCAAACTTTCTTGAAAAACAAGCTCAAGCTATTTTAGACATGCGTTTAAGACGTCTAACAGCTTTAGAGAGAGACAAAATTGAAGCTG										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	1340	1350	1360	1370	1380	1390	1400	1410	1420	1430	1440
F651	AATATAATGAGTTATTAAATTATATTAGTGAATTAGAAGCAATCTTAGCTGATGAAGAAGTGTTATTACAGTTAGTTAGAGATGAATTGACTGAAATTAGAGATCGTTTCG										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	1450	1460	1470	1480	1490	1500	1510	1520	1530	1540	1550
F651	GTGATGATCGTCGTACTGAAATCCAATTAGGTGGATTTGAAGATTTAGAAGATGAAGATCTCATTCCAGAAGAACAAATAGTAATTACTTTGAGCCATAATAACTACATTA										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	1560	1570	1580	1590	1600	1610	1620	1630	1640	1650	1660
F651	AACGTTTGCCGGTATCTACATATCGTGCTCAAAACCGTGGTGGTCGTGGTGTTCAAGGTATGAACACATTGGAAGAAGATTTTGTCAGTCAATTGGTAACTTTAAGTACAC										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	1670	1680	1690	1700	1710	1720	1730	1740	1750	1760	1770
F651	ATGACCATGTATTGTTCTTTACTAACAAAGGTCGTGTATACAAACTTAAAGGTTATGAAGTGCCTGAGTTATCAAGACAGTCTAAAGGTATTCCTGTAGTGAATGCTATTG
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F192 cip 11.0	----------
F651 cip 22.0	----------
F192 cip 22.0	----------
#609	----------

Strain	1780	1790	1800	1810	1820	1830	1840	1850	1860	1870	1880
F651	AACTTGAAAATGATGAAGTCATTAGTACAATGATTGCTGTTAAAGACCTTGAAAGTGAAGACAACTTCTTAGTGTTTGCAACTAAACGTGGTGTCGTTAAACGTTCAGCAT
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F192 cip 11.0	----------
F651 cip 22.0	----------
F192 cip 22.0	----------
#609	----------

Strain	1890	1900	1910	1920	1930	1940	1950	1960	1970	1980	1990
F651	TAAGTAACTTCTCAAGAATAAATAGAAATGGTAAGATTGCGATTTCGTTCAGAGAAGATGATGAGTTAATTGCAGTTCGCTTAACAAGTGGTCAAGAAGATATCTTGATTG
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F192 cip 11.0	----------
F651 cip 22.0	----------
F192 cip 22.0	----------
#609	----------

Strain	2000	2010	2020	2030	2040	2050	2060	2070	2080	2090	2100
F651	GTACATCACATGCATCATTAATTCGATTCCCTGAATCAACATTACGTCCTTTAGGCCGTACAGCAACAGGTGTGAAAGGTATTACACTTCGTGAAGGCGATGAAGTTGTAG										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	2110	2120	2130	2140	2150	2160	2170	2180	2190	2200	2210	2220
F651	GGCTTGATGTTGCTCACGAAAATAGTGTAGATGAAGTTTTAGTTGTTACTGAAAATGGTTATGGTAAACGTACGCCAGTTAATGACTATCGCTTATCAAACCGTGGTGGTA											
F192	----------											
F651 cip 2.8	----------											
F192 cip 2.8	----------											
F192 cip 11.0	----------											
F651 cip 22.0	----------											
F192 cip 22.0	----------											
#609	----------											

Strain	2230	2240	2250	2260	2270	2280	2290	2300	2310	2320	2330
F651	AAGGTATTAAGACTGCAACGATTACTGAACGTAATGGTAATGTTGTTTGTATTACAACTGTTACTGGTGAAGAAGATTTAATGATTGTTACGAATGCAGGTGTCATTATTC										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	2340	2350	2360	2370	2380	2390	2400	2410	2420	2430	2450
F651	GACTAGATGTTGCAGATATTTCTCAAAATGGTCGTGCAGCACAAGGTGTTCGCTTAATTCGCTTAGGCGATGATCAATTTGTTTCAACGGTTGCTAAAGTAAAAGAAGATG										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	2450	2460	2470	2480	2490	2500	2510	2520	2530	2540	2550
F651	CAGAAGATGAAACGAATGAAGATGAGCAATCTACTTCAACTGTATCTGAAGATGGTACAGAGCAACAACGTGAAGCGGTTGTAAATGATGAAACACCAGGAAATGCAATTC										
F192	----------										
F651 cip 2.8	----------										
F192 cip 2.8	----------										
F192 cip 11.0	----------										
F651 cip 22.0	----------										
F192 cip 22.0	----------										
#609	----------										

Strain	2560	2570	2580	2590	2600	2610	2620	2630	2640	2650	2660	2670
F651	ATACTGAAGTGATTGAATCAGAAGAAACTGATGACGATGGACGTATTGAAGTAAGACAAGATTTCATGGATCGTGTTGAAGAAGATATACAACAATCATTAGATGAAGATGAAGAATAA											
F192	----------											
F651 cip 2.8	----------											
F192 cip 2.8	----------											
F192 cip 11.0	----------											
F651 cip 22.0	----------											
F192 cip 22.0	----------											
#609	----------											

Table 19. DNA sequence changes in gyrA

A.) T to C substitution. Discrepancy between published sequence and sequence from isolated strains.

Sequence source	DNA and amino acid sequence							
	Ser	Phe	Leu	Asp	Tyr	Ala	Met	Ser
published sequence*	TCA	TTT	TTA	GAT	TAT	GCG	ATG	AGT
Amino acid position	21	22	23	24	25	26	27	28
HSC sequence	Ser	Phe	Leu	Asp	**Cys**	Ala	Met	Ser
	TCA	TTT	TTA	GAT	TAC	GCG	ATG	AGT

* Margerrison et al,1992

B.) C to T substitution. Change in DNA sequence between wild type (susceptible) and Flq resistant strains.

Strain	DNA and amino acid sequence							
	His	Gly	Asp	Ser	Ser	Ile	Tyr	Glu
wild type (susceptible)	CAT	GGT	GAC	TCA	TCT	ATT	TAT	GAA
Amino acid position	81	82	83	84	85	86	87	88
	His	Gly	Asp	**Leu**	Ser	Ile	Tyr	Glu
F651 cip 11.0	CAT	GGT	GAC	TTA	TCT	ATT	TAT	GAA
F192 cip 11.0	-------	-------	-------	-------	-------	-------	-------	-------
# 603	-------	-------	-------	-------	-------	-------	-------	-------
# 609	-------	-------	-------	-------	-------	-------	-------	-------

a ciprofloxacin MIC of 11.0 μg/ml or higher. The appearance of the T to C transition coincides with the loss of a Hinf I restriction enzyme site in gyrA.

4. Uptake Studies

Experimental conditions were first determined in a series of preliminary experiments. Initial experiments were designed to determine the optimal concentration of radiolabeled fluoroquinolone to use in our uptake assays. Due to the relatively low specific activity of our labeled compounds we determined that 16 μg/ml was the lowest concentration of antimicrobial that consistently yielded reproducible results. Thus this concentration was used for the majority of uptake experiments. The optimal cell density for uptake experiments was determined to be 10^8 cfu/ml. This cell concentration appeared to be the maximum concentration that still allowed rapid flow through the nylon filters. Pre-soaking filters in solutions of non-radiolabeled fluoroquinolones only served to increase non specific binding of the radiolabeled compounds to the filters and therefore filters were pre-soaked in sterile PBS.

The antibacterial effects of the fluoroquinolone agents were evaluated over the course of the uptake assays by plating small aliquots onto blood agar plates and incubating overnight. Over the 30 to 40 minute time frame of the assays, all the fluoroquinolones produced a kill of approximately 1 log. It was felt that this level of kill would not adversely interfere with the accumulation assay.

A number of experiments were designed to study the uptake of fluoroquinolones in S.aureus. Initial experiments were performed to determine how fluoroquinolones

accumulate in the staphylococcal cell; was uptake passive or was an active transport system involved? Secondly, the role of fluoroquinolone accumulation "or lack of accumulation" in resistant cells was examined at both the gross and molecular levels. Finally, studies were performed to ascertain what relationship, if any, existed between uptake of fluoroquinolone antimicrobials and the PAE.

a) Accumulation of fluoroquinolones in logarithmic phase S.aureus.

Fluoroquinolone accumulation in logarithmic phase S.aureus was studied using different concentrations of fluoroquinolones, varying temperature, competition studies, and metabolic inhibitors.

i) Effect of temperature, concentration, and competition studies:

Figure 18 illustrates the typical uptake kinetics of pefloxacin and norfloxacin in logarithmic phase S.aureus. In logarithmic phase cells, a biphasic pattern of accumulation was typically observed with an initial rapid phase of accumulation seen within the first ten seconds. The rapid phase of accumulation was followed by a slow steady increase over the next 50-60 minutes. At the end of this plateau maximal accumulation began to decrease.

The effect of antimicrobial concentration was examined by varying the concentration of radiolabeled fluoroquinolones in the uptake assays. A number of experiments were performed using antimicrobial concentrations between 4 μg/ml and 256 μg/ml (Fig.19). We observed a linear increase in fluoroquinolone accumulation corresponding with increasing concentrations of radiolabeled antimicrobials. This

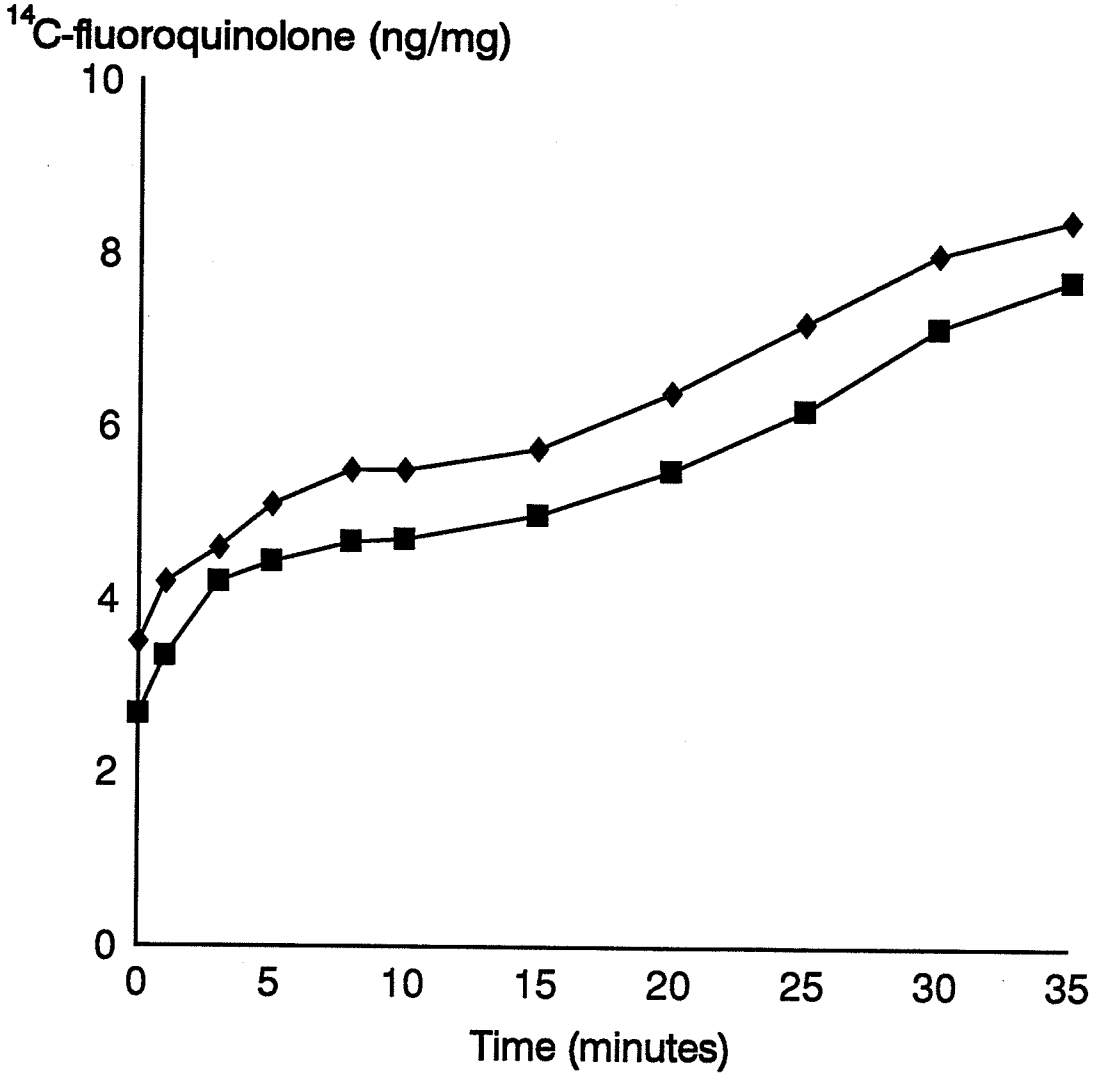
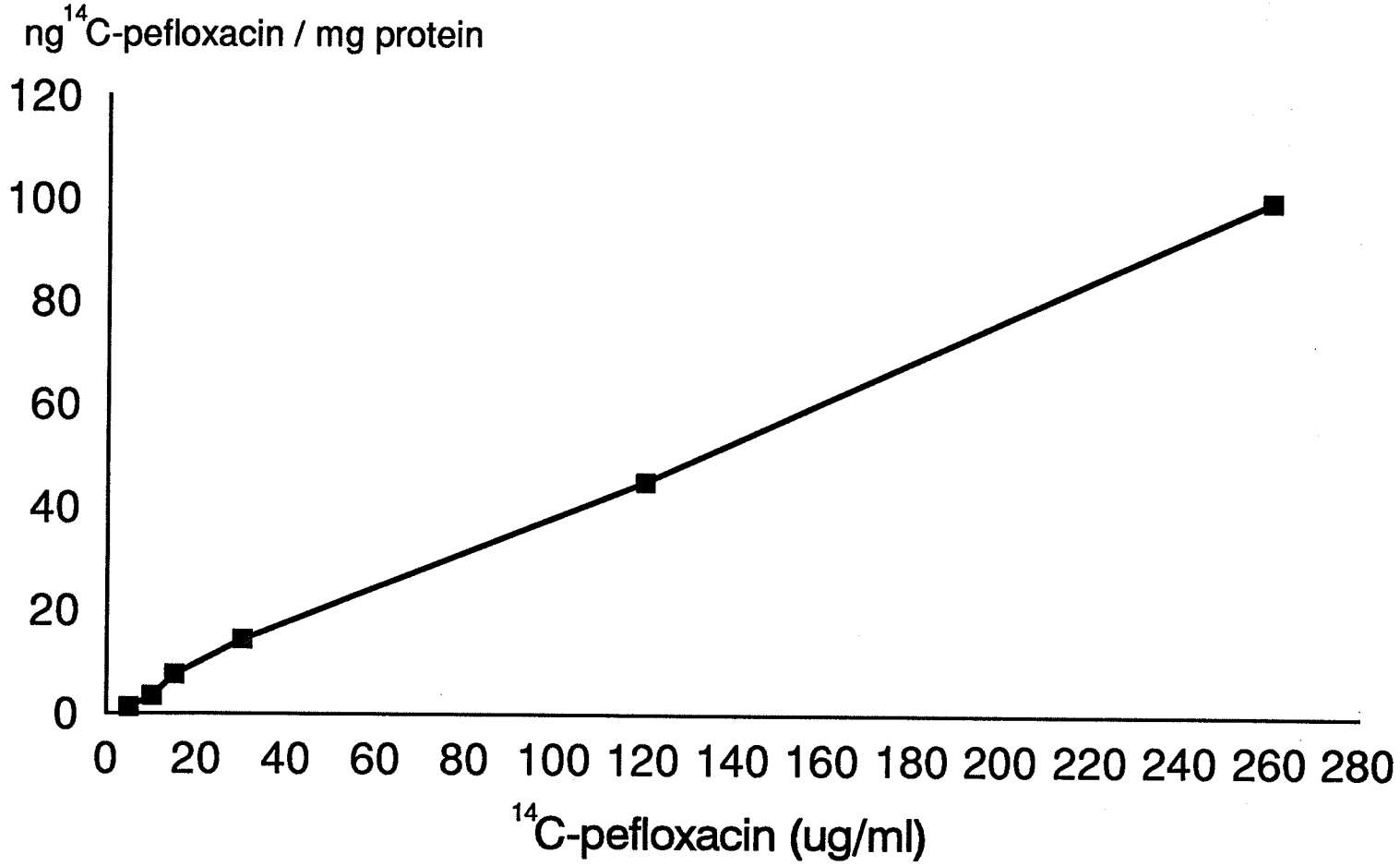

^{14}C-fluoroquinolone (ng/mg)
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Time (minutes)

Figure 18. The uptake kinetics of ^{14}C-pefloxacin and ^{14}C-norfloxacin in logarithmic phase S.aureus F988. Logarithmic phase cultures of S.aureus F988 were exposed to 16 μg/ml ^{14}C-pefloxacin ♦ and 16 μg/ml ^{14}C-norfloxacin ■. ^{14}C-fluoroquinolone accumulation is expressed as ng fluoroquinolone / mg total cell protein.


ng 14C-pefloxacin / mg protein
120
100
80
60
40
20
0
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280
14C-pefloxacin (ug/ml)

135

Figure 19. The Effect of fluoroquinolone concentration on the uptake kinetics of ^{14}C-pefloxacin in S.aureus F651. The uptake of 4 μg/ml to 256 μg/ml ^{14}C-pefloxacin was examined. Results are expressed as ng ^{14}C-pefloxacin / mg total cell protein.

effect was seen with all fluoroquinolones evaluated. Experiments performed with high concentrations of radiolabeled fluoroquinolones (256 μg/ml) did not demonstrate any saturation of the uptake system (Fig.20). To further explore this a number of competition experiments were performed to determine if unlabelled ciprofloxacin could compete for the uptake system with radiolabeled pefloxacin. Unlabelled ciprofloxacin : ^{14}C-pefloxacin ratios of 1:1, 2:1, 3:1, and 4:1 were used in the competition studies. Pefloxacin accumulation was not modified at any ciprofloxacin : pefloxacin ratio evaluated (Table 20).

Experiments were also designed to determine the effect of temperature on fluoroquinolone accumulation (Fig.21). S.aureus cells were pre-incubated at the test temperature for 10 minutes prior to addition of the radiolabeled fluoroquinolone. Accumulation experiments were performed at temperatures ranging from 4°C to 42°C. We observed a linear increase in fluoroquinolone accumulation with increasing temperature over the entire range tested ($p<0.05$). Again, this effect was observed for all fluoroquinolones evaluated.

ii) Effect of metabolic inhibitors:

Accumulation experiments were performed in the presence of the metabolic inhibitors CCCP, 2,4-DNP, KCN, and NaN_3 to determine if fluoroquinolone uptake in S.aureus was an energy dependent or independent process. The inhibitors CCCP and 2,4-DNP are known to destroy the proton-motive force (PMF) across the cytoplasmic membrane, while KCN and NaN_3 are known to inhibit electron transport (Cohen et al.1988; Diver et al,1990). All inhibitors were first evaluated for their bactericidal

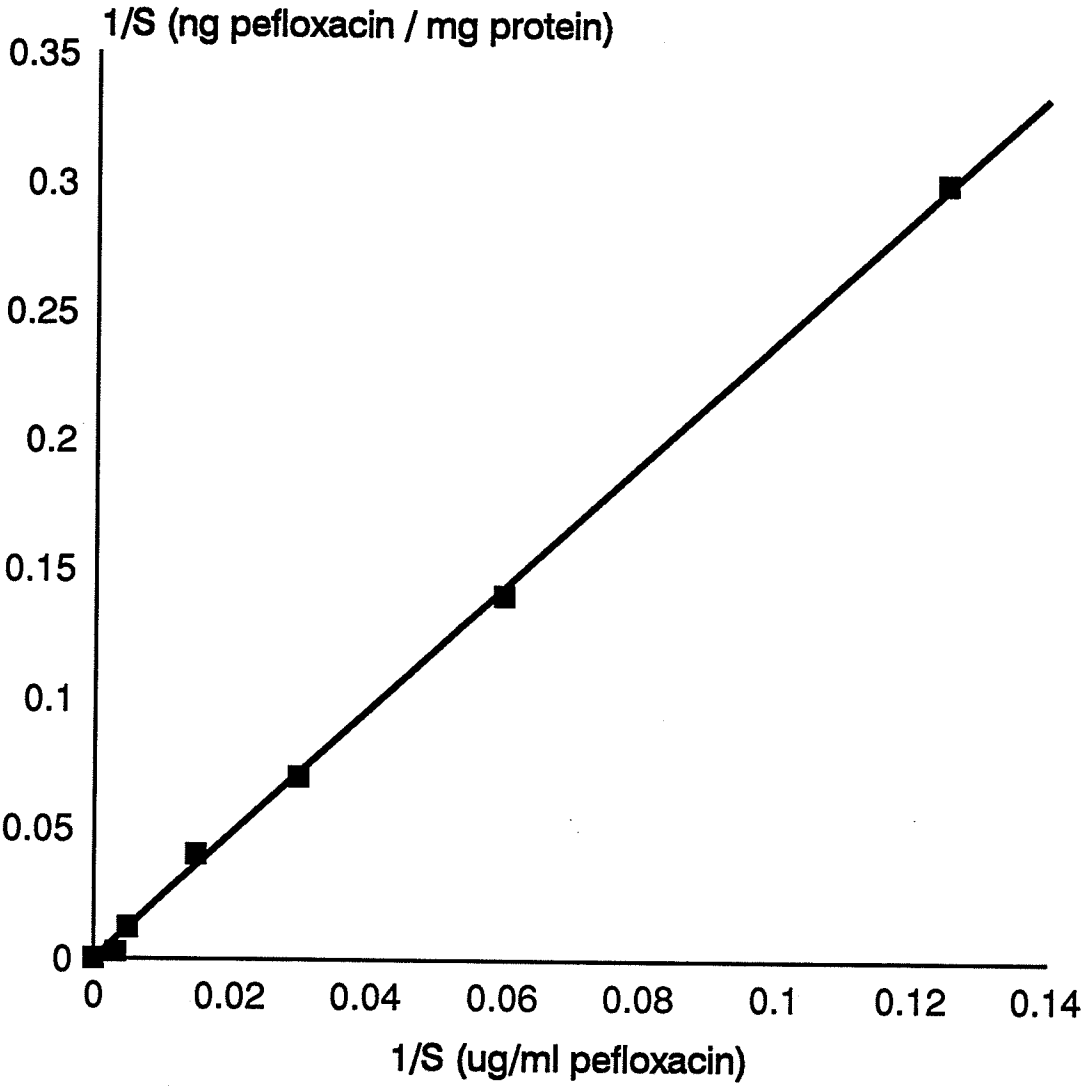

1/S (ng pefloxacin / mg protein)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
1/S (ug/ml pefloxacin)

Figure 20. Lineweaver-Burk plot for the accumulation of ^{14}C-pefloxacin in S.aureus F651 over a concentration range of 4 to 256 μg/ml.

Table 20. Effect of Unlabelled Ciprofloxacin on the Uptake of ^{14}C-Pefloxacin.

	Mean Fluoroquinolone Accumulation (ng/mg)*		
Quinolone	10 minutes	20 minutes	30 minutes
Pefloxacin	5.1	5.7	7.5
Cip : Pfx ratio			
1 : 1	5.25	5.6	7.4
2 : 1	4.9	5.7	7.4
3 : 1	5.1	5.8	7.7
4 : 1	5.3	5.8	7.5

* Accumulation expressed as ng ^{14}C-pefloxacin / mg total cell protein

activity. Concentrations used in experiments throughout our studies were shown not to be bactericidal over the course of the accumulation assays. Inhibitors were added 10 minutes after introduction of the radiolabeled fluoroquinolone.

Figure 22 illustrates the effect of CCCP and 2,4-DNP on the accumulation of ^{14}C-pefloxacin and ^{14}C-lomefloxacin in S.aureus. After addition of these inhibitors a significant increase in the accumulation of both pefloxacin and lomefloxacin was observed ($p<0.05$). The effect of the inhibitors was rapid with increased accumulation apparent soon after addition. At 35 minutes the accumulation of both antimicrobials in treated cells was approximately two fold over the untreated controls. This same trend was seen in all S.aureus strains tested.

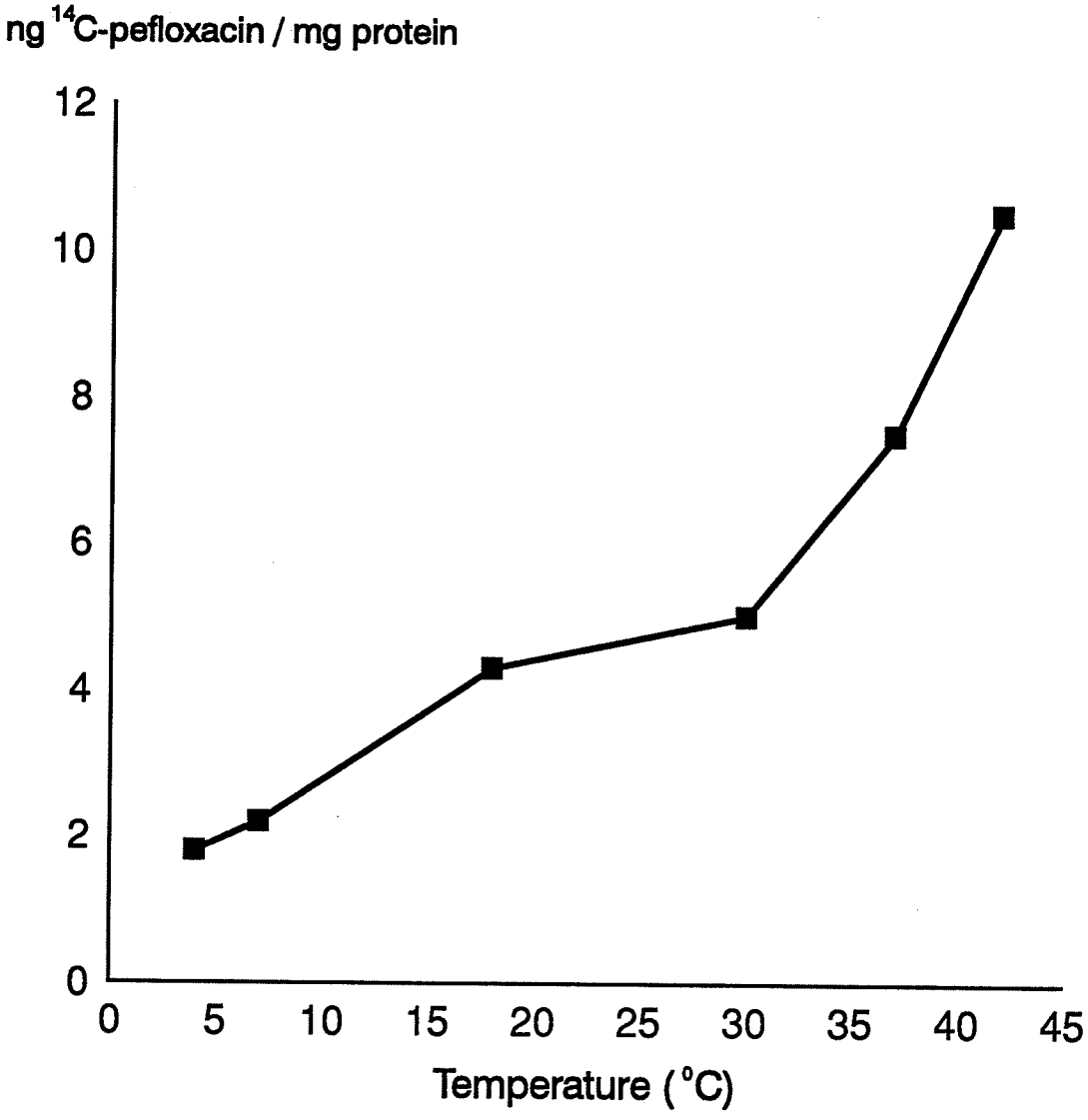

ng ^{14}C-pefloxacin / mg protein
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
40
45
Temperature (°C)

Figure 21. The effect of temperature on the uptake kinetics of ^{14}C-pefloxacin in S.aureus F447. The uptake of ^{14}C-pefloxacin over a temperature range of 4°C to 42°C was examined. Results are expressed as ng ^{14}C-pefloxacin / mg total cell protein.

Similar accumulation kinetics were observed in S.aureus treated with the inhibitors KCN or NaN_3 (Fig. 23). A significant increase in both pefloxacin and lomefloxacin accumulation was observed after addition of both metabolic inhibitors ($p<0.05$). Although both KCN and NaN_3 increased drug accumulation, KCN appeared to be the more effective of the two inhibitors. Accumulation kinetics in the presence of KCN or CCCP, and 2,4-DNP were almost indistinguishable. Again, the same trends in fluoroquinolone accumulation in the presence of KCN or NaN_3 were observed with all S.aureus strains examined.

iii) Fluoroquinolone hydrophobicity:

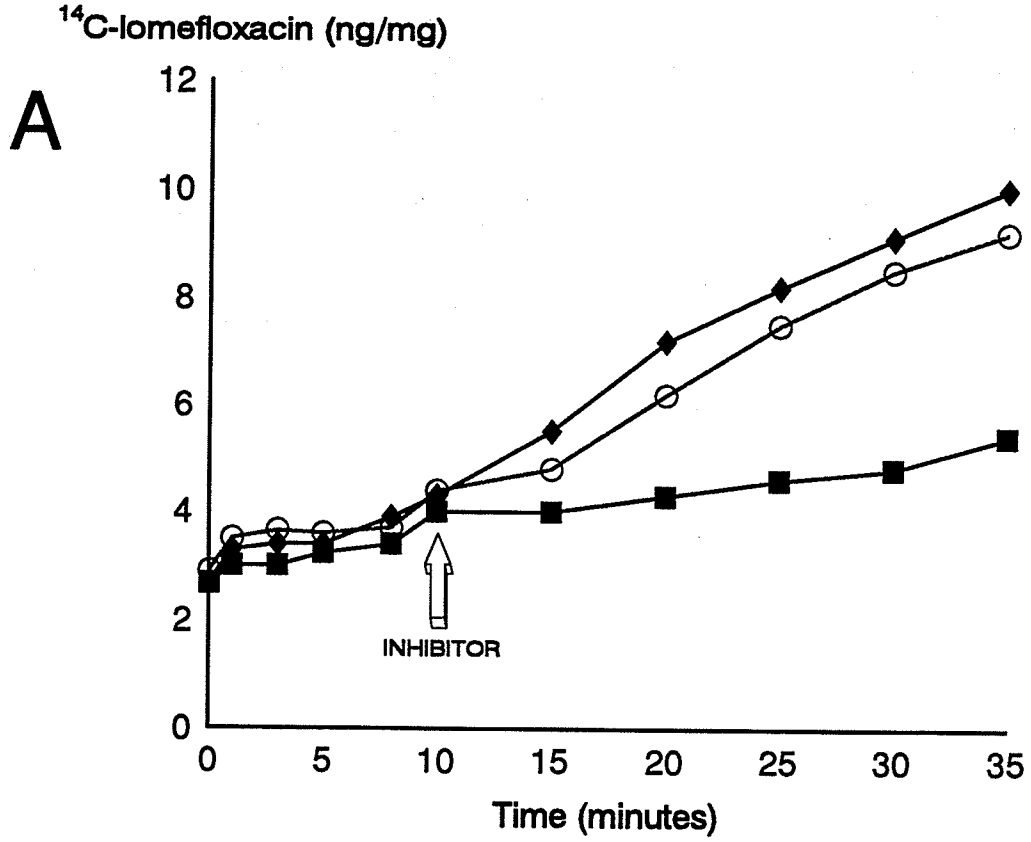
The hydrophobicity profiles of the modern fluoroquinolones agents are known to span the spectrum from relatively hydrophilic molecules to relatively hydrophobic molecules. Given this, and the fact that these agents must cross a biological membrane to reach their target, it is logical to assume that the hydrophobicity of the agent could play a role in its ability to reach the cell interior. We therefore choose to compare the accumulation of four fluoroquinolones of differing hydrophobicity (table 21).

Table 21: The Relative Hydrophobicities of Four Fluoroquinolones

Fluoroquinolone	Partition Coefficient*
Sparfloxacin	0.97 +/- 0.21
Pefloxacin	0.36 +/- 0.11
Norfloxacin	0.12 +/- 0.04
Lomefloxacin	0.07 +/- 0.04

* partition coefficient in n-octanol - 0.1M phosphate buffer pH 7.2

A


^{14}C-lomefloxacin (ng/mg)
12
10
8
6
4
2
0
INHIBITOR
0
5
10
15
20
25
30
35
Time (minutes)

B

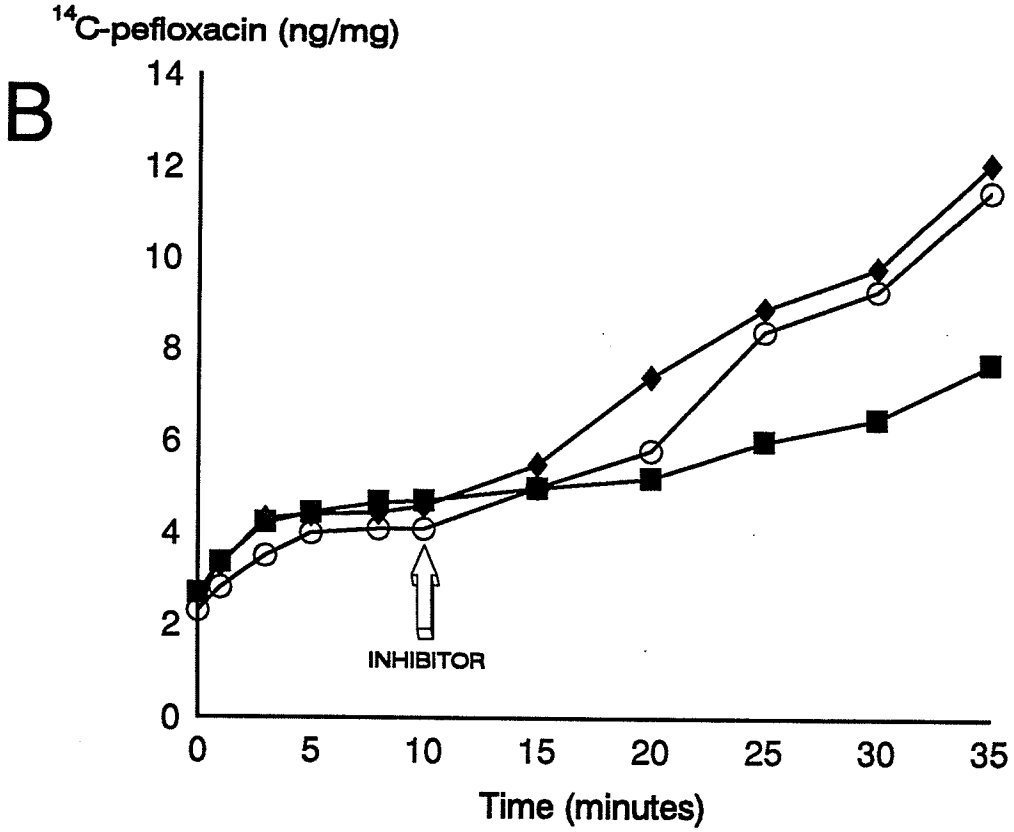

^{14}C-pefloxacin (ng/mg)
14
12
10
8
6
4
2
0
INHIBITOR
0
5
10
15
20
25
30
35
Time (minutes)

Figure 22. The effect of metabolic inhibitors on the accumulation of fluoroquinolones in S.aureus F651. Logarithmic phase cultures of S.aureus ■ were exposed to **A.** 16 μg/ml lomefloxacin or **B.** 16 μg/ml pefloxacin. 10 minutes after introduction of the ^{14}C-fluoroquinolone, CCCP (50 μM) ♦, or 2,4-DNP (2mM) O were added. Results are expressed as ng fluoroquinolone / mg total cell protein.

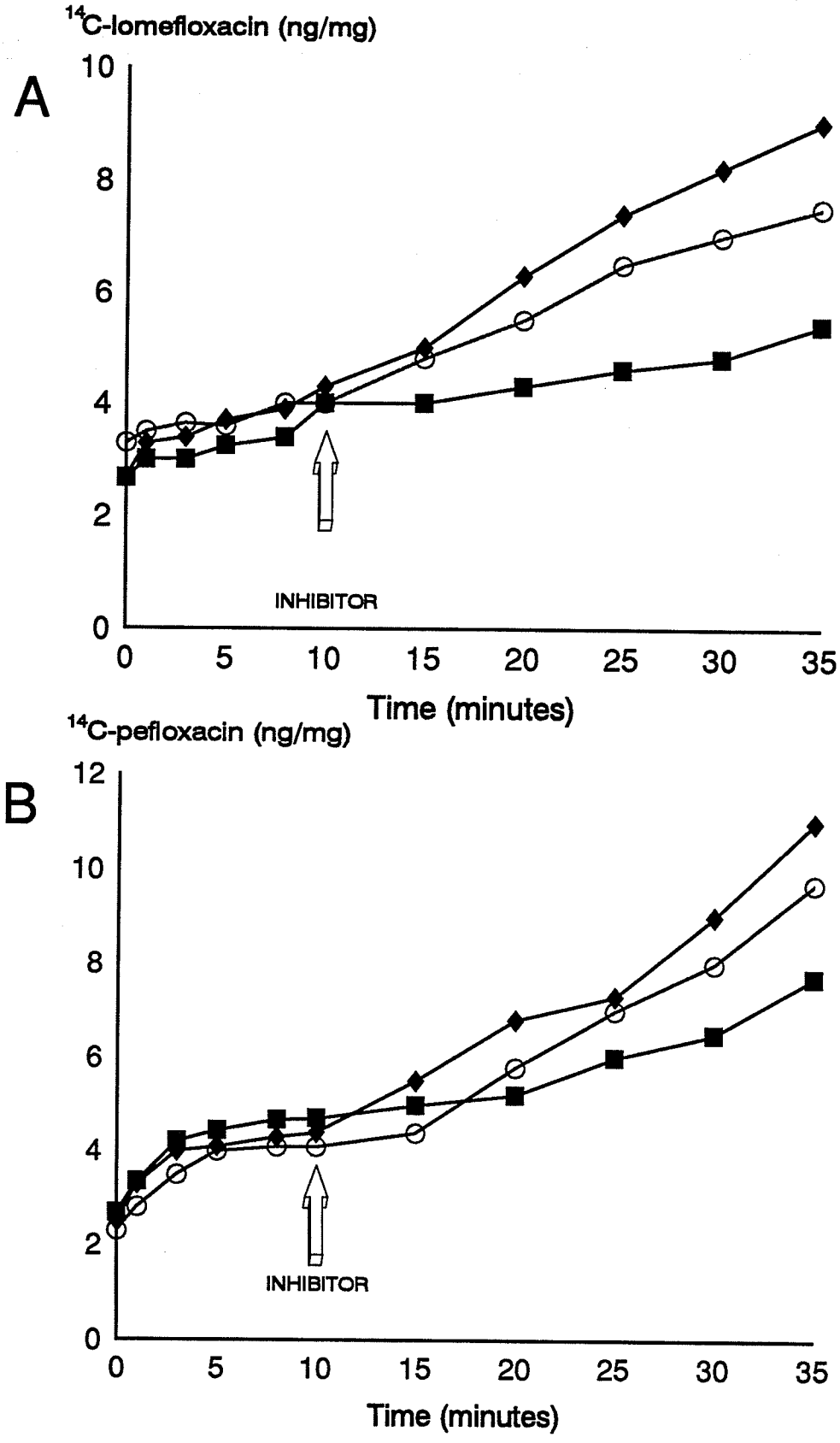

A
^{14}C-lomefloxacin (ng/mg)
10
8
6
4
2
0
INHIBITOR
0
5
10
15
20
25
30
35
Time (minutes)
B
^{14}C-pefloxacin (ng/mg)
12
10
8
6
4
2
0
INHIBITOR
0
5
10
15
20
25
30
35
Time (minutes)

Figure 23. The effect of metabolic inhibitors on the accumulation of fluoroquinolones in S.aureus F651. Logarithmic phase cultures of S.aureus ■ were exposed to **A.** 16 μg/ml lomefloxacin or **B.** 16 μg/ml pefloxacin. 10 minutes after introduction of the ^{14}C-fluoroquinolone, KCN (2 mM) ♦, or NaN_3 (5mM) O were added. Results are expressed as ng fluoroquinolone / mg total cell protein.

A definite trend was observed between the degree of fluoroquinolone hydrophobicity and the amount of antimicrobial accumulation ($p<0.05$). Accumulation of sparfloxacin, the most hydrophobic quinolone tested, was greater than the other three comparator quinolones. Lomefloxacin, having the least hydrophobic profile was accumulated to a lesser extent than the other more hydrophobic agents. These results were seen in all strains tested (Fig. 24). In the majority of strains, accumulation of pefloxacin was significantly greater ($p<0.05$) than norfloxacin, however a significant difference could not be seen in some strains. The relative amounts of norfloxacin and lomefloxacin uptake in most strains were similar, however significantly greater amounts of norfloxacin were seen in some strains.

Of interest is the fact that the MICs of the four fluoroquinolone agents correlate with their relative hydrophobicity and extent of accumulation. Others have reported that no correlation was found between quinolone accumulation and extent of antibacterial activity, however our results indicate otherwise (Piddock et al). It should be noted, however, that due to the small numbers of strains examined in our studies the possibility of coincidence exists. The effect of CCCP was compared with each of the four fluoroquinolones. Figure 25 illustrates the effect of CCCP on sparfloxacin and lomefloxacin. Addition of the metabolic inhibitor significantly increased ($p<0.05$) the accumulation of lomefloxacin but did not significantly increase the accumulation of sparfloxacin ($P>0.05$). A significant increase in the accumulation of pefloxacin and norfloxacin were also seen upon addition of CCCP ($p<0.05$). This trend was observed in all strains examined.

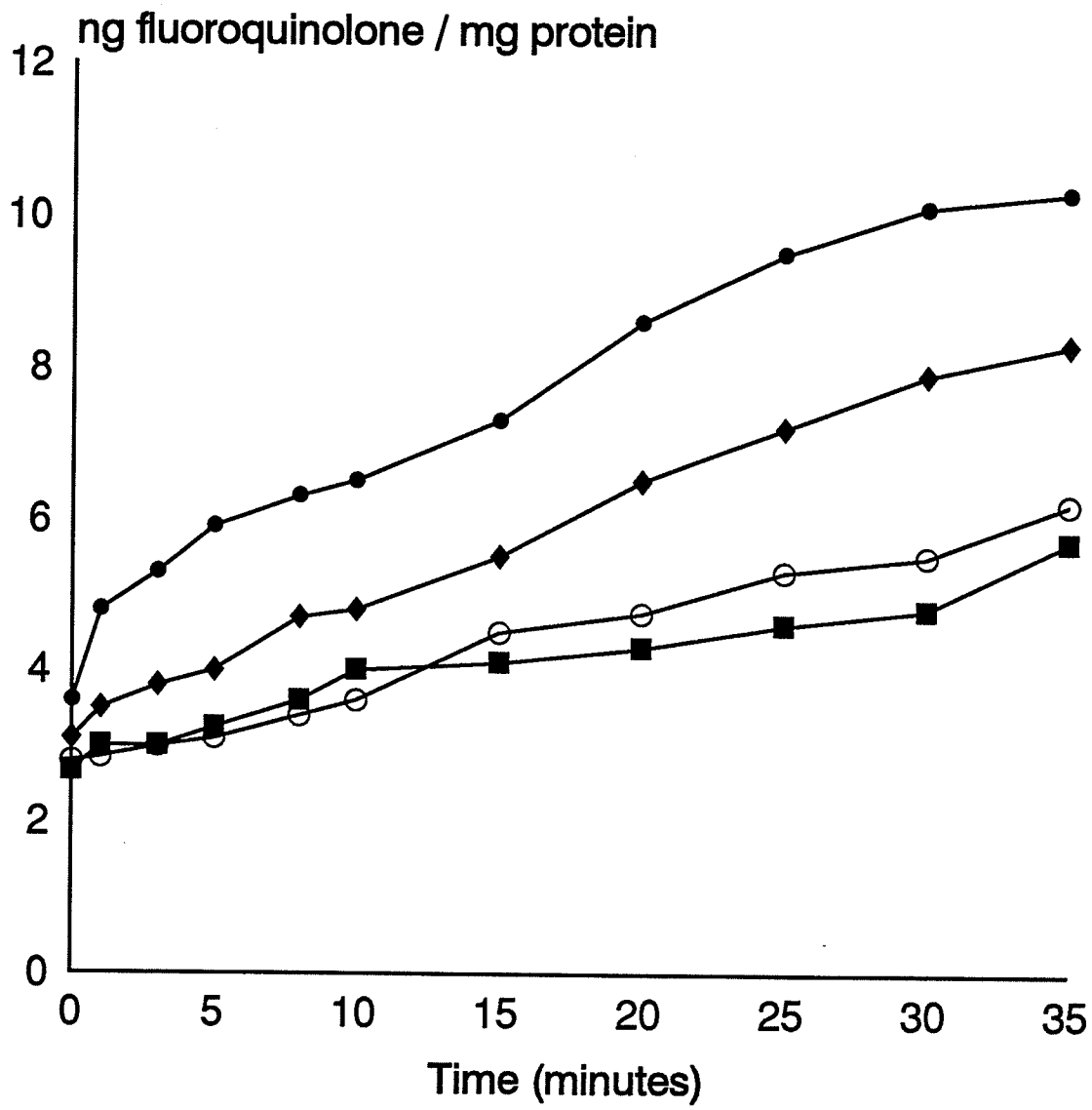

ng fluoroquinolone / mg protein
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Time (minutes)

Figure 24. The effect of fluoroquinolone hydrophobicity on accumulation in S.aureus F651. Logarithmic phase cultures of S.aureus F651 were exposed to 16 μg/ml sparfloxacin ●, pefloxacin ♦, norfloxacin ○, or lomefloxacin ■. Results are expressed as ng fluoroquinolone / mg total cell protein.

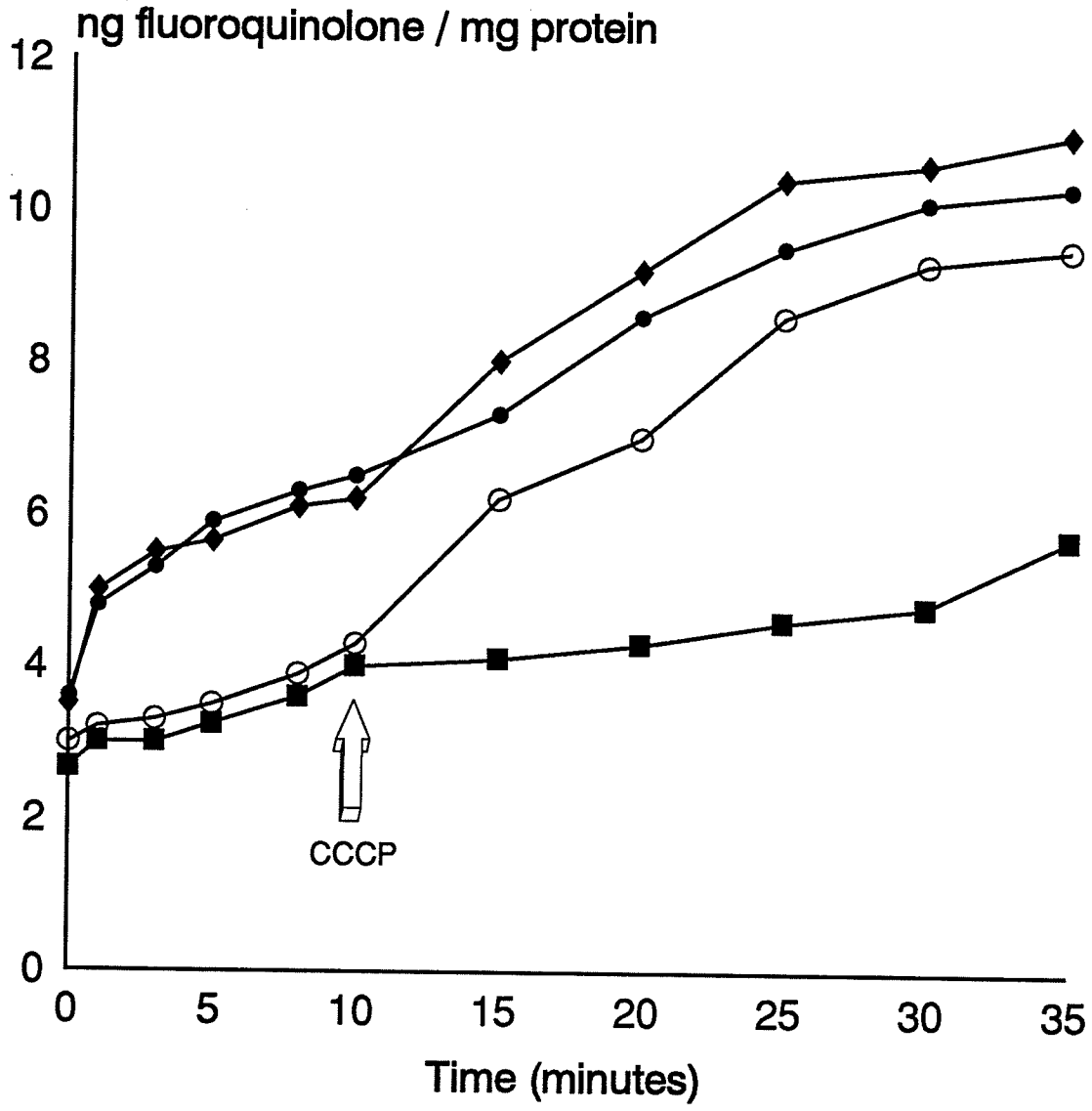

ng fluoroquinolone / mg protein
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Time (minutes)
CCCP

Figure 25. The effect of CCCP on the accumulation of ^{14}C-sparfloxacin and ^{14}C-lomefloxacin in S.aureus F651. Logarithmic phase cultures of S.aureus F651 were exposed to 16 μg/ml sparfloxacin ●, + CCCP (50 μM) ♦, or lomefloxacin ■, + CCCP (50μM) ○. Results are expressed as ng fluoroquinolone / mg total cell protein.

b) Fluoroquinolone uptake during the PAE:

We examined the accumulation of fluoroquinolones during the PAE to determine what effect the PAE might have on antimicrobial accumulation. We believed that fluoroquinolone accumulation would be increased during the effect. Our hypothesis was based on the purported existence of an energy dependent efflux system in S.aureus responsible for the removal of fluoroquinolones from the bacterial cell. Given the growth characteristics of bacterial cells during the PAE we believed that cells would be incapable of generating sufficient energy during this period to drive energy dependent systems at optimal levels. Thus intracellular levels of fluoroquinolone agents would accumulate to greater levels in these cells.

Preliminary experiments had shown that the number of viable cells remaining after induction of the PAE and drug removal by dilution were insufficient to accurately determine fluoroquinolone accumulation. A larger test volume (100ml) was therefore used and removal of antimicrobials changed from dilution to centrifugation and washing. These modifications did not result in any change to the duration of the PAE. PAE determinations were performed simultaneously to ensure cells were in the PAE phase during the accumulation assays.

i) Accumulation kinetics during the PAE:

The accumulation of four fluoroquinolones (sparfloxacin, pefloxacin, norfloxacin, and lomefloxacin) were examined during the PAE and compared to accumulation kinetics in actively growing cells (Fig. 26 and 27). Accumulation of pefloxacin, norfloxacin, and lomefloxacin was significantly increased ($p<0.05$) during the PAE

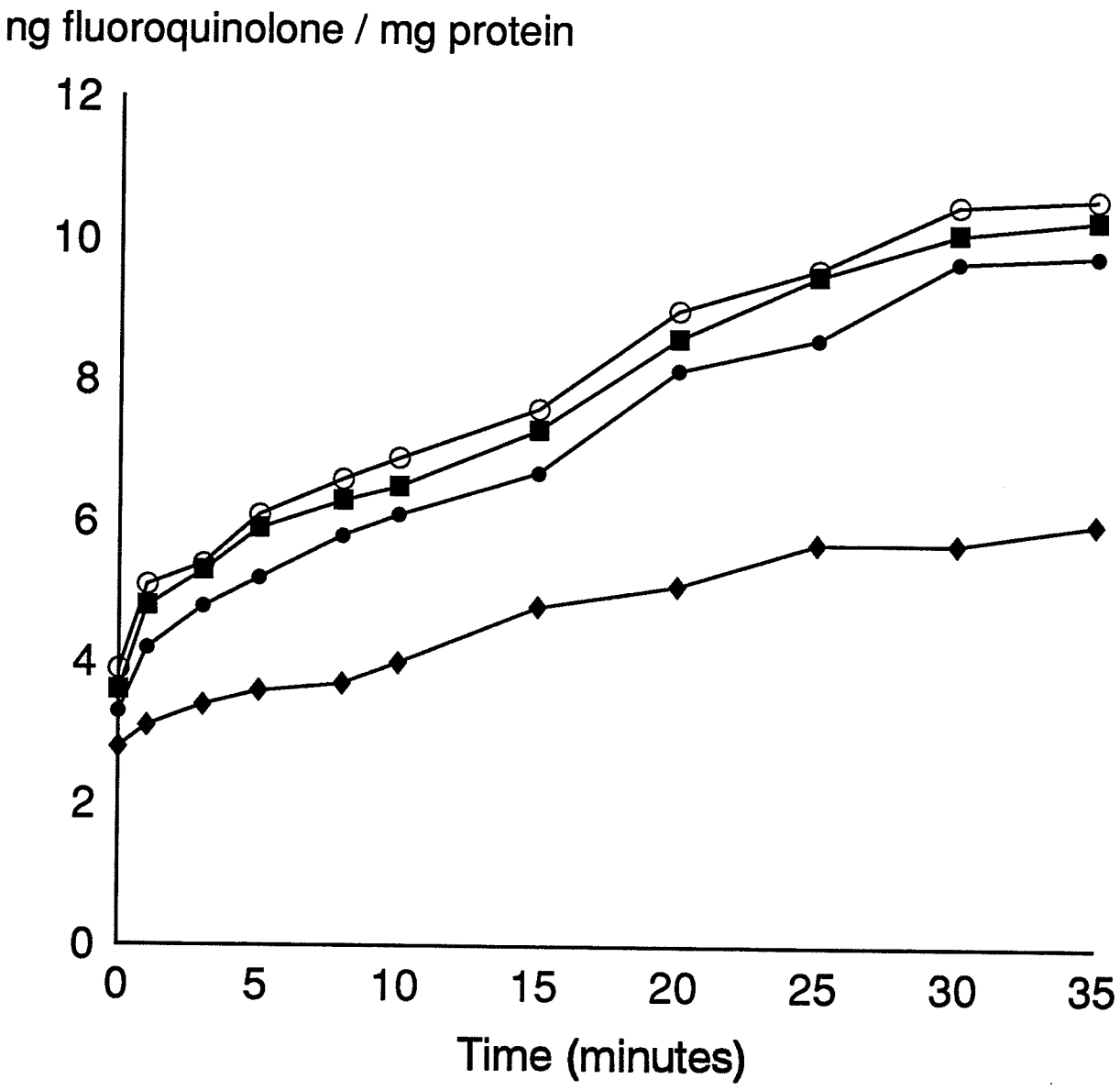

ng fluoroquinolone / mg protein
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Time (minutes)

Figure 26. Accumulation kinetics of ^{14}C-sparfloxacin and ^{14}C-norfloxacin in logarithmic phase and PAE phase S.aureus F651. Logarithmic phase and PAE phase cultures of S.aureus F651 were exposed to 16 μg/ml of ^{14}C-sparfloxacin and ^{14}C-norfloxacin. Log phase cultures: sparfloxacin ■, norfloxacin ♦ PAE phase cultures: sparfloxacin ○, norfloxacin ● The PAE was induced by exposure to 10x MIC of ciprofloxacin. Results expressed as ng fluoroquinolone / mg total cell protein

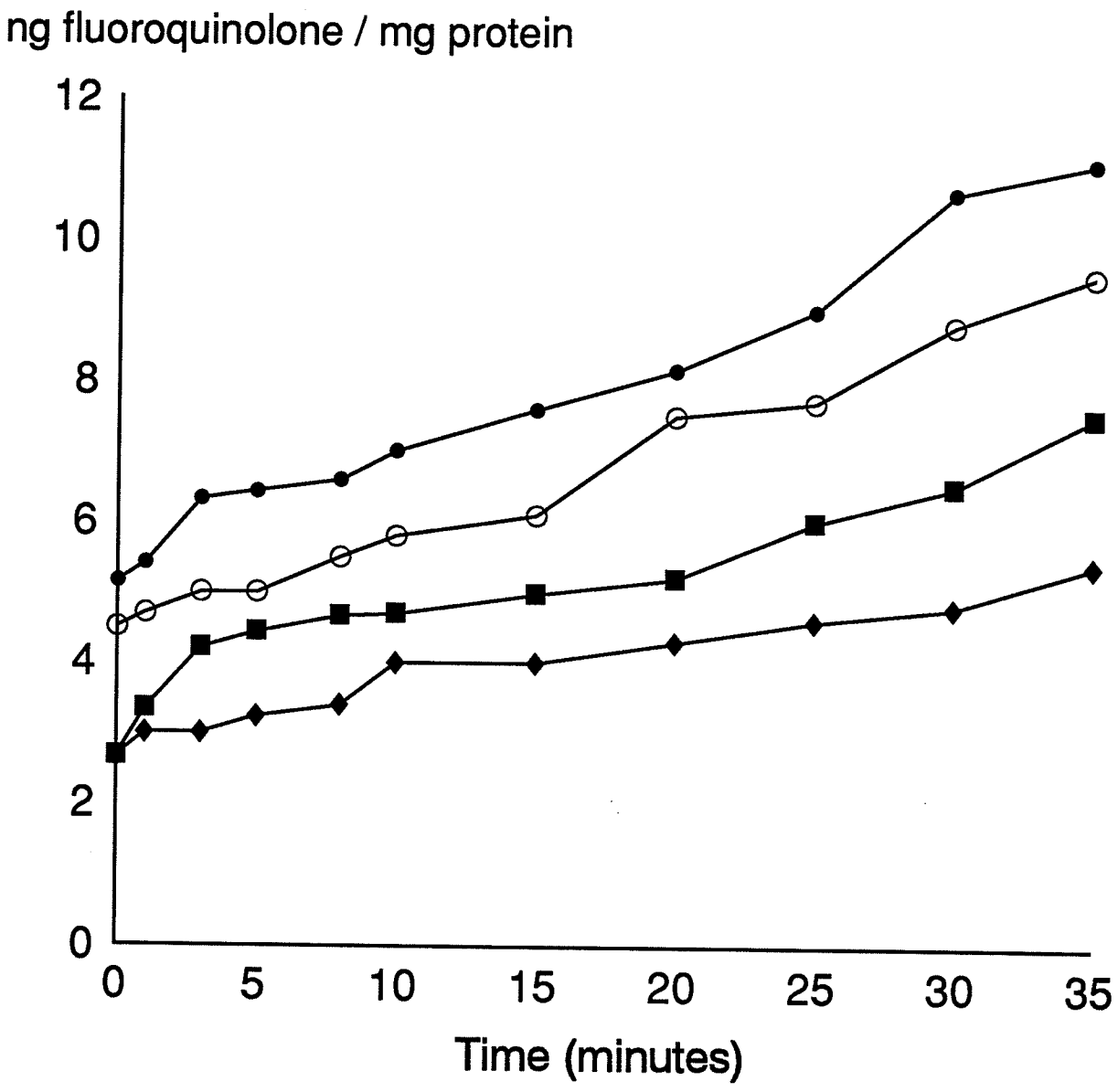

ng fluoroquinolone / mg protein
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Time (minutes)

Figure 27. Accumulation kinetics of ^{14}C-pefloxacin and ^{14}C-lomefloxacin in logarithmic phase and PAE phase S.aureus F651. Logarithmic phase and PAE phase cultures of S.aureus F651 were exposed to 16 μg/ml of ^{14}C-lomefloxacin and ^{14}C-pefloxacin. Log phase cultures: pefloxacin ■, lomefloxacin ♦. PAE phase cultures: pefloxacin ●, lomefloxacin ○. The PAE was induced by exposure to 10x MIC of ciprofloxacin. Results expressed as ng fluoroquinolone / mg total cell protein.

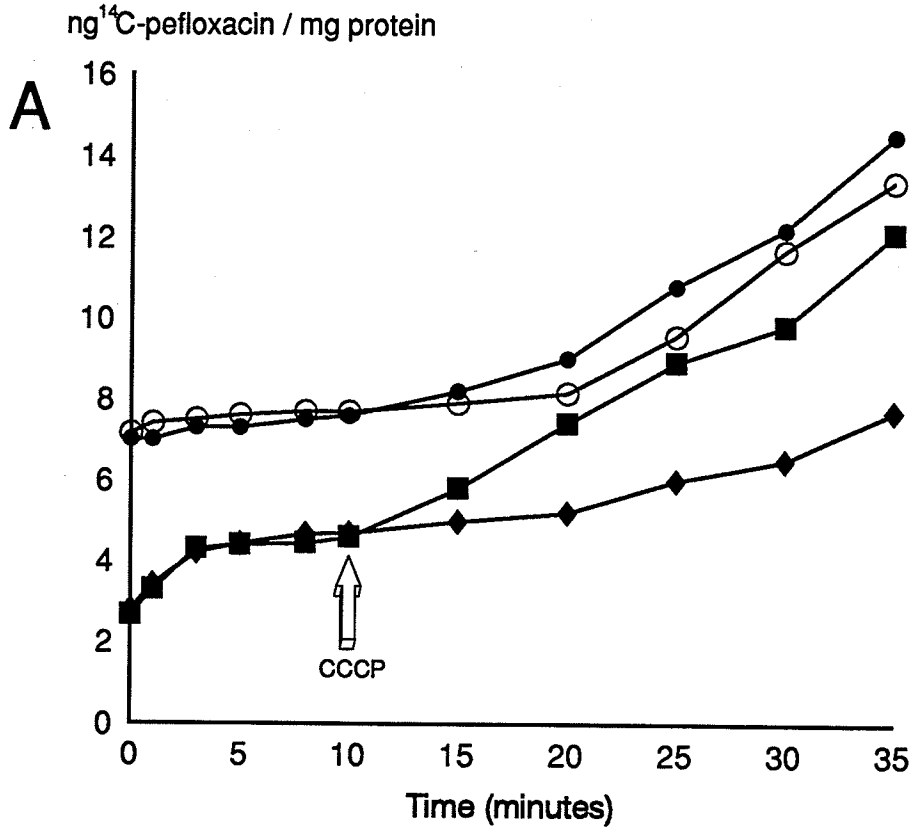
compared to accumulation in logarithmic phase cells. Sparfloxacin, however, did not show a significant increase in accumulation during the PAE ($p>0.05$). This trend was observed regardless of the fluoroquinolone used to induce the PAE and was seen in cells exposed to both 4x or 10x MIC. Cells exposed to 10x MIC did, however, accumulate somewhat higher levels of drug than those cells exposed to 4x MIC. These experiments were repeated in several S.aureus strains with the same result occurring in each strain.

ii) The effect of inhibitors on accumulation of fluoroquinolones during the PAE:

When fluoroquinolone accumulation was examined during the PAE, the relative amount of drug accumulation appeared to be similar to that seen in actively growing cells exposed to CCCP. We therefore examined the uptake of fluoroquinolones during the PAE and in the presence of CCCP (Fig. 28 and 29). The already elevated fluoroquinolone levels found in cells during the PAE phase were not significantly increased ($p>0.05$) in the presence of CCCP. This result was observed in all antimicrobials examined (sparfloxacin, pefloxacin, norfloxacin, and lomefloxacin). Similar results were obtained when fluoroquinolone accumulation was examined in the presence of 2,4-DNP. As well, similar results were obtained in all S.aureus strains examined.

iii) Accumulation of fluoroquinolones post - PAE:

Our original hypothesis stated that fluoroquinolone accumulation would be increased during the PAE. This was based on the growth characteristics of cells during this phase. Using the same model we felt that cells leaving the PAE phase and


ng ^{14}C-pefloxacin / mg protein
A
16
14
12
10
8
6
4
2
0
CCCP
0
5
10
15
20
25
30
35
Time (minutes)

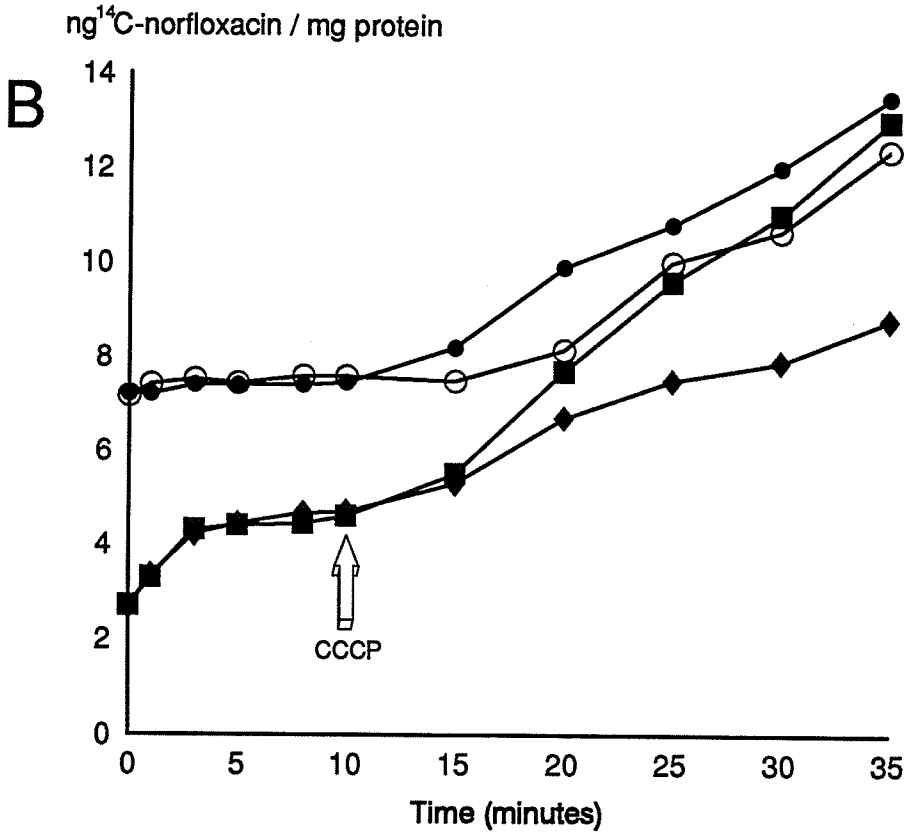
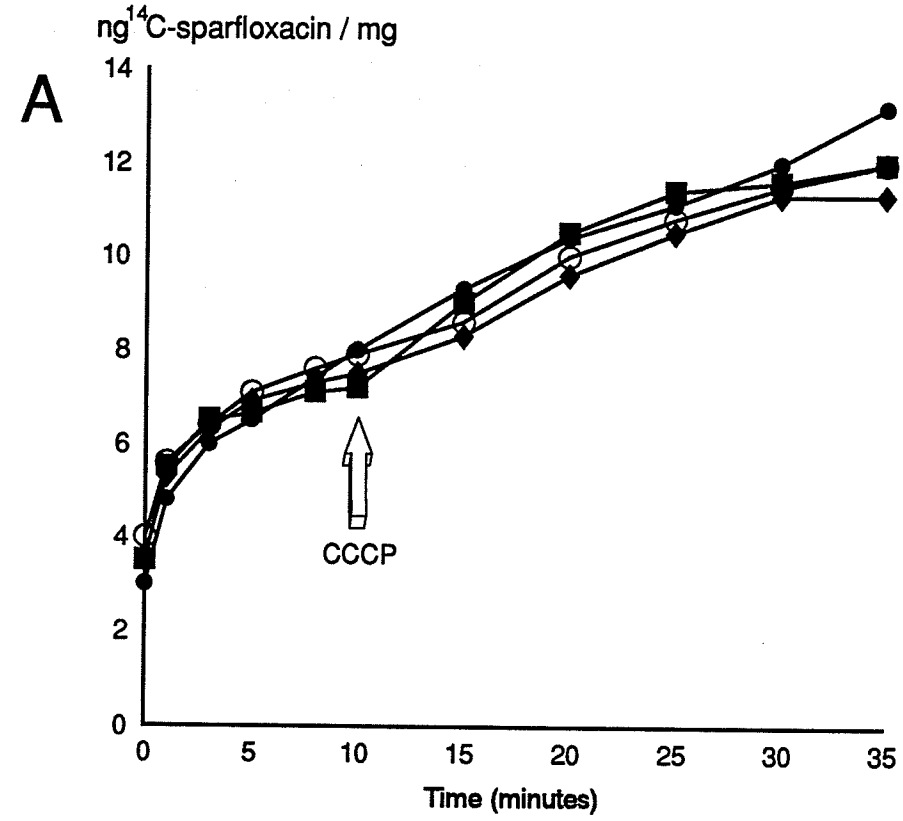

ng ^{14}C-norfloxacin / mg protein
B
14
12
10
8
6
4
2
0
CCCP
0
5
10
15
20
25
30
35
Time (minutes)

Figure 28. The effect of CCCP on the accumulation of ^{14}C-norfloxacin and ^{14}C-pefloxacin during the PAE. Logarithmic phase and PAE phase cultures of S.aureus F988 were exposed to 16 μg/ml of ^{14}C-norfloxacin and ^{14}C-pefloxacin in the presence and absence of 50 μM CCCP. **A.** Norfloxacin **B.** Pefloxacin. Log phase cultures ♦, + CCCP ■. PAE phase cultures O, + CCCP ●. The PAE was induced by exposure to 10x MIC of ciprofloxacin. Results expressed as ng fluoroquinolone / mg total cell protein.


A
ng ^{14}C-sparfloxacin / mg
14
12
10
8
6
4
2
0
CCCP
0
5
10
15
20
25
30
35
Time (minutes)

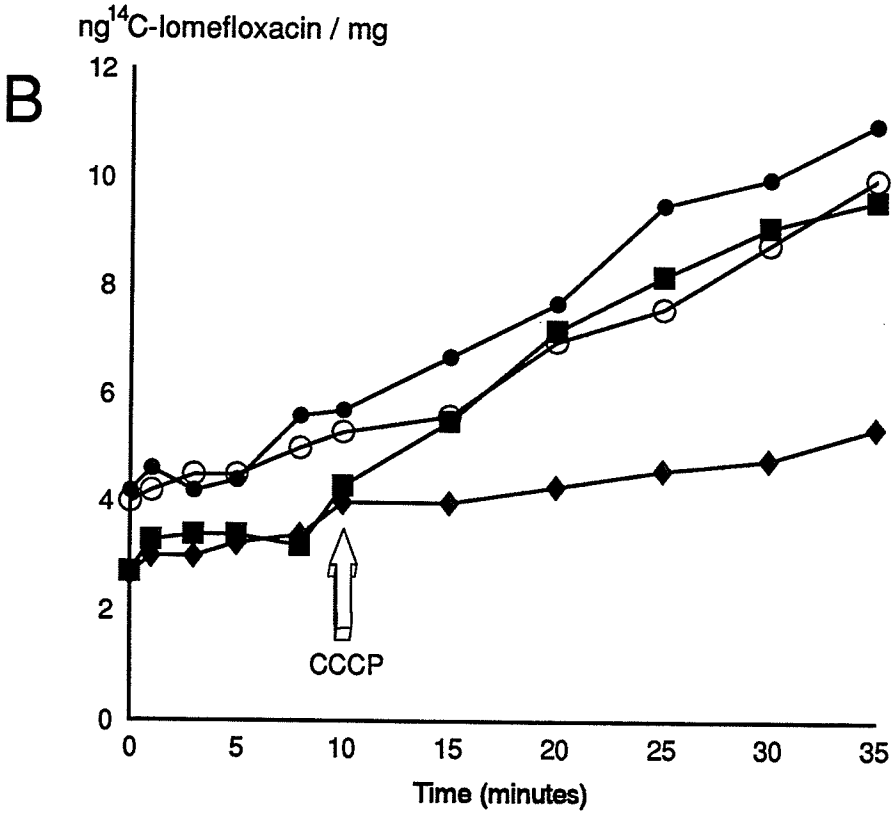

B
ng ^{14}C-lomefloxacin / mg
12
10
8
6
4
2
0
CCCP
0
5
10
15
20
25
30
35
Time (minutes)

Figure 29. The effect of CCCP on the accumulation of ^{14}C-sparfloxacin and ^{14}C-lomefloxacin during the PAE. Logarithmic phase and PAE phase cultures of S.aureus F988 were exposed to 16 μg/ml of ^{14}C-sparfloxacin and ^{14}C-lomefloxacin in the presence and absence of 50 μM CCCP. **A.** Sparfloxacin **B.** Lomefloxacin. Log phase cultures ♦, + CCCP ■. PAE phase cultures ○, + CCCP ●. The PAE was induced by exposure to 10x MIC of ciprofloxacin. Results expressed as ng fluoroquinolone / mg total cell protein.

resuming logarithmic growth, would have accumulation kinetics similar to pre-exposure or untreated cells. We therefore examined fluoroquinolone accumulation in S.aureus at 1, 4, and 6 hours after resumption of logarithmic growth and compared these results with accumulation in untreated logarithmic phase cultures. Just prior to the start of each uptake assay bacterial cells in both the controls and the post-PAE cell cultures were adjusted to approximately 10^8 cfu/ml.

At one hour post-PAE, accumulation kinetics of ^{14}C-pefloxacin were surprisingly similar to those seen during the PAE (Fig.30). Four hours after resumption of logarithmic phase growth, accumulation of ^{14}C-pefloxacin was significantly lower than seen at 1 hour post-PAE, however levels were still significantly higher than seen in untreated cells ($p<0.05$). Accumulation kinetics at 6 hours post-PAE had returned to "control levels" and accumulation was not significantly different than levels seen in control cells ($p<0.05$). This pattern of accumulation was consistent regardless of the fluoroquinolone used to induce the PAE.

c) Accumulation of fluoroquinolones in resistant S.aureus:

Bacteria use a plethora of techniques to acquire resistance to antimicrobial agents. Limiting access of the antimicrobial agent to its site of action by restricting passage of the agent into the cell or immediately removing it from the cell has been demonstrated previously using several classes of antimicrobials. Studies on the accumulation of fluoroquinolones in gram negative bacteria have demonstrated the importance of Omp F in fluoroquinolone passage through the outer membrane (Chapman and Georgopapadakou,1988). These same studies have also shown that low

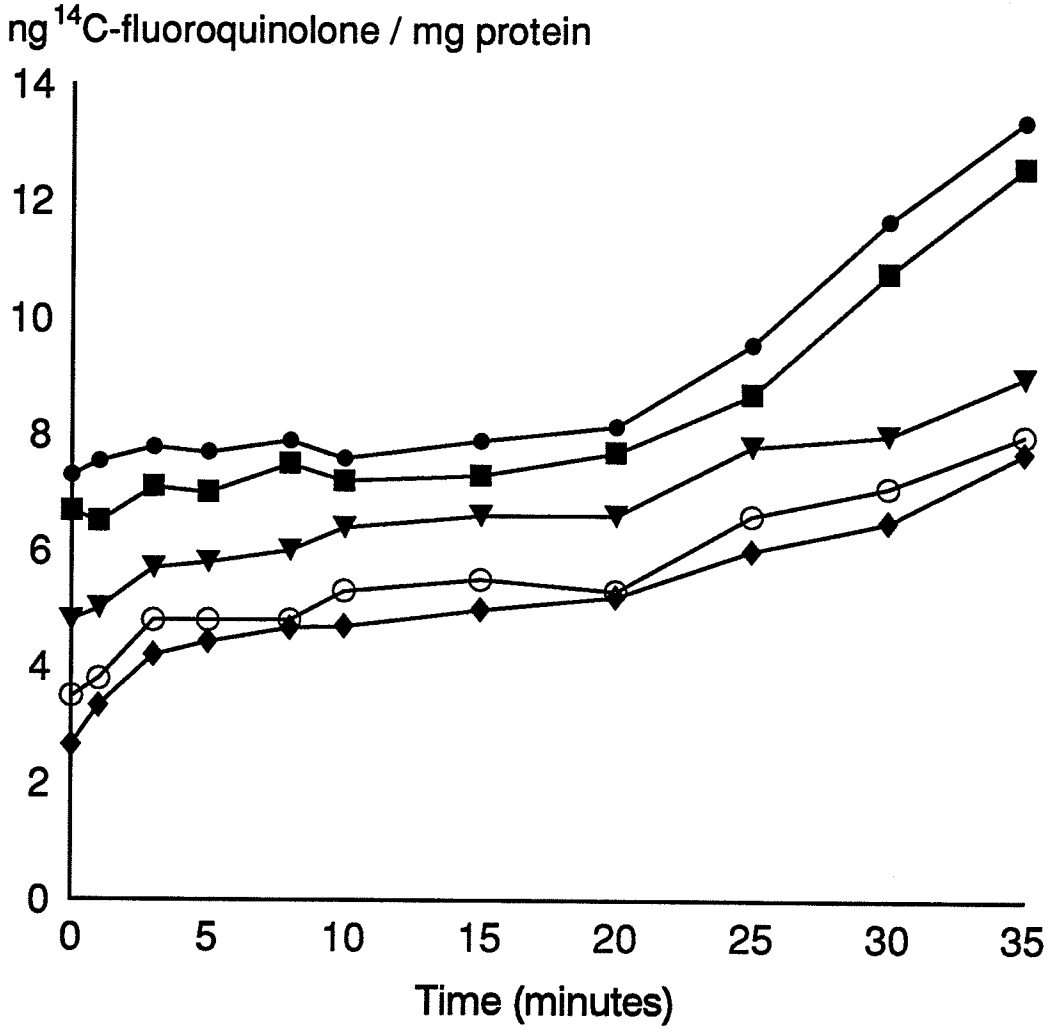
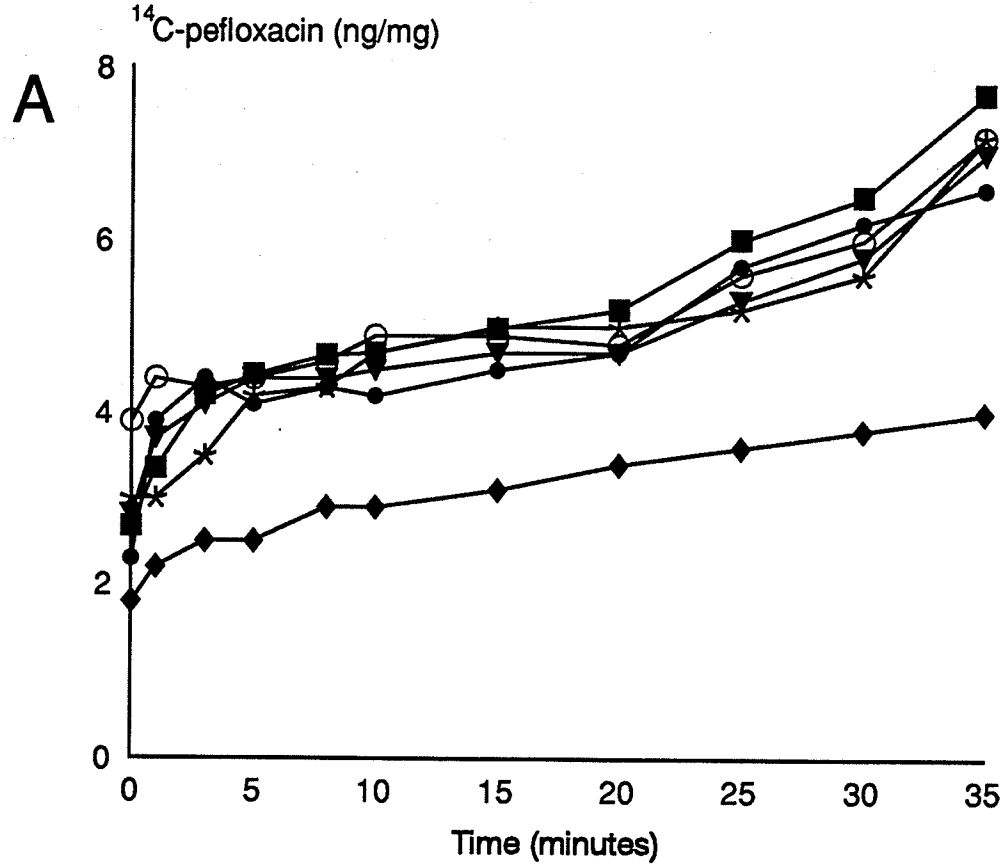

ng ^{14}C-fluoroquinolone / mg protein
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Time (minutes)

Figure 30. The accumulation of ^{14}C-pefloxacin in S.aureus after leaving the PAE phase. The accumulation of 16 μg/ml ^{14}C-pefloxacin was determined in logarithmic phase cells ♦, PAE phase cells ●, and after cells had resumed logarithmic growth 1 hour post-PAE ■, 4 hours post-PAE ▼, and 6 hours post-PAE ○ of S.aureus F651. The PAE was induced by exposure to 10x MIC of ciprofloxacin. Results are expressed as ng fluoroquinolone / mg total cell protein.

level fluoroquinolone resistance can be acquired in E.coli by the loss or reduced expression of Omp F. Little data exist however for the role of permeability in the acquisition of fluoroquinolone resistance in S.aureus. We therefore studied the accumulation of ^{14}C-fluoroquinolones in our low and high level fluoroquinolone resistant laboratory mutants and high level resistant clinical isolates. The accumulation of ^{14}C-pefloxacin, ^{14}C-sparfloxacin, and ^{14}C-norfloxacin in wild type susceptible S.aureus strains were compared with the accumulation of these agents in low level ciprofloxacin resistant mutants (MIC 2.8 μg/ml), mid level resistant mutants (MIC 5.6μg/ml), and high level resistant mutants (MIC 11-22 μg/ml). High level ciprofloxacin resistant clinical isolates (MIC 256-1024 μg/ml) were also examined.

Accumulation of ^{14}C-pefloxacin and ^{14}C-norfloxacin in wild type strains F651 and F192 were similar to accumulation kinetics in low level resistant strains F651 R-3 and F192 R-10 (MIC 2.8 μg/ml) (Fig.31). The mean results over several experiments did not show any significant difference ($p>0.05$). When experiments were repeated using mid level and high level resistant mutants we found reduced accumulation in strains F651 R4-7 at both mid and high levels of ciprofloxacin resistance. Strain F192 R11/12 did not exhibit any difference in uptake kinetics with ^{14}C-pefloxacin or ^{14}C-norfloxacin until its MIC approached 22 μg/ml, (R13). High level fluoroquinolone resistant isolates all showed reduced accumulation of ^{14}C-pefloxacin and ^{14}C-norfloxacin. Accumulation kinetics of ^{14}C-sparfloxacin in resistant strains were not significantly different than accumulation observed in wild type strains, even in those strains that had reduced accumulation with ^{14}C-pefloxacin and ^{14}C-norfloxacin (Fig.32).


A
^{14}C-pefloxacin (ng/mg)
8
6
4
2
0
0
5
10
15
20
25
30
35
Time (minutes)

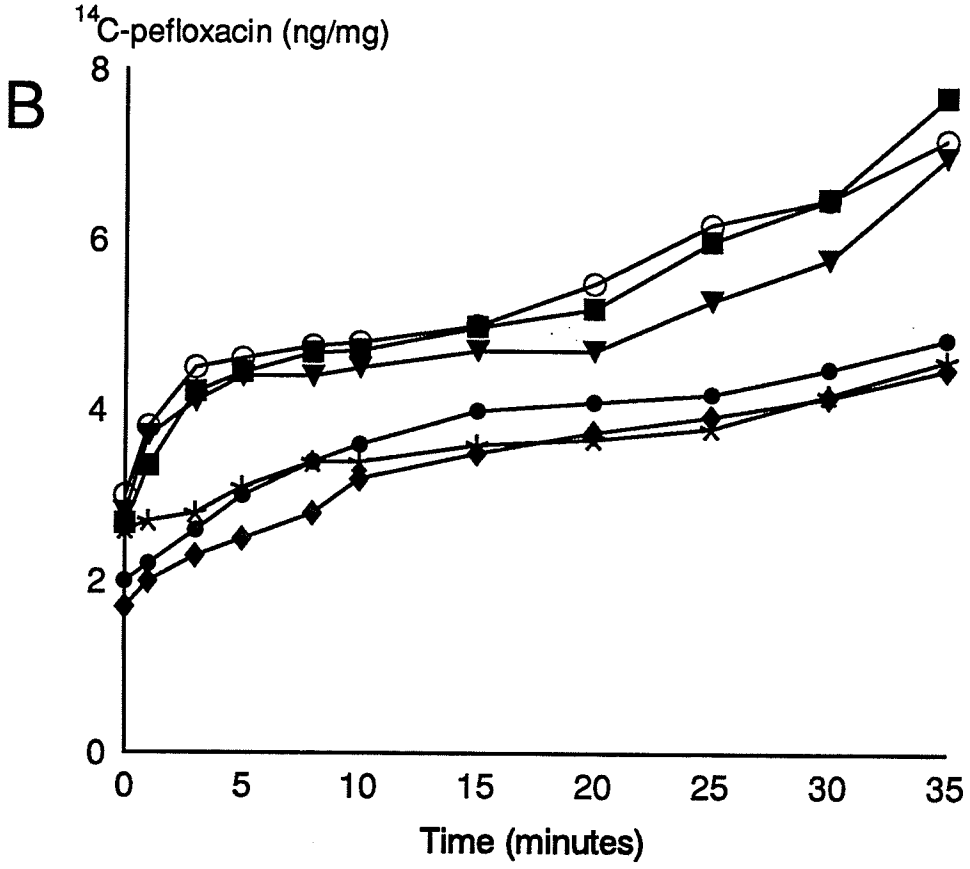

B
^{14}C-pefloxacin (ng/mg)
8
6
4
2
0
0
5
10
15
20
25
30
35
Time (minutes)

Figure 31. Accumulation of ^{14}C-pefloxacin in fluoroquinolone resistant mutants of S.aureus. The uptake of 16 µg/ml ^{14}C-pefloxacin was compared in wild type and ciprofloxacin resistant S.aureus mutants. **A:** F651 (wt) ■, F192 (wt) ▼, F651 R-3 (cip 2.8) ○, F192 R-10 (cip 2.8) ●, F651 R-4 (cip 5.6) ♦, F192 R-11 (cip 5.6) *. **B:** F651 (wt) ■, F192 (wt) ▼, F651 R-5 (cip 11) ●, F192 R-12 (cip 11) ○, F192 R-6 (cip 22) ♦, #609 (cip 1024) *. All results expressed as ng ^{14}C-pefloxacin / mg total cell protein.

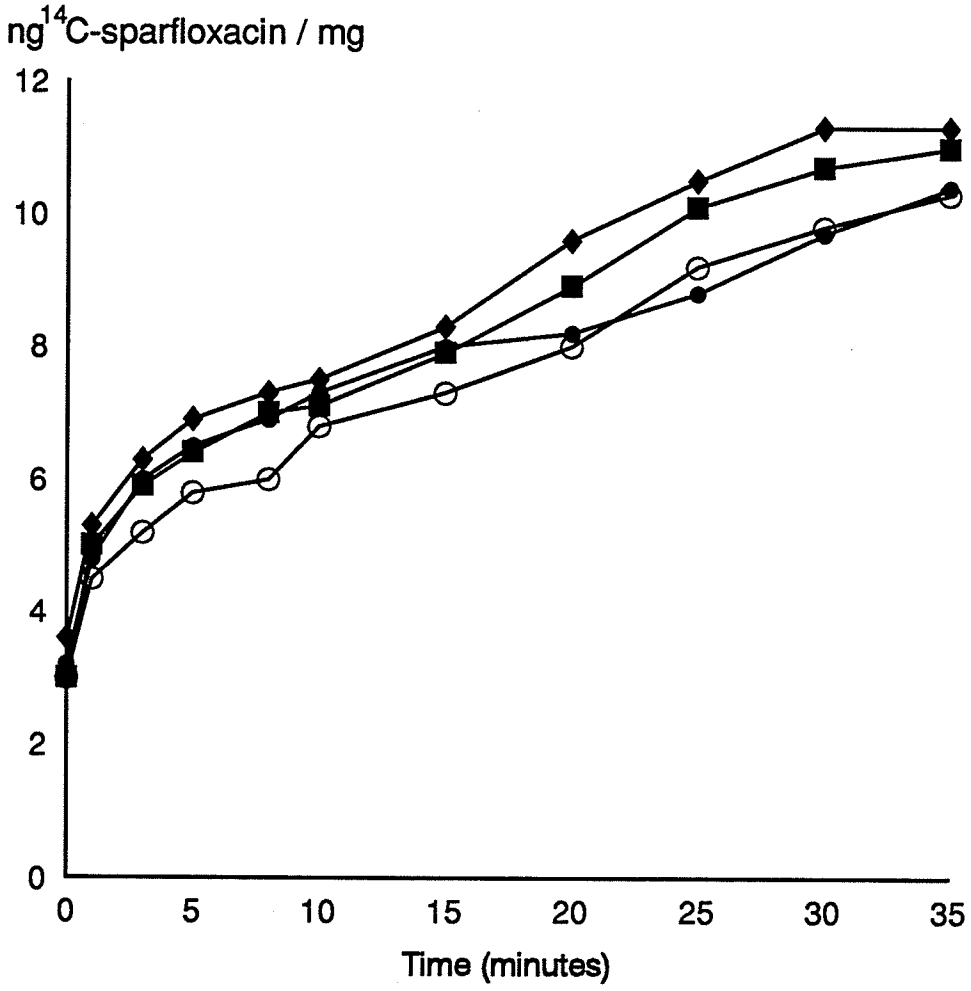
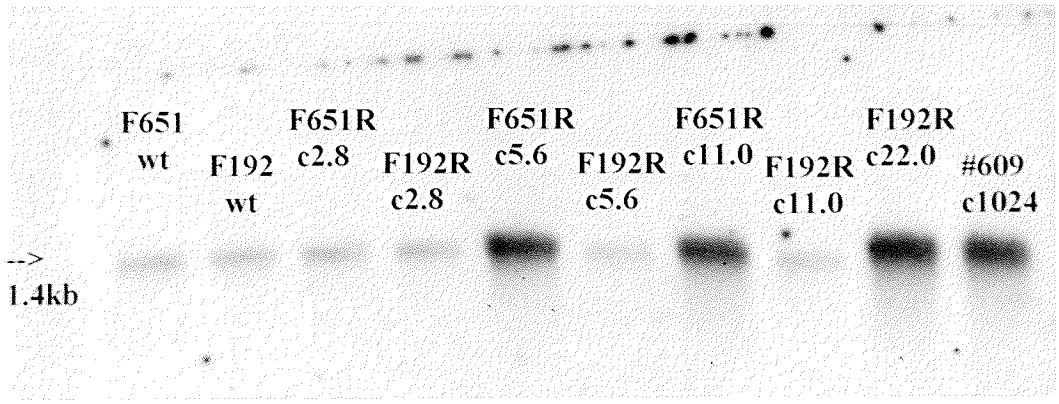

ng 14C-sparfloxacin / mg
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Time (minutes)

Figure 32. Accumulation of ^{14}C-sparfloxacin in fluoroquinolone resistant mutants of S.aureus. The uptake of 16 μg/ml ^{14}C-sparfloxacin was compared in wild type and ciprofloxacin resistant S.aureus mutants. F651 (wt) ♦, F651 R-3 (cip 2.8) ■, F651 R-4 (cip 5.6) O, F651 R-5 (cip 11) ●. All results expressed as ng ^{14}C-sparfloxacin / mg total cell protein.

In an attempt to explain our above findings we examined the DNA sequence in the open reading frame of the norA gene amplified from both susceptible and resistant mutants (Table 16). We were unable to find any DNA sequence changes in the open reading frame between susceptible and resistant isolates. Ohshita et al had reported that a substitution from A to C, resulting in an amino acid change from Asp to Ala near the 3' end of the norA open reading frame, was responsible for quinolone resistance in isolates of S.aureus (Ohshita et al,1990). Our results indicate that an alanine residue resides at this position in all our strains; susceptible and resistant. Given that we could not find any DNA sequence changes in the coding region of the norA gene we considered the possibility that increased expression of the norA was responsible for the reduced uptake seen in our mutant strains. To answer this question, total cellular RNA was isolated from both susceptible and resistant S.aureus strains and a Northern analysis performed (Fig. 33). Our results suggested that several of the strains did have higher expression of norA than others. Results of the Northern analysis correlated well with the uptake data suggesting a strong connection between fluoroquinolone accumulation and expression of norA. Only those strains that had demonstrated reduced uptake of radiolabeled fluoroquinolones were shown to have increased expression of norA by Northern blot.

This data prompted us to sequence the DNA region upstream of the norA start codon and search for sequence changes in the norA promoter region. A 500 bp fragment of DNA was amplified spanning a region 480 bp upstream of the norA start codon and including the first 18 bp of the norA coding region. Results of the DNA

Figure 33. Northern blot of total cellular RNA isolated from susceptible and fluoroquinolone resistant S.aureus. The blot was probed with a ^{32}P-labeled DNA fragment internal to the norA gene coding region. Labels indicate strain and ciprofloxacin MIC.

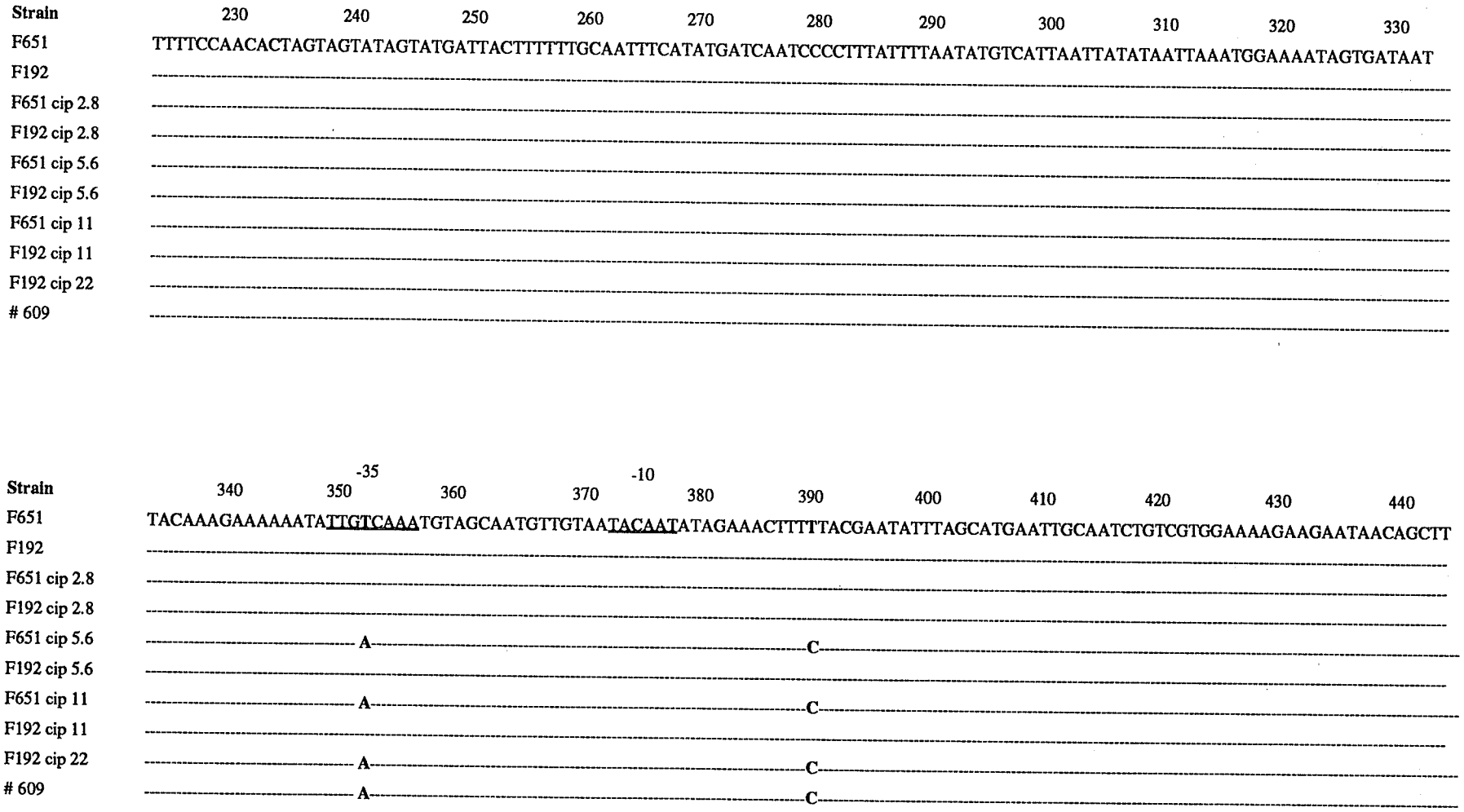


sequencing analysis are found in tables 22 and 23. We found two base substitutions present in all our resistant strains exhibiting reduced fluoroquinolone uptake and increased expression of norA. The first substitution appeared at position 352 where a T was replaced with an A. Position 352 is found within the putative -35 consensus sequence of the norA promoter. The second substitution occurred at position 390 where a T was replaced with a C. Position 390 is located 89 bp upstream from the ATG start codon and is found in a region between the start codon and the -10 and -35 consensus sequences.

Table 22. DNA sequence of a 500 bp fragment spanning a region 480 bp upstream of the norA ATG start codon and including the first 18 bp of the norA coding region. Nucleotide substitutions and the ATG start codon are in boldface. The -10 and -35 sequences are double underlined.

Strain	10	20	30	40	50	60	70	80	90	100	110
F651	GCATGCAAATGTGCAAAATGACCATTGTCTAGACGAGAAATATTACCTAATAAGCTCGTCAATTCCAGTGGCTCAGTAATATGTTTTTCTTCGTATTGTTTCGTTGTTAAA
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F651 cip 5.6	----------
F192 cip 5.6	----------
F651 cip 11	----------
F192 cip 11	----------
F192 cip 22	----------
# 609	----------

Strain	120	130	140	150	160	170	180	190	200	210	220
F651	TTGAAAAATTTTAATACAACGTCATCACATGCACCAATGCCGCTGACAGATGTAAATGTTAAGTCTTGGTCATCTGCAAAGGTTGTTATACATTCAACGATATCTTCTCCT
F192	----------
F651 cip 2.8	----------
F192 cip 2.8	----------
F651 cip 5.6	----------
F192 cip 5.6	----------
F651 cip 11	----------
F192 cip 11	----------
F192 cip 22	----------
# 609	----------


Strain
230 240 250 260 270 280 290 300 310 320 330
F651 TTTTCCAACACTAGTAGTATAGTATGATTACTTTTTTGCAATTTCATATGATCAATCCCCTTTATTTTAATATGTCATTAATTATATAATTAAATGGAAAATAGTGATAAT
F192
F651 cip 2.8
F192 cip 2.8
F651 cip 5.6
F192 cip 5.6
F651 cip 11
F192 cip 11
F192 cip 22
609
-35
-10
Strain
340 350 360 370 380 390 400 410 420 430 440
F651 TACAAAGAAAAAATATTGTCAAATGTAGCAATGTTGTAATACAATATAGAAACTTTTTACGAATATTTAGCATGAATTGCAATCTGTCGTGGAAAAGAAGAATAACAGCTT
F192
F651 cip 2.8
F192 cip 2.8
F651 cip 5.6 A C
F192 cip 5.6
F651 cip 11 A C
F192 cip 11
F192 cip 22 A C
609 A C

Strain	450	460	470	480	490	500
F651	TAAGCATGACATGGAGAAAAAAGAGGTGAGCATATGAATAAACAGATTTTTGTCTT					
F192	----------					
F651 cip 2.8	----------					
F192 cip 2.8	----------					
F651 cip 5.6	----------					
F192 cip 5.6	----------					
F651 cip 11	----------					
F192 cip 11	----------					
F192 cip 22	----------					
# 609	----------					

Table 23. (A and B): Nucleotide changes in the promoter region of norA.The -35 and -10 sequences are underlined.

A: The nucleotide change at position 352 from T to A is in boldface.

Strain	(352) -35 -10
F651	TATTGTCAAATGTAGCAATGTTGTAATACAATATA
F192	----------
F651 R-3 (cip 2.8)	----------
F192 R-10 (cip 2.8)	----------
F651 R-4 (cip 5.6)	----------**A**----------
F192 R-11 (cip 5.6)	----------
F651 R-5 (cip 11)	----------**A**----------
F192 R-12 (cip 11)	----------
F192 R-13 (cip 22)	----------**A**----------
#609	----------**A**----------

B: The nucleotide change at position 390 from T to C is in boldface.

Strain	-10 390
F651	AATACAATATAGAAACTTTTTACGAATATTT
F192	----------
F651 R-3 (cip 2.8)	----------
F192 R-10 (cip 2.8)	----------
F651 R-4 (cip 5.6)	----------**C**----------
F192 R-11 (cip 5.6)	----------
F651 R-5 (cip 11)	----------**C**----------
F192 R-12 (cip 11)	----------
F192 R-13 (cip 22)	----------**C**----------
#609	----------**C**----------

Discussion:

1: Postantibiotic Effect

a) Fluoroquinolone PAE in MSSA and MRSA:

Initial studies on the PAE of fluoroquinolones in S.aureus examined the effect of antimicrobial concentration on the duration of the PAE in both methicillin susceptible (MSSA) and methicillin resistant (MRSA) S.aureus. As expected, a concentration dependent PAE was observed in both MS and MR S.aureus strains. At 1x MIC, a PAE of only 5 minutes duration was observed in most strains. Given that our observations suggest that most PAE experiments probably have a standard error of +/- 5 minutes, the observed values at 1x MIC may in fact be artifactual. At 4x and 10x MIC we observed PAEs of approximately 1 hour and 1-1.25 hours respectively (tables 10 and 11). Although our results generally concur with values previously reported by others, the maximal PAE determinations we observed are considered somewhat low (Bundtzen et al,1981; Chin and Neu, 1987; Craig and Vogelman, 1987). Our slightly lower values may be due in part to the method we used to determine the MICs of our test agents. Most investigators determine MICs using serial two fold dilutions of antimicrobials (Chin and Neu,1987; Hessen et al,1989; Odenholt et al,1989; Rescott et al,1988). Our MICs were determined using a more accurate method. Thus an antimicrobial having a perceived MIC of 1.0 μg/ml might in reality have an MIC of only 0.58 μg/ml. Using MICs determined with serial two fold dilutions, PAE experiments performed at 10x MIC would result in the organism being exposed to 10 μg/ml and not 5.8 μg/ml. The organism would in fact be exposed

to >17x MIC and not 10x MIC. Experiments using drug concentrations above 10x MIC were not used in any of our studies as we felt that using fluoroquinolone concentrations higher than 10x MIC might result in organisms being exposed to drug levels not readily achievable in a clinical setting. Typically, peak serum levels of quinolones approach 0.35-0.4 μg/ml and trough levels approach 0.2 μg/ml.

Based on differences in the mechanisms of action and mechanisms of resistance between fluoroquinolones and methicillin, a β-lactam, we felt that no significant difference would exist between the quinolone PAE in fluoroquinolone susceptible MS and MR S.aureus. Fluoroquinolones are directed against an intracellular target, DNA gyrase. β-lactam antimicrobials exert their effect by inhibiting penicillin binding proteins (PBP) found in the membrane of the bacterial cell. Methicillin resistance is based on the production of a unique PBP (PBP 2a), a 78 Kda protein with low affinity for β-lactam antimicrobials (Hackbarth and Chambers,1989; Tomasz et al,1991). The results supported our hypothesis. When the quinolone PAEs of MSSA and MRSA strains were directly compared, no significant differences were observed (Davidson et al,1990).

b) Reproducibility of the fluoroquinolone PAE in S.aureus:

Present dosing regimens have been designed to maintain serum concentrations above the MIC of common organisms, within the antimicrobials spectrum of activity, for the majority of the dosing interval (Craig and Vogelman,1987). Although these empiric dosing schemes work fairly well in practice, they may not be optimal in terms of efficacy, toxicity, or cost. The PAE in combination with other

pharmacodynamic parameters provides a more accurate description of the duration of antimicrobial activity than does the MIC alone (Zhanel et al,1990). However, if we are to design antimicrobial dosing regimens based on *in-vitro* and *in-vivo* PAE data, reproducibility of the effect must first be demonstrated.

Fluoroquinolone PAEs were evaluated at three concentrations; 1x, 4x, and 10x MIC. Each experiment was repeated a minimum of seven times with the mean and standard deviation calculated after each set of experiments. Our results indicate that the PAE is a reproducible phenomenon. In addition, the reproducibility of the PAE increases with increasing concentration of antimicrobial as evidenced by the decreasing standard deviation at higher drug concentrations (Davidson et al,1990; Zhanel et al,1990). The greater variability of the PAE at or near the MIC may be the result of "population effects". In any dense bacterial population, a minority of organisms will have MICs several fold higher than the experimentally determined MIC. These organisms, able to grow in the presence of low concentrations of drug, could adversely effect the PAE.

c) Fluoroquinolone hydrophobicity:

The newer fluoroquinolones, while significantly less hydrophobic than their progenitor compound nalidixic acid, vary considerably in their degree of hydrophobicity. Methods used in the calculation of fluoroquinolone hydrophobicity vary widely and thus the relative values assigned to each compound also greatly differ in the literature (McCaffrey et al, 1992; Bazile et al,1992; Asuquo and Piddock,1993; Denis and Moreau,1993). The four fluoroquinolones we choose for

study, ranked from most to least hydrophobic, were sparfloxacin, pefloxacin, lomefloxacin, and norfloxacin respectively. The relative ranking of these four agents is well agreed upon in the literature (McCaffrey et al, 1992; Bazile et al,1992; Asuquo and Piddock,1993; Denis and Moreau,1993).

Sparfloxacin, the most hydrophobic quinolone examined produced PAEs of greater duration ($p<0.05$) in all strains than did the other agents. A general trend was observed indicating that the duration of the PAE was related to the hydrophobicity of the quinolone agents with the more hydrophobic agents producing the longest PAEs (Davidson et al ,1994). While this trend was quite readily apparent, statistically significant differences in the PAE of pefloxacin and lomefloxacin or lomefloxacin and norfloxacin were not always established. The reader will note that the fluoroquinolone ciprofloxacin was not included in this comparison. As we were unable to obtain a supply of ^{14}C-ciprofloxacin for use in accumulation experiments it was omitted from these experiments. We can, however, predict that ciprofloxacin would not be accumulated by the Staphylococal cell to the same degree as the more hydrophobic agents. In addition, we know that ciprofloxacin, a relatively hydrophilic quinolone, does produce a PAE in our S.aureus strains similar to that of sparfloxacin. This apparent discrepancy in the duration of the ciprofloxacin PAE and its degree of accumulation would appear to contridict our findings. Thus while hydrophobicity and the amount of fluoroquinolone accumulation may play a role in regulating the duration of the PAE, it is likely that other factors such as the agents affinity for its target play a much larger and more important role.

d) The PAE of fluoroquinolones in serum:

In-vitro experiments involving the PAE are generally performed using standard bacteriological media. Some investigators, in an attempt to bridge the gap between *in-vitro* and *in-vivo* experiments have examined the *in-vitro* PAE in biological fluids such as serum, urine and cerebrospinal fluid (Bundtzen et al,1981; Chin and Neu,1987; Davidson et al,1991; Karlowsky et al,1993; Van der Auwera and Klastersky,1987; Zhanel et al,1991,1992). Bundtzen reported that broth containing 90% heat inactivated human serum abolished the PAE of rifampin and reduced the PAE of tetracycline from 3.7 to 1.7 hours in E.coli. When MICs were determined in serum and PAE experiments repeated, based on the new MICs, the PAE was re-established. Chin and Neu reported that ciprofloxacin produced a 4 hour PAE in both MHB and MHB:serum (50:50).Van der Auwera and Klastersky measured the PAE with S.aureus, Listeria monocytogenes, and Mycobacterium fortuitum in serum samples drawn from patients 1 and 6 hours post-treatment with high dose amikacin. Prolonged PAEs were demonstrated with S.aureus and L.monocytogenes.

Our study was designed to determine and compare the PAEs of three fluoroquinolones in MHB and 100% normal human serum. MICs of the three fluoroquinolones determined in serum were not significantly increased over MICs determined in MHB. This is consistent with the low serum protein binding of these fluoroquinolones agents (Drusano,1989). Growth kinetics of S.aureus were similar in both MHB and serum, however the PAE was significantly increased in serum at both 4x and 10x MIC. Gram stains of cells during the PAE were made in both MHB and

serum to exclude the possibility that serum promoted clumping of the cells thereby artificially lowering the number of CFU and increasing the duration of the PAE. No apparent increase in clumping was observed. Heat treating the serum reduced the PAE of ciprofloxacin from 3.2 hours to 2.4 hours, still a significant increase over the PAE observed in MHB. However, this finding suggests that some heat labile component of serum acts in concert with the antimicrobials to extend the PAE. Other classes of drugs were examined to determine if this effect could be reproduced with other agents. Both the PAEs of gentamicin and cephalexin were increased in serum. The PAE of cloxacillin (a highly protein bound β-lactam) was significantly reduced. The MIC of cloxacillin was also found to be significantly increased in serum. A prolonged PAE of cloxacillin in serum was re-established when experiments were performed with drug concentrations designed to correct for the high protein binding.

e) PAE in fluoroquinolone resistant S.aureus.

A series of experiments were performed to determine and compare the PAEs in fluoroquinolone susceptible "wt" S.aureus and their isogenic fluoroquinolone resistant mutants. We hypothesized that quinolone induced PAEs in these related strains would be similar assuming they were exposed to the same multiple of their respective MICs. In our initial studies we examined the PAE in five susceptible clinical isolates of S.aureus and their corresponding isogenic fluoroquinolone resistant mutants. Ciprofloxacin had MICs of 22 μg/ml in all the resistant mutants. Two of the five mutants (F651 R-6 and F192 R-13) failed to enter a prolonged PAE phase. The other three mutants entered a PAE phase of similar duration to their

susceptible parental strains. The MICs of the two aberrant strains were repeated to ensure that underexposure of drug was not responsible for the apparent loss of PAE. The ciprofloxacin MICs were subsequently confirmed to be 22 μg/ml and additional PAE experiments demonstrated the reproducibility of the effect. Other highly resistant S.aureus strains were examined, but all produced a prolonged PAE.

A series of PAE experiments were then performed using F651-R and F192-R mutant strains with ciprofloxacin MICs of 0.7, 1.4, 2.8, 5.6, 11, and 22 μg/ml. Mutant strains with these MICs had been previously stocked during the process used to select for high level fluoroquinolone resistant mutants. We discovered that strain F651-R entered a PAE phase of similar duration to its parental strain until its MIC approached 22 μg/ml. Strain F192-R failed to produce a significant PAE when its MIC was determined to be 2.8 μg/ml. These results suggested that fluoroquinolone resistance was necessary but not sufficient to cause loss or abrogation of the PAE.

Given the apparent association between loss of PAE and development of resistance, the genes gyrA, gyrB, and norA were selected for examination. Mutations in gyrA are known to confer fluoroquinolone resistance in both gram negative and gram positive bacteria and the norA gene is thought to be responsible for encoding an active efflux pump capable of removing fluoroquinolones from the bacterial cell (Cohen et al,1988; Fasching et al,1991; Goseitz et al,1992; Kaatz et al,1993; Nakamura et al,1989; Ohshita et al,1990; Piddock et al,1991; Sreedharan et al.1990,1991; Ubukata et al,1989). Mutations in the gyrB gene were not until recently considered to confer high level fluoroquinolone resistance (Heisig,1993; Ito

et al,1994), however it was included for study because of its integral association with gyrA.

Examination of RFLP patterns were used as an initial screen in an attempt to first target one of the three genes, however no discernible difference in the RFLP pattern was observed in Southern blots probed for gyrB or norA. Different RFLP patterns were observed between susceptible S.aureus and high level fluoroquinolone resistant S.aureus strains when gyrA PCR fragments were digested with Hinf I. The difference in these patterns was due to the loss of a Hinf I site consistent with a nucleotide substitution at Ser 84. As we were unable to find gross changes between quinolone resistant PAE^+ and quinolone resistant PAE^- strains using RFLP analysis, all three genes were isolated and sequenced.

DNA sequence analysis in the coding regions of the gyrB and norA genes failed to reveal nucleotide changes between any of the strains examined. DNA sequences between susceptible wild type strains, quinolone resistant PAE^+ and quinolone resistant PAE^- strains were all identical. Ohshita et al (1990), had previously reported that a nucleotide substitution in the 3' end of the norA coding region from A to C was responsible for fluoroquinolone resistance in isolates of S.aureus. All our isolates, both susceptible and quinolone resistant strains had a C residue in this position. Other investigators have also shown that a C residue exists at this position in quinolone susceptible isolates of S.aureus (Ng et al,1994). We therefore felt that norA mediated fluoroquinolone resistance is not the result of this nucleotide substitution. DNA sequence analysis of gyrA revealed 1) a sequence change between

susceptible and high level fluoroquinolone resistant isolates and 2) a discrepancy between the DNA sequence of our isolates and the sequence of isolates we obtained from Toronto. The first nucleotide change, predicted by RFLP analysis was the substitution of C for T at nucleotide position 252. This substitution results in an amino acid change from Ser to Leu and has been well documented as one of the primary gyrA mutations responsible for fluoroquinolone resistance in S.aureus (Ito et al,1994; Peterson et al,1993). DNA sequence analysis also revealed that isolates collected from the clinical microbiology laboratory in Winnipeg had a T at position 75 in the gyrA coding region. The high level resistant isolates obtained from Toronto were found to have a C in this position. DNA sequences of gyrA published by Brockbank and Barth and Margerrison et al describe a C in this same position. The nucleotide substitution in our isolates results in an amino acid change from Tyr to Cys, one polar amino acid for another. Given that all S.aureus isolates obtained from the clinical microbiology laboratory in Winnipeg contain this substitution, the biological significance is unclear. It is apparent, however, that this nucleotide substitution alone could not be responsible for the loss of PAE. This substitution may have no biological relevance and simply be a reflection of the relatedness of our isolates. Nevertheless the question remains; does this substitution play a role in loss of PAE? If it does, it appears that this substitution (mutation) would have to act in concert with another yet undescribed mutation. Our DNA sequence analysis has excluded the coding regions of gyrA, gyrB, and norA.

The mechanisms of action and cellular effects of the fluoroquinolones are

complex and as yet are not fully understood. Studies have shown that cellular killing by fluoroquinolones are inhibited by chloramphenicol, rifampin, or amino acid starvation (Crumplin et al,1984; Zeiler,1985). These studies suggest that killing may require the new synthesis of protein(s). It should be noted that the inhibition of protein synthesis only stops the bactericidal effect of quinolones; DNA synthesis remains inhibited.

One of the more interesting observations is the induction of the SOS system in bacteria after exposure to fluoroquinolones (Piddock et al,1987,1990). It is well known that the SOS response includes a variety of changes; the increased transcription of some genes, repression of others, as well as post transcriptional modifications. One role of the SOS system is to inhibit cell division. Presumably this is to prevent the formation of new daughter cells containing damaged DNA. Piddock has speculated that this inhibition of cell division may, in part, be responsible for cell death. Although some investigators have stated that the fluoroquinolone PAE may represent the time required for the drugs to diffuse from the DNA:DNA gyrase complex (Fuursted, 1987), involvement of the SOS system seems plausible. The duration of the PAE may also reflect, in part, the time required for the decay of SOS functions and a return to normal cell metabolism. If the latter hypothesis is true, the observed loss of PAE in our mutants strains may be a result of impaired SOS functions. That is, the SOS system fails to inhibit cell division and therefore the exposed cells resume logarithmic growth shortly after removal of the fluoroquinolone agent. Although this hypothesis was not explored during the course of this thesis, I

believe this avenue should be examined.

2: Fluoroquinolone accumulation in S.aureus:

a) Effect of temperature, concentration:

Our initial studies examined the effects of temperature and concentration on the accumulation of fluoroquinolones in S.aureus. We found uptake of these agents was increased in a linear fashion both with increasing concentration and temperature. These results were similar to previous reports investigating fluoroquinolone accumulation in gram negative organisms (Bedard et al,1987; Diver et al,1990) and more recent reports on the accumulation of these agents in gram positive organisms (Yoshida et al,1991). Accumulation experiments performed with high concentrations of ^{14}C-quinolone failed to show any saturation of the accumulation system. In addition, the accumulation kinetics of ^{14}C-pefloxacin were not altered in the presence of unlabeled ciprofloxacin. These results are consistent with the hypothesis that accumulation of fluoroquinolones proceeds via an energy independent passive diffusion process (Davidson et al,1991).

b) Effect of metabolic inhibitors:

To provide further evidence for the passive diffusion of fluoroquinolone agents into the bacterial cell, accumulation of ^{14}C-quinolones were determined in the presence of the metabolic inhibitors CCCP, 2,4-DNP, NaN_3, and KCN. CCCP and 2,4-DNP are known to destroy the proton motive force (PMF) across the cytoplasmic membrane, while KCN and NaN_3 are known to inhibit electron transport. All metabolic inhibitors failed to impede accumulation supporting the conclusion of a

passive uptake process. However, all intracellular ^{14}C-quinolones concentrations, were significantly increased in the presence of these inhibitors. While our results confirmed that quinolones enter the cell via an energy independent process, the observed increase in fluoroquinolone accumulation suggested the presence of an energy dependent efflux system. This observation has also been noted by other investigators in the field (Okuda et al,1991;Yoshida et al,1991; McCaffrey et al,1992;Denis and Moreau,1993; Piddock,1994). Such a system was first described by Cohen et al (1988) when he reported the presence of an endogenous active efflux system in E.coli. The gene responsible for coding the purported efflux protein, norA, has now been identified and cloned in S.aureus (Ubukata et al,1989; Yoshida et al,1990). The open reading frame of the norA gene encodes a protein of 388 amino acids with an estimated molecular weight of 42,265 daltons. The deduced polypeptide appears to contain 12 hydrophobic membrane-spanning regions (Yoshida et al,1990). The gene has been found in both fluoroquinolone susceptible and resistant strains of S.aureus. Of interest is the fact that this efflux system is the first described for a fully synthetic antimicrobial.

c) Effect of fluoroquinolone hydrophobicity:

The newer fluoroquinolones, while significantly less hydrophobic than their progenitor compound nalidixic acid, vary considerably in their degree of hydrophobicity. This study selected four fluoroquinolones and attempted to correlate the duration of the PAE and the level of fluoroquinolone uptake in S.aureus with the degree of hydrophobicity of each agent. Sparfloxacin, the most hydrophobic

fluoroquinolone we examined induced the longest PAE and achieved higher intracellular levels than did the other more hydrophilic agents. Other investigators had also observed high accumulation levels of sparfloxacin in S.aureus (Okuda et al,1991;Yoshida et al,1991;Piddock and Zhu,1991; Denis and Moreau,1993), however, none have attempted to correlate this with the PAE. We propose two explanations for the observed higher levels of sparfloxacin accumulation. Firstly, the lipophilicity of the more hydrophobic agent should allow easier penetration of the gram positive membrane. Secondly it has been suggested that the norA efflux system is only effective in pumping hydrophilic fluoroquinolones out of the bacterial cell. Our results would support both these ideas. The addition of CCCP resulted in a significant increase in the uptake of the hydrophilic fluoroquinolones but failed to significantly increase the accumulation of sparfloxacin. Denis and Moreau, 1993, also made this observation. Failure of CCCP to significantly increase the accumulation of sparfloxacin was not totally unexpected given the hypothesis that norA efflux favors hydrophilic agents.

We had previously shown that hydrophobic fluoroquinolones tend to induce PAEs of longer duration than do hydrophilic agents. These results alone might suggest that the duration of the PAE could be related to the hydrophobicity of the agent and its ability to achieve and maintain high intracellular levels of drug. However, if the uptake of ciprofloxacin were examined in S.aureus one would predict, based on its relative hydrophilicity, that its accumulation would be significantly lower that that of sparfloxacin. The PAE of ciprofloxacin induced in our

strains is similar to sparfloxacin. Ciprofloxacin's affinity for the gyrase complex may be greater than that of sparfloxacin, hence its effect may be as pronounced as sparfloxacin even with lower intracellular levels of drug accumulation.

Our results also indicated that a relationship did exist between the hydrophobicity of the agents and the degree of accumulation. Yoshida et al 1991, McCaffrey et al 1992, Bazile et al 1992, and Denis and Moreau 1993, all come to the same conclusion. Asuquo and Piddock 1993, were not, however, able to arrive at this conclusion. This may be in part due to the high correlation coefficient limits they set to identify a positive relationship.

We were also able to show a relationship between the MIC and relative amount of fluoroquinolone accumulation. This relationship was not perfect, however, and was based only on a limited number of strains. While Yoshida et al (1991) also reported a positive relationship, Bazile et al 1992, Asuquo and Piddock 1993, and Piddock 1993, observed no relationship between the amount of accumulation and antibacterial activity of fluoroquinolones.

d) Fluoroquinolone uptake during the PAE:

During the PAE, organisms are not actively dividing and may in fact be undergoing self repair. We felt that these cells may be metabolically depressed and therefore unable to generate sufficient energy to drive an energy dependent efflux system at optimal levels. Based on this we hypothesized that S.aureus would accumulate higher levels of fluoroquinolones during the PAE due to the organisms inability to effectively remove the intracellular accumulation of drug.

Three of the four quinolones examined; pefloxacin, norfloxacin, and lomefloxacin, were found to accumulate significantly higher amounts of ^{14}C-quinolone in cells during the PAE than in actively growing cells. These observations were consistent with our hypothesis that the efflux system is depressed during the PAE. The uptake of sparfloxacin was not significantly increased during the PAE. This was an expected result given our previous results suggesting that hydrophobic quinolones are not effectively removed by the norA efflux system. To further test our hypothesis we examined the uptake of radiolabeled quinolones in the presence of CCCP. The addition of the metabolic inhibitor failed to significantly increase the accumulation of the ^{14}C-quinolone agents suggesting that the norA efflux system was already depressed (Davidson et al,1991).

We have previously speculated as to the mechanism of the fluoroquinolone PAE; 1) time required for the quinolone agent to disassociate from the gyrase complex and 2) the time required for the decay of SOS functions. Data collected from our uptake experiments suggest a third possibility. While induction of the PAE is unlikely to be the result of any transport mechanism, the continuation of the PAE may in part be due to the temporary inactivation or suppression of the norA efflux system. The suppression of such a system would allow a relatively high level of fluoroquinolones to remain inside the cell thus providing a pool of drug available for continued binding to the target site. The levels of intracellular drug would slowly dissipate through the membrane by passive diffusion.

Our data appears to support our original hypothesis that the norA efflux system

is depressed during the PAE. Based on this hypothesis, it would be reasonable to assume that organisms leaving the PAE phase and resuming exponential growth would accumulate fluoroquinolones in a similar manner to "normal" untreated cell populations. Our finding that accumulation kinetics were still affected 1 and 4 hours post-PAE was quite unexpected. In fact, cells required an average of 6 hours to resume normal steady state kinetics. Although this finding raises many questions, it clearly demonstrates that effects of antimicrobial exposure last significantly longer than the duration of the PAE (Davidson et al,1994). More importantly it makes us aware that other more subtle cellular changes not taken into consideration in the current definition of the PAE, can occur as a result of antimicrobial exposure.

e) Uptake in fluoroquinolone resistant S.aureus.

Fluoroquinolone accumulation in quinolone resistant S.aureus was examined to determine if the norA efflux system had a role in the development of our resistant mutants. DNA sequence analysis of the gyrA gene in our mutants had revealed a single nucleotide change at position 252 resulting in an amino acid change from Ser to Leu. However, this change did not occur until the ciprofloxacin MICs were determined to be 11 μg/ml. Thus we were interested in exploring the possibility of altered quinolone transport in the development of our lower level quinolone resistant mutants.

The kinetics of ^{14}C-pefloxacin uptake between susceptible wild type strains and low level resistant mutants (cip. MIC 2.8 μg/ml) were found to be similar. Accumulation of ^{14}C-pefloxacin in strains F651-R with ciprofloxacin MICs of 5.6,

11, and 22 μg/ml were significantly reduced ($p>0.05$). Uptake kinetics in strains F192-R were similar to susceptible strains until the ciprofloxacin MIC approached 22 μg/ml. The highly fluoroquinolone resistant S.aureus strains obtained from Toronto also exhibited reduced uptake kinetics.

Ohshita et al (1990) had previously reported the existence of a single nucleotide change in the open reading frame of norA attributed to fluoroquinolone resistance in S.aureus. However, DNA sequence analysis of the norA gene coding region failed to reveal any nucleotide changes between our susceptible and resistant mutants. Given these results we explored the possibility that reduced accumulation of fluoroquinolones in our resistant strains was the result of increased expression of norA. Isolation of total cellular RNA and subsequent Northern analysis revealed an apparent increase in steady state norA mRNA levels. Kaatz et al, 1993, has also shown that increased expression of norA correlates with increased fluoroquinolone MICs. This observation prompted us to amplify a region of DNA upstream from the norA coding region containing the promoter sequences of the norA gene. Comparisons of the DNA sequences between strains having normal and reduced uptake kinetics revealed two nucleotide substitutions in the DNA of all strains having reduced accumulation of fluoroquinolones. The two nucleotide substitutions were detected at position #352 and position #390. At position #352 we detected the replacement of a T residue with an A and at position #390 we detected the replacement of another T residue with a C. The T to A transversion at position #352 resides in the -35 consensus sequence of the promoter whereas the T to C transition

occurred at a position 89 nucleotides upstream from the norA ATG start codon. Recently, Ng et al (1994) also reported a base change at position #390 associated with increased norA expression, however they observed a T to G transversion.

The bacterial RNA polymerase complex is known to bind to the -35 and -10 consensus sequences of the promoter. Mutations in the consensus regions have previously been shown to increase or decrease the efficiency of transcription. A mutation in the -35 consensus sequence of the promoter as described could serve to increase affinity of the bacterial promoter for the RNA polymerase complex thereby increasing the efficiency of transcription. As well, the appearance of the second nucleotide substitution just downstream from the -10 sequence could have similar effects. In addition, other factors such as consensus binding sites for other regulatory proteins can significantly change the efficiency of RNA transcription. The possibility exists that the described mutations affect the binding of another regulatory protein.

3. Future work:

DNA sequence analysis of the gyrA, gyrB, and norA coding regions failed to detect any mutation which might explain the observed loss of fluoroquinolone PAE in the quinolone resistant S.aureus mutants. The hypothesis that the PAE of fluoroquinolones may be due in part to the time required for the decay of SOS functions is worth exploring. Initial work might concentrate on examination of SOS functions in the mutant S.aureus strains to determine if loss of PAE is due to impaired SOS functions. Specifically, studies should concentrate on the genes or gene products responsible for inhibition of cell division. In addition, depression of the

norA efflux system during the PAE appears to be a plausible explanation for sustaining the fluoroquinolone PAE. Binding studies examining the relative affinities of different fluoroquinolones for the gyrase complex would however strengthen this hypothesis. Given that the exact mechanism of the fluoroquinolone PAE and subsequent cell recovery has not been elucidated, it is likely to be a multi-factorial process and considerable more work will undoubtedly be required to fully understand the mechanism of the fluoroquinolone PAE.

The finding of two nucleotide substitutions in the promoter region of the norA gene that correlate with reduced accumulation of fluoroquinolones in resistant organisms strongly suggest that increased expression of norA is responsible, in part, for fluoroquinolone resistance. Future studies might include comparing the wild type and mutant promoters in a reporter system or the use of a complementation system in $norA^-$ organisms and subsequent examination of fluoroquinolone accumulation. Finally, while the results are strongly suggestive of increased synthesis or stability of norA transcripts, the possibility of other post-transcriptional events occurring should not be ignored.

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APPENDIX A

1. Determination of the postantibiotic effect:

Phosphate buffered saline

a) Stock solution

Na_2HPO_4	2.74g
NaH_2PO_4	7.87g
H_20	QS to 1000ml

b) Working solution

NaCl	8.5g
stock solution	40ml
H_20	QS to 1000ml

2. Protein determination by the method of Lowry:

Lowry protein reagents

reagent A: 2% Na_2CO_3 in 0.1N NaOH

reagent B: 0.5% $CuSO_4$-$5H_2O$ in 1% tartaric acid

reagent C: 50ml reagent A with 1ml reagent B (discard each day)

reagent D: same as reagent C but omit NaOH

reagent E: Diluted folin reagent

3. Preparation of Southern blots:

20x SSC

NaCl	175.3g
Sodium citrate	88.2g
H_2O	QS to 1000ml

pH 7.0

4. End labeling oligonucleotides with $\gamma^{32}P$:

10x PNK buffer

Tris-HCl	7.9g
$MgCl_2$	0.95g
Dithiothreitol	0.78g
Spermidine	0.025g
EDTA	0.037g
H_2O	QS to 100ml

pH 7.6

5. Southern hybridization:

Hybridization buffer

	STRINGENCY		
	HIGH	MODERATE	LOW
BSA	0.1g	0.1g	0.1g
H2O	3ml	----	1.5ml
1M NaP*	2ml	5ml	5ml
Formamide	1.5ml	1.5ml	----
0.5M EDTA	20µl	20µl	20µl
20% SDS	3.5ml	3.5ml	3.5ml

1M NaP*

Na_2HPO_4-$7H_2O$	134g
H_3PO_4	4ml
H_2O	QS to 1000ml

pH 7.2, autoclave

6. Polymerase chain reaction:

10x PCR reaction buffer

NaCl	200mM
Tris-HCl pH 8.9	100mM
$MgCl_2$	25mM
gelatin	0.05%
Triton X-100	0.5%

5x TBE buffer

Tris base	121.1g
Boric acid	61.8g
EDTA	7.4g
H_2O	QS to 1000ml

pH 8.3

7. Preparation of competent DH5α E.coli:

SOB broth

Tryptone	20g
Yeast extract	5g
NaCl	0.5g
H_2O	1000ml
KCl (0.2M, pH7)	10ml

Autoclave and add 5ml 2M $MgCl_2$

SOC broth

To SOB broth, add glucose to a final concentration of 20mM.

FSB buffer

KOAc	0.49g	Glycerol	50ml
RbCl	6.05g	pH to 6.4 with dilute HCl	
$MnCl_2$-H_2O	4.45g	H_20	QS to 500ml
$HaCoCl_2$	0.40g		
$CaCl_2$-H_2O	0.74g		

8. Boiled plasmid preparations:

BPB buffer

Sucrose	8%
Triton X-100	0.5%
EDTA pH 8.0	50mM
Tris-HCl pH 8.0	10mM

9. Large scale preparation of plasmid DNA:

Solution I

Glucose	50mM
Tris-HCl (pH 8.0)	25mM
EDTA	10mM

Solution II

NaOH	0.2N
SDS	1%

Solution III

KOAc	60ml
Glacial acetic acid	11.5ml
H_2O	28.5ml

10. RNA isolation:

Lysis buffer

Tris-HCl pH 7.4	30mM
NaCl	100mM
EDTA	5mM
SDS	1%
Proteinase K	100µg/ml

DNase digestion buffer

Tris-HCl pH 8.0	20mM
$MgCl_2$	10mM

11. Northen analysis:

RNA hybridization buffer

1M KPO_4 (pH 7.4)	12.5ml
20x SSC	125ml
100x Denhardts solution	25ml
Formamide	250ml
H_2O	82.5ml

Denhardts solution

Ficoll 400	10g
polyvinylpyrrolidone	10g
BSA	10g
H_2O	QS to 500ml