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## Molecular Characterization of Qin: A Novel Gene of *Pseudomonas Aeruginosa* Involved in Quinolone Sensitivity

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MOLECULAR CHARACTERIZATION OF QIN:  
A NOVEL GENE OF PSEUDOMONAS AERUGINOSA  
INVOLVED IN QUINOLONE SENSITIVITY

by

Debra A. Tonetti

A Dissertation Submitted to the Faculty of the Graduate  
School of Loyola University of Chicago in Partial  
Fulfillment of the Requirements for the Degree of  
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May

1990

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I am most grateful to my family and friends, without whom this project would not be completed. Their invariable support, understanding, and belief in my abilities enabled me to move forward.

## DEDICATION

This dissertation is dedicated to my parents, Mario and Josephine Tonetti, who have helped me in more ways than I can enumerate.

## VITA

Debra A. Tonetti was born August 27, 1958 in Chicago, Illinois and is the daughter of Josephine and Mario Tonetti.

Debra graduated from Riverside-Brookfield High School in June of 1976. Following one year of study at Triton Community College in River Grove, Illinois, Debra transferred to Northern Illinois University in DeKalb, Illinois. She received a Bachelor of Science degree in August of 1980 with a major in biology and a minor in chemistry.

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Debra entered the Master of Science degree program in the Biology Department at Loyola University Lake Shore Campus in the Spring of 1983 and conducted her thesis research in the laboratory of Dr. Howard Laten. The degree

of Master of Science was conferred in May of 1985. Debra returned to the Department of Biochemistry and Biophysics at Loyola in 1984 to enter the doctoral program. She completed her research in the laboratory of Dr. Robert Miller. During this time, she was awarded a Basic Science Fellowship in 1984, a Research Assistantship in 1985, 1986, and 1987, and a University Dissertation Fellowship in 1988. She was accepted as an associated member of Sigma Xi in 1987.

Debra was awarded the Enrico Fermi Scholar Postdoctoral Fellowship from Argonne National Laboratory in 1989, where she presently is pursuing postdoctoral training in the laboratory of Dr. Eleizer Huberman.

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## CHAPTER I

### INTRODUCTION

The initial aim of this project was to clone the gene coding for the DNA gyrase "A" subunit from Pseudomonas aeruginosa. Based on the characteristics of P. aeruginosa nalA mutants (65,113), the nalA gene encodes this subunit. The intent was to characterize this gene in order to gain insight into the interaction of quinolone antibiotics with their target, the DNA gyrase "A" subunit, as well as extend our basic understanding of P. aeruginosa gene organization. P. aeruginosa is more resistant to quinolones than other Gram-negative bacteria (125). This elevated resistance is due to two factors (i) membrane impermeability and (ii) inherent higher resistance of purified P. aeruginosa DNA gyrase to inhibition by quinolones in vitro (reviewed in 56, 158).

The construction and screening of a P. aeruginosa chromosomal library for the gyrA gene is described. The

library was established in both E. coli and P. aeruginosa nalidixic acid resistant ( $\text{Nal}^r$ ) genetic backgrounds. Since the nalidixic acid sensitive ( $\text{Nal}^s$ ) phenotype is dominant to  $\text{Nal}^r$  in Escherichia coli (51), the screening was based on the identification of cells that became  $\text{Nal}^s$  due to the acquisition and expression of a P. aeruginosa chromosomal DNA fragment. Such a clone was identified and was designated gin, for sensitivity to quinolones. gin was characterized physiologically for its ability to complement various quinolone-resistant mutations of E. coli and P. aeruginosa. These data were compared with the ability of the E. coli gyrA gene to complement a P. aeruginosa nalA mutation.

The minimal DNA fragment defining gin was obtained by subcloning and  $\text{Tn}3$  mutagenesis. This fragment was shown to retain the quinolone-sensitive phenotype. The minimal fragment delineating gin was used as a probe for Southern hybridization to determine the degree of homology between gin and E. coli gyrA.

The chromosomal map position of gin was obtained to determine whether this gene maps near any known locus associated with quinolone resistance in P. aeruginosa. To this end, gin, along with an antibiotic-resistance gene to serve as a selective marker, was integrated into the P.

aeruginosa chromosome by homologous recombination. Transduction and conjugation experiments are described which identified the map position of gin.

The gin gene was delineated and the nucleotide sequence was obtained. The protein product was expressed in E. coli mini-cells and compared with the deduced amino acid sequence from the gin gene. Northern hybridization contributed information regarding the expression of this gene. The information from these experiments was used to formulate a hypothesis regarding the identity of gin.

## CHAPTER II

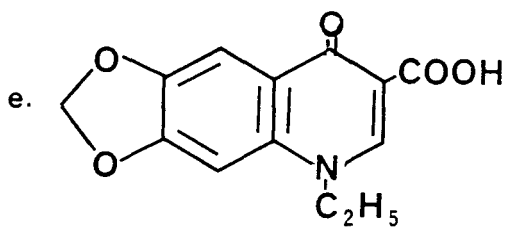
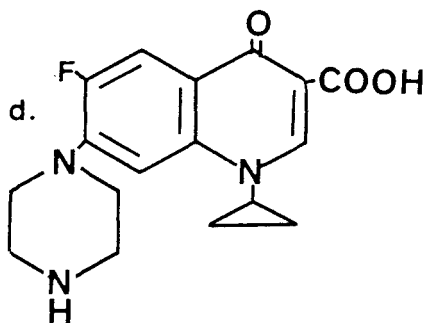
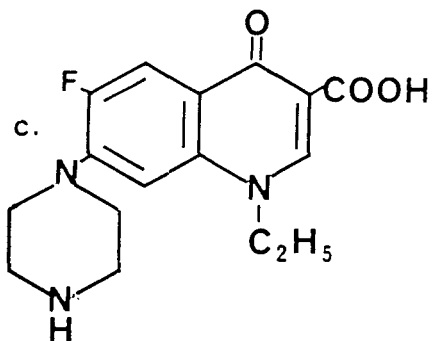
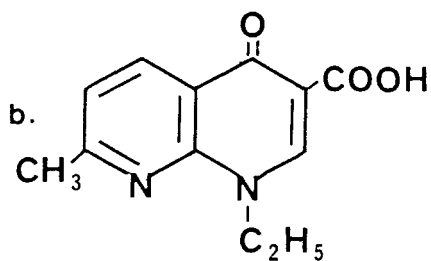
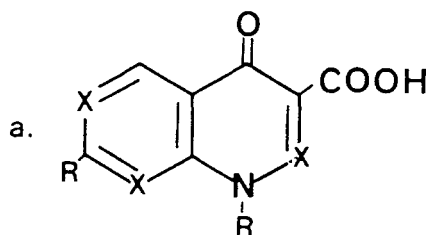
### REVIEW OF THE RELATED LITERATURE

#### *Introduction*

Pseudomonas aeruginosa is a Gram-negative bacterium which is primarily found in soil (140). However this organism is also an opportunistic pathogen known to colonize burn wounds (3) and the lungs of cystic fibrosis patients (24). These infections are difficult to eradicate due to the high level of resistance to many antibacterials. The development of a synthetic class of antibacterials known as quinolones has improved the management of both Gram-positive and Gram-negative bacterial infections. These chemically synthesized compounds are structurally related derivatives of the parent molecule, nalidixic acid (Figure 1).

P. aeruginosa is more resistant to quinolone antibiotics than other Gram-negative bacteria (125). This elevated resistance is due to two factors (i) membrane

Figure 1. Chemical structures of selected quinolones: a. Basic quinolone structure b. Nalidixic acid c. Norfloxacin d. Ciprofloxacin e. Oxolinic acid



impermeability (111) and (ii) the inherent higher resistance of the drug target, DNA gyrase (89).

The target of the quinolone antimicrobials is the topoisomerase type II enzyme, DNA gyrase (37). This enzyme is responsible for introducing negative supercoils into the circular bacterial chromosome in an ATP-dependent reaction. The activity of DNA gyrase opposes the activity of topoisomerase I within the cell, which relaxes negatively supercoiled DNA in ATP-independent manner (143). These opposing activities maintain the bacterial chromosome in a negative superhelical state which is necessary for the proper expression of genes. Quinolones interfere with the DNA gyrase supercoiling activity by forming a drug-gyrase-DNA complex which effectively shuts down DNA replication (31).

The clinical advantage of quinolone therapy is not only its broad-spectrum activity, but also the lack of plasmid-mediated resistance, as is often a problem with the beta-lactam and aminoglycoside antibacterials (96). Quinolone-resistance is associated with chromosomal mutations, many of which have been mapped to the DNA gyrase structural genes and to several loci affecting membrane permeability (reviewed in 56,158).



*DNA gyrase: structure and function*

DNA gyrase was initially discovered in Escherichia coli as an essential element in integrative recombination of phage lambda (38). Although DNA gyrase has since been described and purified from other bacterial species such as Micrococcus luteus (75), Bacillus subtilis (129), Citrobacter freundii (1), Staphylococcus aureus (134), and P. aeruginosa (89,5), the E. coli enzyme has been the most extensively studied. The E. coli enzyme is a tetramer which is composed of two "A" subunits and two "B" subunits with molecular weights of 105,000 and 95,000 respectively (92). The structural genes which encode the subunits, gyrA and gyrB, are widely separated on the E. coli chromosome. The activities associated with DNA gyrase include (103):

1. ATP-dependent negative supercoiling
2. ATP-independent relaxation of negative supercoils
3. Catenation and decatenation of DNA rings
4. Knotting and unknotting of DNA
5. Quinolone induced cleavage of DNA with covalent attachment between the "A" subunit and free 5' ends of DNA
6. DNA wrapping in a positive sense
7. DNA-dependent ATP hydrolysis

Different activities for each subunit have been determined. The "A" subunit contains the nicking-closing activity while the "B" subunit possesses an ATP binding site and ATPase activity. The assignment of these activities was facilitated by the discovery of specific inhibitors of the individual subunits which are represented by two classes of drugs. The quinolone antibacterials, such as nalidixic acid and oxolinic acid, interact with the "A" subunit of DNA gyrase and interfere with the nicking-closing activity (17,35). Novobiocin and coumermycin specifically compete for the ATP binding site located on the "B" subunit (130).

*P. aeruginosa DNA gyrase*

P. aeruginosa is several-fold more resistant to nalidixic acid than E. coli (125). There is evidence to suggest that both membrane impermeability and DNA gyrase resistance contribute to this phenomenon. It was shown that inhibition of replicative DNA synthesis by nalidixic acid in permeabilized wild-type P. aeruginosa cells resulted in ID<sub>50</sub> values that were comparable to ID<sub>50</sub>s reported for permeabilized E. coli and S. marcescens cells (111). These results suggested that the P. aeruginosa cell envelope is responsible for the higher resistance observed

as compared to other bacteria. However, a more direct study was made possible by the purification of DNA gyrase from P. aeruginosa strain PAO (89). This study demonstrated that P. aeruginosa DNA gyrase is three-fold more resistant to nalidixic acid than E. coli DNA gyrase as measured by in vitro supercoiling activity in the presence of quinolones.

The P. aeruginosa subunit molecular weights were reported to be 92,000 +/- 3000 and 108,000 +/- 3,000 (5). This is in agreement with the initial molecular weight of 360,000 +/- 30,000 reported for the holoenzyme (89), and the subunit molecular weights later reported by Inoue et al. of 77,000 and 110,000 that correspond to the "A" and "B" subunits respectively (65). These data are also consistent with the tetrameric conformation of the enzyme,  $A_2B_2$ , as found in E. coli.

The hypothesis that the stability of the gyrase-DNA-drug complex may contribute to the differences in the minimal inhibitory concentrations (MIC) and minimal bacteriocidal concentrations (MBC) observed between E. coli and P. aeruginosa was explored. The apparent inhibition constants ( $K_i$ ) for various quinolones were measured for the relaxation reaction using purified P. aeruginosa DNA gyrase (5). The antimicrobial potency of the individual

quinolones correlated with the  $K_i$ s obtained for the inhibition of the relaxation reaction (5). Nalidixic acid had the highest reported  $K_i$  ( $10.8 \times 10^{-3}$  M), oxolinic acid had a lower  $K_i$  ( $3.7 \times 10^{-3}$  M), and norfloxacin demonstrated the lowest  $K_i$  ( $2.0 \times 10^{-3}$  M).

*DNA gyrase: mechanism of activity*

The currently accepted model for the introduction of negative supercoils is a strand passage mechanism (17). In this model, a transient double-stranded break is made in the circular DNA through which a DNA segment is passed, followed by resealing on the opposite side of the same DNA segment. A reduction of two in the linking number of the molecule occurs in this process. ATP binding induces a conformational change in the enzyme which drives a single round of supercoiling. The hydrolysis of ATP is required for enzyme turnover, readying the enzyme for another round of supercoiling (130).

*Interaction of DNA gyrase and DNA*

It has been shown that DNA wraps around the outside of the DNA gyrase molecule with approximately 120-150 base pairs (bp) in direct contact with the enzyme (31,76). Several attempts have been made to identify a consensus

sequence for the binding of DNA gyrase (30,36,78,94,99). Examination of the cleavage breakpoint of pBR322 DNA, induced by oxolinic acid followed by SDS treatment, revealed a 20 bp consensus sequence that is essential for gyrase cleavage (78). The consensus sequence alone, however, is not sufficient. At least 117 bp of flanking DNA on one or the other side of the cleavage consensus sequence is also necessary for cleavage by DNA gyrase (30). The flanking regions of DNA do not display any sequence-specific requirements.

It was recently reported that DNA gyrase preferentially binds to the prokaryotic family of repetitive extragenic palindromic (REP) sequences (155). Hundreds of copies of these sequences occur throughout the prokaryotic genome and were estimated to account for approximately 0.5 % of the chromosomal DNA (155). It is postulated that the REP sites may be involved in the maintenance of supercoiling and the higher-order structure of the bacterial chromosome as mediated through DNA gyrase. DNA gyrase was also shown to bind to the partition locus (par) of the pSC101 plasmid (141), which also displays homology with areas of the REP sequence.

*Quinolones: Interaction with their target*

The availability of drug-resistant mutants has greatly advanced the understanding of the physiological role which DNA gyrase plays in the bacterial cell. Initially, drug-resistant mutants for both drug classes were mapped to the genetic loci now known to be the structural genes for the individual subunits. Both nalidixic acid and coumermycin are known to cause rapid cessation of DNA replication (25). However the mechanism by which this occurs differs for the two types of drugs. Coumermycin and novobiocin cause a decrease in the superhelical density of the bacterial chromosome which parallels inhibition of DNA synthesis (27). Nalidixic acid and oxolinic acid appear to modulate their effects on DNA synthesis by a different mechanism. Treatment of E. coli cells with oxolinic acid does not appreciably change the superhelical density of the chromosome (29). However, upon addition of SDS, DNA cleavage occurs with a concomitant formation of a protein-DNA-drug complex (37,131). DNA gyrase subunit "A" becomes covalently attached to a free 5' phosphoryl group in a phosphotyrosine linkage (62), similar to the linkage between topoisomerase I and DNA (138). It has been proposed that this trapped reaction complex intermediate acts as a physical barrier to DNA synthesis by

preventing replication fork migration (26). This is consistent with the reported dominance of the nalidixic acid sensitive ( $\text{Nal}^s$ ) phenotype to the nalidixic acid resistant ( $\text{Nal}^r$ ) phenotype (51), since the presence of wild-type (sensitive) "A" subunits would halt DNA replication fork migration upon ternary complex formation.

The question as to whether quinolones bind directly to DNA or to DNA gyrase has been a subject of conflicting reports (74,105,124). However, a recent report describes a cooperative drug-DNA binding model (122,123) that has clarified this issue. The basis of this model is the formation of a DNA gyrase-induced DNA binding site during the initial strand breakage reaction. The quinolone drug then may bind via hydrogen bonding to the unpaired bases in this region. Subsequent binding of quinolone molecules becomes cooperative due to strong intermolecular drug-drug interactions. This proposed model is in agreement with the structure-activity data available for the various quinolone derivatives. Drug-resistant mutations which alter the DNA gyrase enzyme may also alter the gyrase-induced configuration of the DNA, thus reducing the ability of quinolones to bind.

*Role of DNA gyrase in transcription,  
recombination and DNA repair*

The levels of DNA gyrase and topoisomerase I in the cell, and hence the extent of superhelicity of the bacterial chromosome, can affect transcription, recombination and DNA repair (17). Several studies have focused on the involvement of topoisomerases in these processes (25).

A correlation between supercoiling and transcription in general has been documented (25,143). The partial unwinding of the DNA helix caused by negative supercoiling is thought to promote and stabilize the RNA polymerase-DNA initiation complex. Several examples of differential gene expression in response to alteration of chromosomal superhelicity by DNA gyrase inhibitors (69,82,109,142,154), as well as specific examples of altered gene expression (7,108), have been reported. One study reported conflicting results which showed dramatic sensitivity in vitro of the E. coli tRNA<sup>TYR</sup> gene to superhelicity, while experiments in vivo showed no difference in expression with varying superhelicity (71).

Rudd and Menzel demonstrated that constitutive mutants of the hisW gene of E. coli and Salmonella typhimurium are alleles of the gyrA gene (117). This study



represents an excellent example of superhelicity affecting gene expression. They hypothesized that the promoter of the hisR gene of S. typhimurium can only be efficiently transcribed from a supercoiled template. The reduced negative superhelicity of DNA in a gyrA mutant, led to reduced levels of tRNA<sup>HIS</sup> (117).

DNA gyrase participates in several types of recombination. Illegitimate recombination takes place between short non-homologous regions of DNA and is recA-independent (63,64,95). As mentioned previously, DNA gyrase was first identified as a required component in integrative recombination of phage lambda in a cell free-system (39), which is dependent on a negatively supercoiled substrate (90). Other examples of site-specific recombination involving DNA gyrase include the replicative transposition of bacteriophage Mu (116) and Tn<sub>5</sub> transposition (66). It has been proposed that illegitimate recombination is mediated by the exchange of subunits at two gyrase cleavage sites (64). Inhibitors of DNA gyrase also reduce general recombination in UV-irradiated, superinfecting bacteriophage lambda DNA (52). The RecA protein and DNA gyrase are involved in general recombination which takes place between long homologous regions of DNA (33).

Treatment of E. coli with nalidixic acid induces the SOS repair system (147). This system is probably induced by the gyrase-DNA-drug complex and subsequent DNA strand cleavage. Coumermycin has also been shown to induce RecA synthesis in a recF-dependent manner (128). Engle et al. (29) described recA, lexA-dependent secondary DNA synthesis following oxolinic acid induced enzyme-DNA-drug complex formation. The observed secondary synthesis was postulated to be due to a DNA damage-inducible repair system.

A similar recovery synthesis has been observed following quinolone treatment of P. aeruginosa. This DNA synthesis was shown to be dependent on continued RNA and protein synthesis (5). Benbrook and Miller (5) hypothesized that ongoing RNA and protein synthesis is required for recovery synthesis because DNA gyrase itself may be induced.

#### *Regulation of DNA gyrase expression*

Following the cloning of the structural genes for the "A" and "B" subunits in E. coli (91), the regulation of DNA gyrase expression by the level of chromosomal superhelicity was investigated. An abundance of evidence suggests that cellular levels of DNA supercoiling are controlled by a balance between relaxing activity

(topoisomerase I) and negative supercoiling activity (DNA gyrase) (25). A mechanism describing the homeostatic control of DNA supercoiling was first put forth by Menzel and Gellert (84). They were able to show the increased synthesis of both DNA gyrase subunits by novobiocin and coumermycin treatment as well as by heat inactivation of a temperature sensitive gyrase mutant. Experiments carried out in vitro demonstrated that supercoiled DNA is a poor template for transcription of the gyrA and gyrB messages, while relaxed DNA is a favorable substrate (85). Conversely, the E. coli topoisomerase I gene (topA) requires a supercoiled DNA template for efficient transcription from the topA promoter (137). In the presence of mutant genes coding for either gyrase subunit, Tse-Dinh observed a reduction in transcription from topA. These experiments were carried out by fusing the control region of topA to the galactokinase reporter gene. This experimental design was also utilized to study the induction of the gyrA and gyrB genes (86). Menzel and Gellert concluded from these studies that both gyrA and gyrB are induced following treatment with coumermycin. They found that only a small 20 bp region located around the -10 TATA consensus sequence of the promoter and including the first few transcribed bases was necessary for relaxation

stimulated transcription of the gyrA and gyrB genes.

*Structural genes encoding DNA gyrase*

The gyrA and gyrB genes encoding the "A" and "B" subunits of DNA gyrase respectively have been cloned from both E. coli (91) and B. subtilis (72,73). The gyrA and gyrB genes of E. coli are widely separated on the chromosome. The gyrA and gyrB loci of B. subtilis are adjacent to each other, but are independently transcribed. Although the gyrA gene of P. aeruginosa has not been cloned, there is sufficient evidence to conclude that the nalA locus located at 40 min on the chromosome (101) (52 min, old map, 57) does encode the "A" subunit of DNA gyrase and corresponds to the gyrA loci of E. coli and B. subtilis (65,113). Two independent investigators have specifically observed alterations in the DNA gyrase "A" subunit purified from nalA mutants, while purified "B" subunit appeared to be unaltered. The "A" and "B" subunits of DNA gyrase were purified from a nalidixic acid-resistant mutant of P. aeruginosa and it was shown that an alteration of the "A" subunit, but not the "B" subunit, caused the bacteria to be quinolone-resistant, and mapped to the nalA locus (65). Two independently isolated quinolone-resistant mutants containing the norfloxacin-resistant mutation nfxA (56) and

the ciprofloxacin-resistant mutation cfxA (113) were shown to be alleles of nalA and to alter the "A" subunit of gyrase. A chromosomal locus for the structural gene encoding the "B" subunit of P. aeruginosa has not yet been identified.

The B. subtilis gyrA and gyrB genes were translated in E. coli maxi-cells and produced proteins of molecular weight 67,000 and 77,000, respectively (73). The B. subtilis DNA gyrase subunits are significantly smaller than those of E. coli gyrase (105,000 and 95,000). Despite the size differential, all of the enzyme activities associated with the E. coli enzyme are also present in the B. subtilis enzyme (102,129). However, in vivo, both the B. subtilis gyrA and gyrB genes must be present to complement an E. coli gyrA mutant, suggesting that the B. subtilis "A" subunit cannot functionally interact with the E. coli "B" subunit. Interspecies mixing experiments carried out in vitro demonstrated that the B. subtilis "A" subunit and the E. coli "B" subunit do not reconstitute supercoiling activity, while the E. coli "A" subunit and the B. subtilis "B" subunit do produce an active enzyme (102). The dominant nature of the E. coli  $\text{Nal}^s$  phenotype could not be confirmed in B. subtilis because gene conversion prevented the construction of a merodiploid (73).

Under low stringency conditions, Southern hybridizations revealed that a similarity in nucleotide sequence exists between the E. coli and B. subtilis gyrA and gyrB genes (73). A direct comparison of the deduced amino acid sequences of E. coli gyrA and B. subtilis gyrA revealed a 50% overall identity with certain regions showing greater than 90% identity (93,132,158). The deduced amino acid sequences derived from the gyrB genes displayed 68% identity (158).

*Quinolone resistance: mutations affecting DNA gyrase*

As mentioned previously, quinolone resistance is rarely plasmid-mediated, and almost exclusively involves chromosomal mutations affecting either the primary quinolone target, DNA gyrase, or membrane permeability. The infrequent nature of plasmid-mediated quinolone resistance has been attributed to several factors (149): i) mutations in gyrA which cause resistance to quinolones are recessive ii) quinolones reduce plasmid conjugation iii) elimination of plasmids in growing cells is facilitated by quinolones.

A number of mutations associated with high level quinolone resistance have been mapped to the gyrA structural gene. Quinolone-resistant mutants can be selected in vitro by serial plating to higher

concentrations of drug, and have also been clinically isolated from patients undergoing quinolone drug therapy. In *E. coli*, the nalA, nfxA (59), norA (54), cfxA (58) and ofxA (150) mutations all map to the gyrA gene. Yoshida *et. al.* examined nucleotide alterations of four spontaneous *E. coli* quinolone-resistant (gyrA) mutants (157). They found that all four mutations were located in the highly conserved hydrophilic N-terminal region of the GyrA polypeptide, and were very close to the tyrosine amino acid at position 122, that attaches to DNA in the presence of oxolinic acid (138).

Although most quinolone-resistant mutations reside in the gene coding for the drug target, the "A" subunit of DNA gyrase, the nal-24 and nal-31 alleles, mapped within the gyrB structural gene (152,153). Novobiocin resistance is generally associated with gyrB mutations, however nalA-24 and nalA-31 are not novobiocin-resistant. Cozzarelli postulated that two domains are present in the GyrB protein (17). The v domain interacts with the GyrA protein, while the other domain contains the ATP binding site. The gyrB gene was cloned from these mutants and the nucleotide sequence was analyzed (153). nalA-24 and nalA-31 mutations caused single amino acid changes at positions 426 and 447, respectively, which are located in the v domain of the GyrB

protein. Therefore, the nalA-24 and nalA-31 mutations alter the interaction between the gyrA and gyrB gene products, resulting in quinolone resistance.

As previously discussed, the DNA gyrase "A" and "B" subunits have been purified from P. aeruginosa (5,65,89), and it has been determined that nalA encodes the "A" subunit (65,113). However, since the gyrA and gyrB genes of P. aeruginosa have not yet been cloned, specific alterations have not been determined.

#### *The Gram-negative cell envelope*

Many antibiotic-resistant mutants of Gram-negative bacteria have alterations in the proteins and lipopolysaccharides of the outer membrane, which result in reduced drug entry (11,50,135). It has been demonstrated that several quinolone-resistance mutations occur in proteins of the bacterial outer membrane.

The complex structure of the Gram-negative cell envelope consists of several layers of membranes which act as an impediment to antibiotics (Figure 2) (6,77). The outermost layer is the capsule which is composed of polysaccharides, and often is strain-specific. The capsule

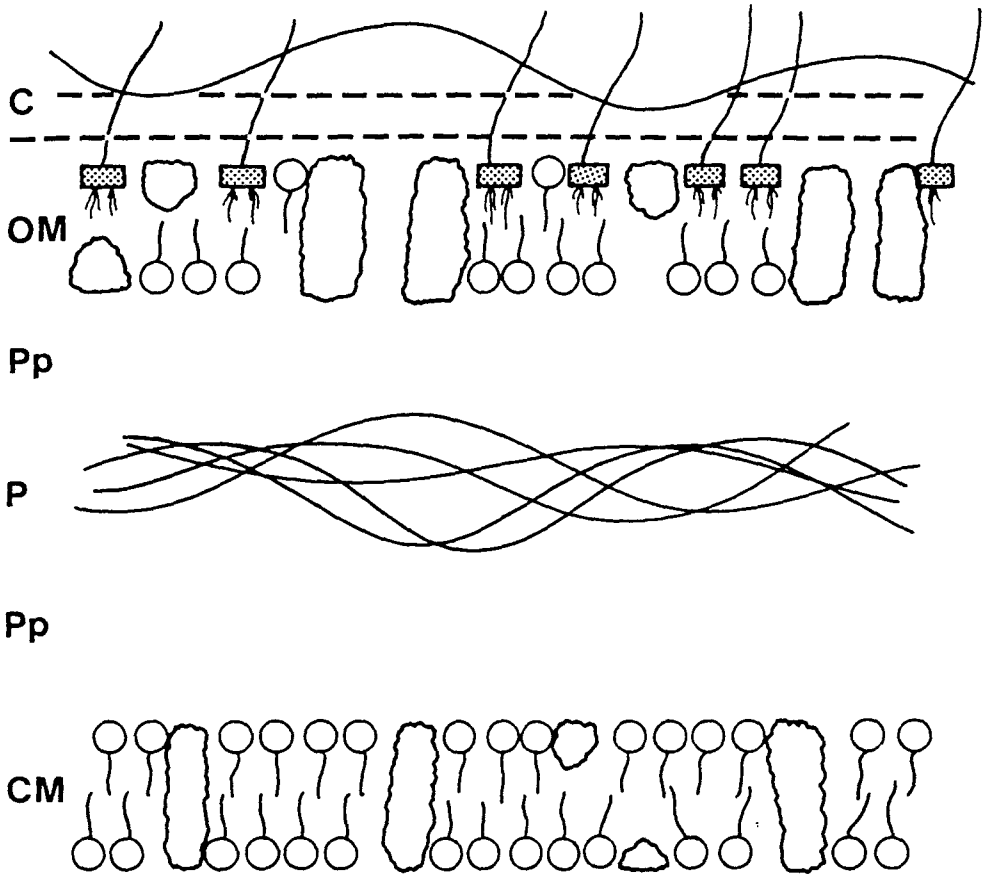


Figure 2. The Gram-negative cell envelope.

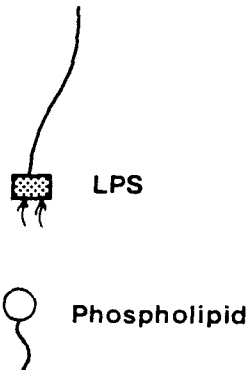
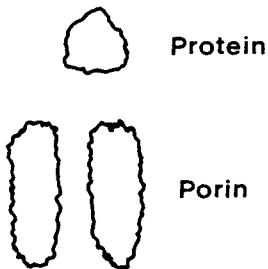
C - Capsule; OM - Outer membrane; Pp - Periplasmic space;

P - Peptidoglycan; CM - Cytoplasmic membrane

### Cell envelope of Gram-negative bacteria



#### Legend



can be quite extensive in certain strains of P. aeruginosa, particularly in isolates from patients suffering from cystic fibrosis. Directly beneath the capsule is the outer membrane which consists of proteins, phospholipids, and lipopolysaccharides. The outer membrane has an asymmetrical pattern, the inner surface of the membrane is predominantly phospholipid-lined, while the outer surface is lipopolysaccharide-rich.

The proteins of the outer membrane either have a structural function or are involved in the formation of the porin channel. Porins are transmembrane diffusion channels that are intimately associated with the peptidoglycan layer, and function in the passage of hydrophilic molecules, including certain hydrophilic antibiotics, into the cell. Between the outer and inner membranes, lies the periplasmic space. The peptidoglycan layer is within the periplasmic space, and is composed of two subunits, N-acetylmuramic acid and N-acetylglucosamine. These two subunits alternate to form a high molecular weight polymer which contains chains of certain rare amino acids attached to the N-acetylmuramic acid molecules. The inner membrane separating the periplasm from the cytoplasm is typical of most biologic membranes.

Outer membrane proteins C and F (OmpC, OmpF) are the major porin proteins found in E. coli K-12, with a molecular weight exclusion between 500-800 (6). The outer membrane permeability of P. aeruginosa is reported to be as much as 100-times lower than E. coli (160). Yoshihara and Nakae recently demonstrated that the porins in the outer membrane of P. aeruginosa form very small diffusion pores with a molecular weight exclusion of less than 250, permitting only small solutes to diffuse through the membrane (159). The major outer membrane proteins of P. aeruginosa that have been identified include the C, D1, D2, E, F, G, H1, H2, I and P proteins (6,46,47,49,159) (Table 1). The proteins that compose the small diffusion porin channels are proteins C, D, and E of molecular weights 70,000, 46,000, and 43,000, respectively (159). Hancock and Nikaido initially reported that protein F constituted the major porin protein of P. aeruginosa forming a channel with a molecular weight exclusion of 6000-9000 (48). The protein F structural gene, oprF was recently cloned (28). It was determined that protein F does not form a porin, but is involved in maintaining the structural integrity of the outer membrane of P. aeruginosa, similar in function to the

TABLE 1

P. aeruginosa outer membrane proteins

protein	Molecular Weight	Function	Reference
C	70,000	porin	(159)
P	49,000	inducible porin (phosphate-transport)	(49)
D1	47,000	porin	(159)
D2	46,000	porin	(159)
E	43,000	porin	(159)
F	35,000	structural	(148)
G	25,000	possible porin	(156)
H1	19,500	N.D.	(4)
H2	17,000	N.D.	(47)
I	10,500	N.D.	(47)

N.D. - not determined

OmpA protein of E. coli (42,148).

The expression of many outer membrane proteins are known to be modulated by nutrient conditions. Protein H1 is overexpressed in response to conditions of divalent cation depletion and is correlated with resistance to polymyxin B, aminoglycosides, and EDTA (121). It is postulated that protein H1 replaces divalent cations at lipopolysaccharide binding sites, thus inhibiting entry of the antibiotics. The ophR gene encoding protein H1 was recently cloned and sequenced (4). The amino acid sequence of protein H1 displays little similarity with any previously sequenced outer membrane proteins. Protein G may be overexpressed in response to iron and down-regulated in response to low concentrations of iron (156). Although the function of protein G has not yet been determined, it is thought that it may act as a low-affinity uptake system.

*Quinolone resistance:*

*mutations affecting membrane permeability*

Several quinolone-resistant mutations in E. coli map to chromosomal loci other than the structural genes for DNA gyrase. Several mutants possess reduced levels of the OmpF porin protein, which suggests that quinolones enter E. coli

through the OmpF porin as does chloramphenicol, tetracycline, and the beta-lactams (53,54,56,59). A spontaneous norfloxacin-resistant isolate containing the norB mutation exhibited cross-resistance to tetracycline, chloramphenicol, and cefoxitin and mapped to about 34 min on the E. coli K-12 chromosome (54). The norC mutation was found to cause low-level resistance to both ciprofloxacin and norfloxacin, but was hypersusceptible to the more hydrophobic quinolone, nalidixic acid, and mapped to 8 min on the chromosome (54). Both the norB and norC mutations were associated with a reduction in OmpF. In addition, norC mutants displayed an altered lipopolysaccharide structure. Hirai et. al. postulated that norB may regulate OmpF protein production (54). It is unclear what the function of the norC allele may be. Hooper et al. (58) also described a norfloxacin-resistant mutation, nfxB and a ciprofloxacin-resistant mutation cfxB which mapped to 19 and 34 min, respectively, and were also OmpF-deficient. The structural gene encoding OmpF maps at 21 min on the E. coli K-12 chromosome, however nfxB was shown to be nonallelic with ompF. The multiple antibiotic resistance (marA) locus maps at 34 min (40) which suggests that it may be allelic with norB. It can be concluded that the OmpF protein of E. coli may play an important role in quinolone entry.

Studies of spontaneous norfloxacin-resistant mutants isolated from both Klebsiella pneumoniae and Serratia marcescens also revealed decreases in specific outer membrane proteins (56). Two K. pneumoniae outer membrane proteins of molecular weights 39,000 and 41,000 were either reduced or completely absent in the quinolone-resistant strains when compared to the wild-type parent. The level of a 41,000 outer membrane protein was also decreased in the S. marcescens mutant. All three of these proteins have been implicated as possible porin proteins in K. pneumoniae and S. marcescens (56).

Genes coding for outer membrane proteins of P. aeruginosa also appear to be the target of mutations which effect quinolone-resistance. The nfxB mutation displayed a 16-fold increased resistance to norfloxacin, yet was hypersusceptible to aminoglycoside and beta-lactam antibiotics (55). This mutation mapped between 4 and 8 minutes on the PAO chromosome, a region flanked by pro-9031 and ilv-9023. The nfxB mutation correlated with the appearance of a 54,000 Dalton protein, unlike the case of the nfxB mutation of E. coli which produced a decline in the OmpF porin protein. Similarly, the nalB mutation, which was a spontaneous mutation selected in the presence of nalidixic acid, also produced an additional protein of



molecular weight 49,000 (56). In addition, strains carrying the nfxB and nalB mutations appear to have diminished levels of a 25,000 Dalton protein (56).

Another example of a decrease in outer membrane proteins associated with ciprofloxacin-resistance was demonstrated by Daikos et al. (19). Two clinical isolates exhibited a loss of a 31- to 32-kilodalton protein. The loss of a 25,500 Dalton protein identified as the outer membrane protein G and a 40,000 Dalton outer membrane protein was demonstrated in pefloxacin-resistant strains of P. aeruginosa isolated from experimental aortic endocarditis (13). It is speculated that protein G may be a porin-forming protein involved in the entry of quinolone antibiotics.

## CHAPTER III

### MATERIALS AND METHODS

#### *Bacterial strains and bacteriophage*

All P. aeruginosa and E. coli strains used in these studies and their relevant genotypes are listed in Table 2. A list of bacteriophages can be found in Table 3.

#### *Plasmids*

Cosmid pCP13\* is a derivative of pCP13 (20) which carries the tetracycline-resistance ( $Tc^r$ ) gene. This broad-host-range cosmid vector was used to construct a P. aeruginosa chromosomal library and to subclone the E. coli gyrA gene. pTML8 has a single 25 kilobase pair (kbp) HindIII P. aeruginosa chromosomal insert which contains the gin sequence cloned into pCP13\*. pTML86 was derived from pTML8 and possesses a single PstI-HindIII fragment which also contains the gin gene.

pBEU30 is an Flac:Tn3 plasmid that was used to deliver transposon insertions to pTML8 and pTML86.

TABLE II  
Bacterial Strains

Strain	Plasmid	Relevant genotype <sup>a</sup>								Other markers <sup>a</sup>	Source or ref.
		<i>arg</i>	<i>leu</i>	<i>lys</i>	<i>met</i>	<i>nal</i>	<i>phe</i>	<i>pro</i>	<i>trp</i>		
<i>P. aeruginosa</i>											
PAO1		+	+	+	+	+	+	+	+		(88)
PAO25		<i>F10</i>	-10	+	+	+	+	+	+		(34)
PAO303		<i>B21</i>	+	+	+	+	+	+	+		(88)
PAO514		<i>F10</i>	+	+	+	<i>A2</i>	+	+	+		(111)
PAO515		+	+	+	-9011	<i>A5</i>	+	+	+		(111)
PAO660		+	+	+	+	+	-2	+	+		(18)
PAO6002		+	+	+	-9011	<i>B4</i>	+	+	+		(111)
PAO6004		+	+	+	+	+	+	+	+	<i>pip6003</i>	(111)
PAO6005		+	+	+	+	<i>B8</i>	+	<i>C130</i>	+		(111)
PAO6006		+	+	+	+	<i>B9</i>	+	<i>C130</i>	+		(111)
JC3600		+	+	-12	-28	+	+	+	-C6	<i>Sm</i> <sup>r</sup>	
RM2116	FP5	<i>B21</i>	+	+	+	+	+	+	+	<i>Hg</i> <sup>r</sup>	PAO303
RM2163	pTML8	+	+	+	-9011	<i>A5</i>	+	+	+	<i>Tc</i> <sup>r</sup>	PAO515
RM2193	pCP13*	+	+	+	-9011	<i>A5</i>	+	+	+	<i>Tc</i> <sup>r</sup>	PAO515
RM2197	pTML90	+	+	+	-9011	<i>A5</i>	+	+	+	<i>Tc</i> <sup>r</sup>	PAO515
RM4275	pTML86	<i>F10</i>	+	+	+	<i>A2</i>	+	+	+	<i>Tc</i> <sup>r</sup>	PAO514
RM4276	pTML86	+	+	+	-9011	<i>A2</i>	+	+	+	<i>Tc</i> <sup>r</sup>	PAO515
RM4277	pTML86	+	+	+	-9011	<i>B4</i>	+	+	+	<i>Tc</i> <sup>r</sup>	PAO6002

TABLE II continued

## Bacterial Strains

Strain	Plasmid	Relevant genotype <sup>a</sup>								Other markers <sup>a</sup>	Source or ref.
		<i>arg</i>	<i>leu</i>	<i>lys</i>	<i>met</i>	<i>nal</i>	<i>phe</i>	<i>pro</i>	<i>trp</i>		
<i>P. aeruginosa</i>											
RM4278	pTML86	+	+	+	+	+	+	+	+	<i>pip6003</i> Tc <sup>r</sup>	PA06002
RM4279	pTML86	+	+	+	+	<i>B8</i>	+	<i>C130</i>	+	Tc <sup>r</sup>	PA06005
RM4280	pTML86	+	+	+	+	<i>B9</i>	+	<i>C130</i>	+	Tc <sup>r</sup>	PA06006
RM4281	PA01:: pTML822	+	+	+	+	+	+	+	+	Tc <sup>r</sup>	PA01
RM4282	PA01:: pTML822 FP5	+	+	+	+	+	+	+	+	Tc <sup>r</sup> Hg <sup>r</sup>	PA01

TABLE II continued

## Bacterial Strains

Strain	Plasmid	Relevant genotype <sup>a</sup>								Other markers <sup>a</sup>	Source or ref.
		<i>his</i>	<i>hsd</i>	<i>leu</i>	<i>pro</i>	<i>thr</i>	<i>thi</i>	<i>sup</i>	<i>gyr</i>		
<i>E. coli</i>											
DH1		+	<i>R17</i>	+	+	+	-1	<i>E44</i>	<i>A96</i>	<i>recA1</i>	(44)
N4186	pMK90	+	+	+	+	+	+	+	+	<i>recA</i> <i>Ap<sup>r</sup></i>	(91)
HB101		+	<i>S20</i>	-	<i>A2</i>	+	-1	<i>E44</i>	+	<i>recA13</i>	(10)
RM1068		+	<i>R-</i>	+	+	+	+	<i>F</i>	+		J. Pemberton
1488		-53	<i>R2</i>	+	+	+	-1	+	+	<i>minA1</i> <i>minB2</i>	(15)
AB1157		-4	+	<i>B6</i>	<i>A2</i>	+	-1	<i>E44</i>	+		A. J. Clark
HB101	F <sup>+</sup>	+	<i>S20</i>	-	<i>A2</i>	+	-1	<i>E44</i>	+	<i>recA13</i>	M. R. Kelley
JC12717	pBEU30	-4	+	<i>B6</i>	<i>A2</i>	+	-1	<i>E44</i>	+	<i>Km<sup>r</sup></i> <i>Ap<sup>r</sup></i>	A. J. Clark
RM1048	pRK2013	+	<i>S20</i>	-	<i>A2</i>	+	-1	<i>E44</i>	+	<i>recA13</i> <i>Km<sup>r</sup></i>	A. Chakrabarty
RM1220	pTML8	+	<i>R17</i>	+	+	+	-1	<i>E44</i>	<i>A96</i>	<i>recA1</i> <i>Tc<sup>r</sup></i>	DH1
RM2196	pTML90	+	<i>R17</i>	+	+	+	-1	<i>E44</i>	<i>A96</i>	<i>recA1</i> <i>Tc<sup>r</sup></i>	DH1
RM4222	pCP13 <sup>*</sup>	+	<i>R17</i>	+	+	+	-1	<i>E44</i>	<i>A96</i>	<i>recA1</i> <i>Tc<sup>r</sup></i>	DH1
RM4225	pTML86	+	<i>R17</i>	+	+	+	-1	<i>E44</i>	<i>A96</i>	<i>recA1</i> <i>Tc<sup>r</sup></i>	DH1

TABLE II continued

## Bacterial Strains

Strain	Plasmid	Relevant genotype <sup>a</sup>								Other markers <sup>a</sup>	Source or ref.
		<i>his</i>	<i>hsd</i>	<i>leu</i>	<i>pro</i>	<i>thr</i>	<i>thi</i>	<i>sup</i>	<i>gyr</i>		
<i>E. coli</i>											
RM4256	pTML69 F <sup>+</sup>	+	S20	-	A2	+	-1	E44	+	Km <sup>r</sup> , Ap <sup>r</sup> <i>recA13</i>	HB101
RM4257	pTML96 F <sup>+</sup>	+	S20	-	A2	+	-1	E44	+	Km <sup>r</sup> , Ap <sup>r</sup> <i>recA13</i>	HB101
RM4268	pTML86	-53	R2	+	+	+	-1	+	+	<i>minA1</i> <i>minB2, Tc<sup>r</sup></i>	1488
RM4269	pCP13 <sup>*</sup>	-53	R2	+	+	+	-1	+	+	<i>minA1</i> <i>minB2, Tc<sup>r</sup></i>	1488

<sup>a</sup>Genotype symbols follow the conventions recommended by Demerec et al. (23). Designations are as specified by Bachmann (2).

TABLE 3  
Bacteriophages

Phage	Source or Reference
R408	M. R. Kelley (118)
$\lambda$ <u>cI857</u> <u>Sam7</u>	B. H. Brownstein
F116L	G. Jacoby (68)

plasmid pMK90 contains the E. coli gyrA gene (91) and was generously provided by Dr. Martin Gellert, NIH, Bethesda, MD. The 9.2 kbp BamHI fragment of pMK90 which contains the E. coli gyrA gene was subcloned into pCP13\*. This construct was designated pTML90 and was used to move the E. coli gyrA gene into P. aeruginosa.

pRK2013 (119) was used to provide tra functions in trans in all triparental matings. pBR325 (9) was used to subclone a PstI fragment containing gin from pTML8 which was designated pTML822. This construct was used to create single crossovers within the P. aeruginosa chromosome and enabled mapping of the gin gene.

The conjugative plasmid FP5 (83) was used to mobilize the chromosome to determine the time of entry of the Tc<sup>r</sup> gene in the mapping of gin.

The PstI-HindIII gin containing fragment of pTML86 was subcloned into the Bluescript phagemid KS+ and SK+ vectors (Stratagene, La Jolla, CA) for the recovery of single-stranded DNA. The polylinker of the KS+ and SK+ forms is inverted, permitting the cloning of gin in opposite orientations as represented by pTML69 and pTML96 respectively.



*Media, antibiotics and amino acids*

The initial screening of the P. aeruginosa chromosomal library in E. coli DH1 was carried out using nutrient broth (NB) (Difco, Detroit, MI). Mueller-Hinton broth (Difco) was used for the determination of the liquid minimal inhibitory concentration (MIC). All subsequent experiments requiring rich medium were performed using Luria broth (LB) (88) purchased from GIBCO Laboratories, Madison, Wis. for the growth of P. aeruginosa and E. coli. Those experiments requiring growth of P. aeruginosa in minimal media were performed using Pseudomonas minimal media (PMM) (88) supplemented with 0.4 % glucose and the appropriate amino acids (50 ug/ml). For the preparation of solid media, Bacto-agar (Difco) was added to a final concentration of 1.2 %. Tryptone broth [0.1 % (w/v) Bacto-tryptone; 0.5 % (w/v) NaCl; 0.2 % (w/v) maltose; 0.2 ug thiamine/ml; 0.01 M MgCl<sub>2</sub>] was used to grow E. coli strains prior to infection with bacteriophage lambda. Top agar (LB containing 0.7 % agar) was used for the preparation of phage lysates and titering. Lambda phage was titered on trypticase agar [1.0 % (w/v) trypticase, (BBL Microbiology Systems, Cockeysville, MD); 0.5 % (w/v) NaCl; 1.0 % (w/v) agar] overlaid with top agar, all other phages were titered on 1.2 % (w/v) agar in LB (L-agar). Pseudomonas Isolation

Agar (PIA) (Difco) containing 2 % glycerol and the appropriate antibiotic was used to select P. aeruginosa exconjugates resulting from triparental matings. E. coli HB101(F<sup>+</sup>) containing the Bluescript subclone was grown in 2X YTG medium [1.6 % (w/v) tryptone; 1.0 % (w/v) yeast extract; 0.5 % (w/v) NaCl; 0.1 % (w/v) glucose] during infection with helper phage R408 (118).

Stock solutions of nalidixic acid (Sigma, St. Louis, MO) and norfloxacin (gift of Merck, Sharpe, and Dohme, Rahway, NJ) were prepared in 0.01 N NaOH at 50 mg/ml and 1.0 mg/ml respectively. Antibiotics used for selection of E. coli were added at the following concentrations: ampicillin, 50 ug/ml; chloramphenicol, 100 ug/ml; kanamycin, 50 ug/ml; tetracycline, 12.5 ug/ml; nalidixic acid 50 ug/ml. Antibiotics used for selection in P. aeruginosa were used at the following concentrations: mercury hydrochloride, 5 ug/ml; streptomycin, 750 ug/ml; tetracycline, 200 ug/ml; nalidixic acid, 500 ug/ml.

#### *Growth of cells*

The growth of P. aeruginosa and E. coli in liquid culture was monitored using Klett-Summerson flasks which permitted reading the Klett<sub>660</sub> value at selected times during the growth curve. The log of this value was plotted

against time to determine the lag, log and stationary phases that are characteristic of a bacterial growth curve.

*Isolation of P. aeruginosa chromosomal DNA*

Chromosomal DNA was isolated from the nalidixic acid-sensitive P. aeruginosa strain PA0303 by the method of Marmur (81). Cells were harvested from a 100 ml culture of PA0303 grown to 95 Klett<sub>660</sub> units in LB. The cells were washed with 10 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.0), recentrifuged and resuspended in 10 ml of NET buffer (100 mM Tris, pH 8.0; 200 mM NaCl; 10 mM EDTA). Four milliliters of a solution of lysozyme (10 mg/ml) was added and incubated for 10 min at 37°C with gentle shaking. A 1 % (w/v) solution of sodium dodecyl sulfate (SDS) in NET buffer was added and the incubation was continued for 10 min, followed by the addition of 0.6 ml RNase (1 mg/ml) and 15 min incubation. Proteinase K was added at a final concentration of 2 mg/ml and the mixture was further incubated for 1 h at 37°C. Saturated NaCl was added (3.0 ml), inverted once, and extracted with 16 ml chloroform:isoamyl alcohol (24:1). The extraction was repeated and the upper phase was dialyzed for 72 h against 5.5 l TES (50 mM Tris, pH8.0; 5 mM EDTA; 50 mM NaCl). The amount of DNA was quantitated spectrophotometrically by measuring the A<sub>260</sub>.

*Preparation of plasmid DNA*

For the purpose of cloning the *P. aeruginosa* chromosomal library, pCP13\* was isolated from *E. coli* HB101 by the large scale alkaline lysis procedure (80) prior to purification by cesium chloride density equilibrium centrifugation using the method of Matsumura (personal communication). Briefly, HB101 cells containing pCP13\* were grown overnight in 500 ml LB containing tetracycline. The plasmid DNA pellet obtained by the large scale alkaline lysis procedure was resuspended in 8.0 ml TE (10 mM Tris, pH 8.0; 10 mM EDTA), solid cesium chloride (1 g/ml) and ethidium bromide (EtBr) (0.1 ml of a 5 mg/ml solution per ml of DNA solution) were added. The gradients were centrifuged in a Beckman Ti50 rotor at 40,000 RPM at 20°C for 48 h. The lower plasmid band was removed by side puncture and the EtBr was extracted with isopropanol saturated with 20X SSC buffer (3 M NaCl; 0.3 M sodium citrate, pH 7.0) until the EtBr was no longer visible under long-wave UV light. The DNA was precipitated by the addition of two volumes of ice-cold ethanol, placed on dry ice for 10 min and centrifuged in an Eppendorf microfuge for 10 min. The DNA was resuspended in a small volume of TE and a sample was removed for quantitation by measuring

the absorbance at 260 nm.

All other plasmid DNAs for cloning were prepared either by mini-plasmid or large-scale alkaline lysis procedures as described by Maniatis (80). All plasmid DNAs were restricted with the appropriate restriction endonucleases and analyzed by agarose gel electrophoresis to determine sample purity and restriction pattern and were quantified by measuring the absorbance at 260 nm.

#### *Conditions for restriction endonuclease digestion*

Restriction enzymes were purchased from Boehringer Mannheim, or Amersham, Arlington Heights, IL. The reaction conditions recommended by the manufacturer for each enzyme were followed using buffers provided by the manufacturer. The reactions were terminated by the addition of tracking dye [30 % (v/v) glycerol, 7 % (w/v) SDS, 0.07 % (w/v) bromphenol blue] prior to agarose gel electrophoresis. Restricted DNA to be used for ligation was phenol:chloroform (1:1) extracted and ethanol precipitated as previously described.

#### *Agarose gel electrophoresis*

DNA fragments were separated and identified by electrophoresis through 0.7 % (w/v) agarose gels. Solid

agarose (Seakem LE, FMC Bioproducts, Rockland, ME) was melted by boiling in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). After slight cooling, an EtBr solution (5 mg/ml) was added to a final concentration of 1 ug/ml and the agarose solution was poured into a horizontal gel casting tray. The DNA sample containing tracking dye was loaded into the wells and a constant voltage of 50 V was applied until the bromphenol blue dye migrated to the bottom of the gel. The gel was then photographed on a UV transilluminator (Ultraviolet Products, Inc., San Gabriel, CA). Lambda DNA restricted with HindIII was also electrophoresed and served as a size marker.

#### *Electroelution of DNA fragments*

Restriction endonuclease-digested DNA fragments were purified by electroelution for the preparation of radioactive probes or for the purpose of cloning. The DNA bands of interest were excised following agarose gel electrophoresis and electroeluted using an IBI analytical electroeluter. The agarose gel piece containing the desired DNA band was chopped finely and placed in the electroeluter filled with 0.5 X TBE buffer. Electroelution was carried out at 100 V for 30 min into a V-shaped tunnel containing a high salt cushion (8.0 M ammonium acetate).

The migration of the EtBR-stained DNA into the tunnel was monitored using a hand held long-wave UV lamp. Following the completion of the electroelution, the salt cushion was collected and the DNA was recovered by ethanol precipitation.

#### *Conditions for ligations*

T4 DNA ligase was purchased from Boehringer Mannheim and used at 1.0 unit/ligation reaction. The ligation reactions were allowed to proceed at 15<sup>o</sup>C overnight and were terminated by heat inactivation of the enzyme at 70<sup>o</sup>C for 10 min. The ligation buffer used was that recommended by the manufacturer.

#### *Construction of a P. aeruginosa chromosomal library*

Approximately 100 ug of chromosomal DNA was digested with HindIII (20 U/ug) for 1.5 h at 37<sup>o</sup>C, extracted with phenol:chloroform (1:1) and ethanol precipitated. The DNA pellet was resuspended in 0.3 ml TE buffer and layered on a 10-40% sucrose gradient prepared in buffer A (1.0 M NaCl; 0.1 M EDTA; 0.2 M Tris, pH 8.0) that was formed using a gradient maker. The gradient was centrifuged for 21 h in a Beckman SW40.1 rotor at 25,000 RPM. The bottom of the tube was punctured and fractions were collected. The DNA

fragments present in each fraction were sized by removing a sample for electrophoresis on a 0.7% agarose gel. Those fractions containing 18-23 kbp fragments were pooled and ligated into the unique HindIII site of the pCP13\* cosmid vector. The vector was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) prior to ligation to prevent recircularization of the vector (80). An in vitro packaging kit was purchased from Amersham to package the ligation mixture into lambda phage capsids. The packaged recombinant molecules were then used to infect E. coli DH1 (gyrA96 hsdR17) cells. Following transfection, the cells were plated onto nutrient agar containing tetracycline (200 ug/ml) to select those cells containing a cosmid. The library was established by pooling all of the tetracycline-resistant ( $Tc^r$ ) colonies in LB. Several vials were frozen and stored at  $-70^{\circ}C$ .

#### *Transformations*

DNA transformations of E. coli were done either by the calcium chloride method of Davis (22) or by the rubidium chloride method described by Hanahan (45). P. aeruginosa was transformed with DNA by the  $MgCl_2$  procedure (87).



### *Triparental matings*

Triparental matings were performed as specified by Ruvkun and Ausubel (119). *P. aeruginosa* recipients were grown overnight at 43°C to inactivate the host restriction system (114). The helper plasmid, pRK2013, provided Tra functions in trans permitting mobilization of pCP13\*. The helper strain, HB101 containing pRK2013, and the donor strain were grown to mid-log phase. The matings were initiated by mixing equal volumes of the donor, recipient and helper strains. The mating mix was then filtered onto a 0.2 um (average pore size) filter (Nalgene) and the filter was placed on the surface of an LB plate and incubated overnight at 37°C. The following day, the filter was resuspended in a small volume of LB and aliquots were spread onto PIA with antibiotic selection and incubated 1-2 days at 37°C.

### *Conjugations*

Donor and recipient cultures were grown to mid-log phase, harvested by centrifugation, and resuspended in half volume LB:PMM (1:1). Donors and recipients were mixed at a ratio of 1:2 and incubated at 37°C for 2 h without shaking. For interrupted matings, samples were removed at selected times and vortexed at high speed for 2 min to

disrupt mating pairs. The cells were collected by centrifugation, resuspended in saline (0.85% NaCl) and dilutions were plated onto selective medium. Plates were incubated for 2 days at 37°C.

### *Transductions*

Transducing phage lysates were prepared by mixing 0.1 ml of an overnight culture of *P. aeruginosa* with 0.1 ml of  $10^8$  plaque forming units (PFU)/ml of phage. Three milliliters of top agar were added and the mixture was immediately poured onto fresh L-agar plates. After 16 h incubation at 37°C, the top agar was scraped off and placed in a screw-capped tube. The L-agar was rinsed with 2.0 ml LB and added to the top-agar. One-tenth volume of chloroform was added to the screw-cap tube to lyse any remaining cells. After vigorous shaking, the tubes were centrifuged for 10 min at 5000 RPM and the supernatant fluid was collected and titered. Transductions were performed by infecting mid-log phase cultures with a transducing phage lysate at multiplicities of infection (MOI) of 10.0, 1.0, and 0.1. Transductants were plated on selective media and incubated for 1 to 2 days at 37°C. Cotransduction of linked genes was tested by replica plating the primary transductants onto secondary selective

medium.

#### *Drug survival curves*

Cultures to be tested for growth in the presence of quinolones were grown in the absence of quinolones to mid-log, then diluted 1:10 into media containing various concentrations of the drug. At selected times, samples were removed, diluted and plated onto L-agar to determine the surviving colony forming units (CFU)/ml remaining in the culture.

#### *Determination of plate MICs*

Plate MICs were determined by patching isolated colonies of the strain to be tested onto L-agar. The patches were allowed to grow up overnight and the resulting patches were replica plated onto plates containing various concentrations of quinolones. The plate MIC is defined as the minimum concentration of drug which inhibits the growth of the replicated patches.

#### *Determination of MIC's in liquid culture*

Strains to be tested were grown overnight at 37°C in LB with shaking. Klett tubes containing Mueller-Hinton broth and various concentrations of drug were inoculated

with approximately  $10^6$  cells of the overnight culture, and incubated at  $37^{\circ}\text{C}$  with shaking for 20 h. The MIC in liquid culture is defined as the lowest concentration of drug which inhibits visible growth as assessed by turbidity.

#### *Southern hybridization*

Southern transfer of DNA from agarose gels to nitrocellulose was performed as described by Maniatis (80). The DNA was fixed to nitrocellulose filters (Schleicher & Schuell, Keene, NH) by baking in a vacuum oven at  $80^{\circ}\text{C}$  for 2.5 hours. The prehybridization solution [1 M sodium chloride; 10 mM sodium phosphate, pH 6.5; 0.05% (w/v) sodium pyrophosphate; 5 x Denhardt's; 5 ug/ml heat denatured calf thymus DNA; 1% (w/v) SDS] was added to a heat-sealable plastic bag containing the nitrocellulose filter and was incubated at  $65^{\circ}\text{C}$  with gentle agitation for at least 2 h.  $[\alpha]-^{32}\text{P}$ -labeled DNA probes were generated using a nick translation kit (Boehringer Mannheim) and the instructions from the kit were followed.  $[\alpha]-^{32}\text{P}$ -dCTP (3000 Ci/mole) was purchased from New England Nuclear (Wilmington, DE). Unincorporated label was separated from labeled DNA by the spun-column technique (80). The prehybridization solution was withdrawn from the bag and the heat denatured radioactively labeled DNA probe was

added to 10.0 ml of the prehybridization solution and replaced in the bag. Low stringency hybridizations were performed at 55°C overnight while high stringency hybridizations were carried out at 65°C overnight. Under high stringency conditions, filters were washed in 0.2 X SSC, 0.5% (w/v) SDS for 2 h at 65°C, with four changes of wash solution. Under low stringency conditions, the filters were washed at room temperature using 2 X SSC, 0.1% (w/v) SDS with four changes of wash solution during a 2 h period. Autoradiography was performed by placing the filters in an X-ray cassette with intensifying screens and Kodak XAR-5 film at -70°C.

#### *Tn<sub>3</sub> mutagenesis of plasmids*

The method followed for Tn<sub>3</sub> mutagenesis of plasmid DNA was that of White et al. (146). The E. coli strain JC12717 containing pBEU30 was mated with DH1 containing the target plasmid. The DH1 transconjugates were selected by plating onto L-agar containing ampicillin (to select the Tn<sub>3</sub> element) and tetracycline (to select the target plasmid). Approximately 500 colonies were pooled, grown to mid-log phase, collected by centrifugation and resuspended at 1/6 volume of medium. These cells were infected with  $5 \times 10^7$  PFU of phage  $\lambda$ CI857Sam7 and incubated 15 min at

37°C to allow absorption of the phage. Ten volumes of medium were added and incubated at 42°C for 20 min to induce the lytic cycle. The culture was then returned to 37°C and incubation was continued for 2-3 h. A transducing phage lysate was prepared by incubating the culture with 0.1 volume of chloroform for 15 min at 37°C. Following centrifugation to remove cellular debris, the lysate was decanted to a sterile tube and titered on RM1068. DH1 was infected with this transducing phage lysate at MOIs of 0.1, 1.0 and 10.0 and expressed for 1.0 h at 30°C. The entire mixture was plated onto L-agar containing tetracycline alone, ampicillin and tetracycline, or ampicillin alone. Resulting colonies were tested for the Tc<sup>r</sup> and ampicillin resistant (Ap<sup>r</sup>) phenotypes to select for the target plasmid and the Tn<sub>3</sub> element respectively. These Tn<sub>3</sub>-containing isolates were screened for sensitivity to nalidixic acid, and the position of the Tn<sub>3</sub> insertion was mapped by restriction enzyme analysis of plasmid DNA as previously described.

#### *Preparation of single-stranded DNA*

Single-stranded DNA was prepared by infecting E. coli HB101(F<sup>+</sup>) containing the Bluescript subclones with the fl helper phage R408 (118). Colonies of HB101(F<sup>+</sup>)

containing the Bluescript subclones were patched onto L-agar containing ampicillin (100 ug/ml) and kanamycin (50 ug/ml). Following overnight incubation, a loopful of cells was used to inoculate 1.5 ml of 2 x YTG medium containing ampicillin (100 ug/ml). Phage R408 ( $1-5 \times 10^9$  PFU) was added to the tubes and the cultures were shaken vigorously in an environmental shaker for 4 h at 37<sup>0</sup>C. Single-stranded DNA was isolated according to the method of Kidd et al. (118). Each single-stranded DNA preparation was resuspended in 20 ul of TE buffer and a 5.0 ul sample was electrophoresed in a 0.7% (w/v) agarose gel. The visualization of a distinct, brightly stained EtBr band following electrophoresis was qualitatively estimated to be a sufficient yield for DNA sequencing.

#### *Sanger dideoxy sequencing*

The Sanger dideoxy sequencing method (120) was performed using the T7 DNA polymerase enzyme as described by Tabor and Richardson (133). Complementary oligonucleotide primers, synthesized by the Loyola University Macromolecular Facility, were annealed to a single-stranded DNA template and extended using the T7 DNA polymerase enzyme, Sequenase 2.0 (United States Biochemicals, Cleveland, OH). Deoxynucleotides (dNTPs)

(dATP, dCTP, dGTP and dTTP), dideoxynucleotides (ddNTPs) (ddATP, ddCTP, ddGTP, and ddTTP), and the dGTP analog, dITP, were purchased from Pharmacia (Piscataway, NJ).  $^{35}\text{S}$ -dATP (1000-1500 Ci/mMole) was purchased from New England Nuclear.

The sequencing procedure involved three steps: annealing the template to the primer; labeling by extension of the primer in the presence of all four dNTPs, one of which is radioactively labeled; termination in four separate reactions, each containing all four dNTPs and a single ddNTP. Because of the high GC content of *P. aeruginosa* (67%) (104), four additional reactions using the dGTP analog, dITP, was also carried out. This analog reduces the band compressions on a sequencing gel that are problematic in sequencing high percentage GC DNAs. The concentration ratio of dNTPs to ddNTPs in the termination reaction was adjusted to 10:1 to read sequences close to the primer (within 50-200 bases) and to 30:1 to read sequences further away from the primer (within 150-300 bases). These conditions are referred to as "read short" and "read long" respectively.

Single-stranded DNA template (5 ul) was annealed to 0.5 pmoles of primer DNA in a total volume of 10 ul of annealing buffer (40 mM Tris-HCl, pH 7.5; 20 mM  $\text{MgCl}_2$ ; 50



mM NaCl) at 80°C for 5 min. The annealed mixture was then placed at room temperature for 30 min. The labeling reaction was initiated by the addition of 1 ul of 0.1 M dithiothreitol, 2 ul of labeling mix (1.5 uM dCTP; 1.5 uM dTTP; 1.5 uM dGTP or 3.0 uM dITP), 1 ul (10 uCi) of <sup>35</sup>S-dATP, and 3.0 units of Sequenase 2.0 enzyme. After 2-5 min at room temperature, 3.5 ul of the labeling reaction was transferred to four separate wells of a microtiter plate each containing 2.5 ul of the appropriate dideoxy termination mixture (read short conditions: 80 uM all four dNTPs, 8 uM ddNTP; read long conditions: 80 uM all four dNTPs, 2.5 uM ddNTP). In those reactions using dITP, ddGTP was used at one fifth of the usual concentration in the termination reaction since T7 DNA polymerase shows a 10-fold preference for the incorporation of ddGTP over dITP (133). The termination reaction was incubated for 2-5 min at 37°C and 4 ul of stop solution (95% formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05% xylene cyanol) was added to each well. The reactions were stored frozen at -20°C. Immediately before loading onto a sequencing gel, the reactions were heated to 75-80°C for 5 min.

An International Biotechnologies, Inc. model STS 45 sequencing gel apparatus was used. Electrophoresis was in a 6% (w/v) acrylamide/0.3% N,N'-methylenebisacrylamide 7.56

M urea gel made in running buffer (100 mM Tris borate, pH 8.9; 1 mM EDTA). Polymerization was initiated by the addition of 210 ul of a 25% ammonium persulfate solution and 85 ul TEMED to 90 ml acrylamide solution. The gel was immediately poured between the plates separated by 0.4 mm spacers. Two sharktooth combs were inserted and the gel was allowed to polymerize overnight. The sequencing reactions were loaded (2.5 ul per lane) using a triple loading method. Reactions carried out under "read long" conditions were loaded first and electrophoresed at approximately 2000 volts, constant current (60 milliamperes) until the xylene cyanol dye front ran off the bottom of the gel. The "read short" reactions were loaded next and electrophoresis was continued until the xylene cyanol dye front migrated 75% of the length of the gel. At this point, the "read short" reactions were loaded again and electrophoresed until the bromophenol blue dye front ran off the bottom of the gel plus an additional 20 min. The gel was fixed for 20 min in a 10% (v/v) glacial acetic acid, 10% (v/v) methanol solution and dried down onto filter paper using a BioRad model 583 slab gel dryer (Rockville Center, NY). Autoradiography was done using Kodak XAR-5 film.

The DNA sequence was read, transcribed and entered into a Zenith IBM compatible personal computer. The DNAsis, Prosis and University of Wisconsin GCG software packages were used in the sequence analysis.

#### *RNA Extraction*

Total RNA was extracted from *P. aeruginosa* using a modification of a mini-RNA procedure (M. Kelley, personal communication). Cells were grown to mid-log phase (45-55 Klett units) in 40 ml of LB. The culture was then mixed with an equal volume of frozen and crushed buffer A (50 mM Tris, pH 8.0; 50 mM EDTA; 150 mM NaCl) and harvested by centrifugation at 7000 RPM for 5 min in a GSA rotor. The cell pellet was resuspended in 250 ul GIT buffer (4 M guanidine isothiocyanate; 25 mM sodium acetate, pH 6; 120 mM 2-mercaptoethanol) and 250 ul buffer B [10 mM Tris, pH 8.0; 10 mM EDTA; 100 mM NaCl; 1% (w/v) SDS], transferred to a microcentrifuge tube and boiled for 5 min to assist in cellular lysis. To the lysed mixture, 40 ul of 2 M sodium acetate (pH 4.0) and 600 ul of phenol:chloroform (5:1) was added, mixed, and placed on ice for 15 min. Following centrifugation for 20 min in an Eppendorf microfuge, the supernatant fluid was transferred to a new microcentrifuge tube. An equal volume of isopropanol was added, and the

mixture was placed in a dry ice/methanol bath for 30 min, and re-centrifuged as before. The pellet was resuspended in 100 ul of GIT buffer and placed in a 65°C heating block for 5 min. After the pellet was completely resuspended, 1/10 volume of 3 M sodium acetate (pH 5.2) and 100 ul of isopropanol were added, and the mixture was precipitated on dry ice for 15 min. The pellet was collected by centrifugation for 20 min, washed with 70% ethanol, re-centrifuged, and resuspended in 50 ul of 0.2% (v/v) diethylpyrocarbonate (DEPC)-treated water. RNA was quantitated by reading the absorbance at 260 nm.

#### *Northern hybridizations*

Total RNA was electrophoresed through a 1% (w/v) agarose, 0.66 M formaldehyde gel prepared in MOPS buffer [20 mM 3-(N-morpholino) propansulfonic acid] (32). The RNA sample (20 ug/lane) was resuspended in electrophoresis sample buffer [0.75 ml deionized formamide, 0.15 ml 10X MOPS, 0.24 ml formaldehyde, 0.1 ml deionized RNase-free H<sub>2</sub>O, 0.1 ml glycerol, 0.08 ml 10% (w/v) bromophenol blue], heated to 65°C for 5 min, 1 ul of EtBr (1 mg/ml) was added, and the sample was loaded onto the gel. A 0.24-9.5 kbp RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) was run in parallel to identify the size of the messenger

RNA. The running buffer was MOPS/EDTA (20 mM MOPS; 50 mM sodium acetate; 10 mM EDTA, pH 7.0), and electrophoresis was carried out at 80 V for 5 h. Following electrophoresis, the gel was rinsed in 2X SSC for 1 h prior to transfer to nitrocellulose exactly as was described for Southern hybridization (see above). RNA was fixed to the filter using a UV Stratalinker (Stratagene) following the procedures recommended by the manufacturer. Radioactive probes were prepared using a nick translation kit (Boehringer Mannheim) as described above. Pre-hybridization and hybridization solutions were the same as described for Southern hybridizations except for the addition of 25% (v/v) formamide. Pre-hybridization (3.5 h) and hybridization (overnight) were both carried out at 42°C. The filter was washed in 1 % (w/v) SDS, 0.2 X SSC for 15 min at room temperature followed by four 30 min washes at 65°C in the same buffer. The filter was autoradiographed using Kodak XAR-5 film at -70°C with intensifying screens.

#### *Protein expression in E. coli mini-cells*

The E. coli mini-cell-producing strain  $\chi$ 1488 (15) was transformed with the appropriate plasmids using the calcium chloride procedure described previously. The purification of mini-cells was as described by Clark-

Curtiss and Curtiss (15) and Goldberg and Mekalanos (41) with minor modifications. Each plasmid-containing  $\chi$ 1488 strain was grown overnight with vigorous agitation in 500 ml LB including antibiotic selection for the plasmid. The cells were harvested by centrifugation using a GSA rotor at 6000 RPM for 5 min at 20°C. The supernatant fluid was retained and centrifuged again using the GSA rotor at 9000 RPM for 15 min. The resulting pellet was resuspended in 1.5 ml BSG buffer (8.5 g NaCl, 0.30 g  $\text{KH}_2\text{PO}_4$ , 0.60 g  $\text{Na}_2\text{HPO}_4$ , 10 ml 1.0% gelatin, deionized water to 1.0 l) and vigorously vortexed for 2 min before layering onto a 35 ml 5-40% (w/v) sucrose gradient. The gradient was made by freezing a 22% (w/v) sucrose solution in BSG buffer followed by gradual thawing at 4°C overnight. The gradients were centrifuged in an HB4 swinging bucket rotor for 15 min at 4500 RPM at 20°C. The center of the top mini-cell band was withdrawn using a pasteur pipette and layered onto a second identical sucrose gradient, re-centrifuged and collected. The mini-cells were harvested by centrifugation in an SS34 rotor at 11,000 RPM for 15 min. The mini-cells were resuspended in 1 ml of M9 medium (6 g  $\text{Na}_2\text{HPO}_4$ , 3 g  $\text{KH}_2\text{PO}_4$ , 0.5 g NaCl, 1 g  $\text{NH}_4\text{Cl}$  per liter of deionized water) supplemented with 0.4% glucose (w/v), 25 ug histidine/ml of medium, and 0.2 ug thiamine/ml of

medium. The absorbance at 595 nm was adjusted to 1.0 and the mini-cells were incubated for 30 min at 37°C. <sup>35</sup>S-methionine (New England Nuclear) (1146 Ci/mmol) was added at 50 uCi/ml, incubated for 45 min at 37°C, and chased with cold methionine (100 ug/ml) for 5 min. The labeled cells were collected by microfuge centrifugation and resuspended in 1/40 volume TE. An equal volume of 2 x sample buffer [125 mM Tris-HCl, (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.007% (w/v) bromophenol blue] was added. The mixture was boiled for 5 min prior to loading onto an SDS-polyacrylamide gel.

*SDS-polyacrylamide gel electrophoresis  
and detection of <sup>35</sup>S-labeled proteins*

SDS-polyacrylamide gels were run using the method of Laemmli (70). A resolving gel of 12.5% (w/v) acrylamide, 2.7 % (w/v) N,N'-methylenebisacrylamide, 375 mM Tris (pH 8.8), 0.1% (w/v) SDS, 0.5% (w/v) ammonium persulfate, 0.5% (v/v) TEMED and a stacking gel of 4% (w/v) acrylamide, 2.7% (w/v) N,N'-methylenebisacrylamide, 125 mM Tris (pH 6.8), 0.1% (w/v) SDS, 0.5% (w/v) ammonium persulfate, 0.02% (v/v) TEMED were used. Pre-stained protein molecular weight standards (Amersham) were run simultaneously for the determination of molecular mass. The standards used and

their molecular mass are: myosin, 200,000; phosphorylase b, 97,400; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; trypsin inhibitor, 21,500; lysozyme, 14,300. A BioRad mini protean II cell electrophoresis unit was used and gels were run at constant current (200 mA) for 1 h. To enhance the detection of radioactivity, gels were soaked in a 1 M solution of the fluor, sodium salicylate, pH 7.0 (12) for 30 min with gentle shaking. The gels were dried under vacuum at 80°C in a BioRad model 443 slab dryer and autoradiographed with intensifying screens at -70°C using Kodak XAR-5 film.



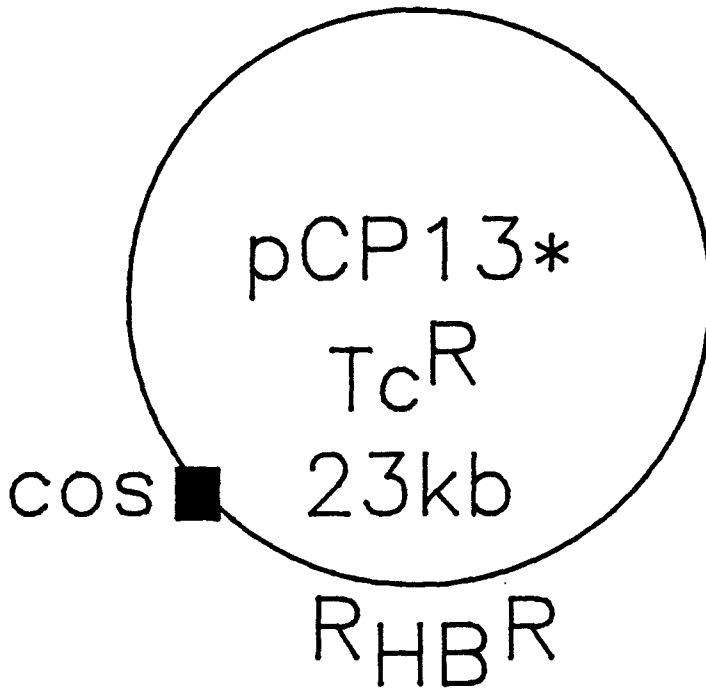
## CHAPTER IV

### RESULTS

#### *Construction of the P. aeruginosa chromosomal library*

A P. aeruginosa chromosomal library was constructed using the broad-host-range cosmid cloning vector, pCP13\* (Figure 3) (20). Chromosomal DNA was partially restricted with HindIII and the fragments were sized on a sucrose gradient. Fractions from the gradient containing fragments ranging from 18-25 kbp were ligated into the unique HindIII site of pCP13\*. The recombinant molecules were packaged in vitro and transfected into E. coli DH1 cells. To select the tet gene of pCP13\*, the cells were plated on NB containing tetracycline (200 ug/ml) to select only those cells harboring the vector. Furthermore, since the vector is 23 kbp, it is too small to be packaged into phage capsids alone, therefore all cells expressing the Tc<sup>r</sup> gene must have acquired an inserted chromosomal sequence in addition to pCP13\*. The likelihood of concatamerization of

Figure 3. pCP13\* cosmid cloning vector. pCP13\* was derived from pCP13 (20) by the excision of the BamHI polylinker followed by religation (gift from S. Farrand). This plasmid is mobilizable in the presence of a Tra<sup>+</sup> plasmid and is capable of replication in both E. coli and P. aeruginosa. Tc<sup>r</sup> - tetracycline resistance gene; cos - cos site; R - EcoRI; H - HindIII; B - BamHI.



the vector is greatly reduced since pCP13\* was treated with alkaline phosphatase. The resulting chromosomal library in DH1 consisted of approximately two thousand independent Tc<sup>r</sup> isolates representing at least twice the size of the P. aeruginosa chromosome.

*Screening the P. aeruginosa nalA libraries*

The library was mobilized into the P. aeruginosa nalA (Nal<sup>r</sup>) strains PA0514 and PA0515 by triparental mating, and was screened for complementation of the nalA (Nal<sup>r</sup>) mutation by replica plating onto L-agar containing nalidixic acid (500 and 1000 ug/ml) or norfloxacin (2, 3, and 4 ug/ml). Wild-type (Nal<sup>s</sup>) strains of P. aeruginosa are sensitive to 100 ug nalidixic acid/ml while Nal<sup>r</sup> strains are resistant to greater than 5000 ug/ml (Table 4). Because nalidixic acid is highly insoluble at these concentrations, norfloxacin, which can be used at much lower concentrations, was utilized in these screens. The library established in PA0515 (nalA5) was screened for Nal<sup>s</sup> and norfloxacin-sensitive (Nor<sup>s</sup>) clones. No Nal<sup>s</sup> clones were found. Twenty-four Nor<sup>s</sup> isolates were recovered by replica plating onto L-agar containing norfloxacin (3 ug/ml). All of these isolates could be grouped into five restriction patterns. Southern hybridization under low

TABLE 4

## MIC - Liquid Culture

STRAIN	GENOTYPE	NOR	MIC ( $\mu\text{g/ml}$ )	NAL
PAO25	wild-type	0.5		250
PAO303	wild-type	0.5		500
PAO514	<u>nalA2</u>	8		>1000
PAO515	<u>nalA5</u>	8		>1000
PAO6002	<u>nalB4</u>	2		1000
PAO6005	<u>nalB8</u>	1		500
PAO6006	<u>nalB9</u>	4		1000
PAO6004	<u>pip6003</u>	0.5		100

stringency conditions resulted in no detectable homology between these cloned fragments and the E. coli gyrA gene (results not shown). Further characterization of these clones was abandoned because: (i) the Nor<sup>S</sup> phenotype of these clones was unstable (ii) they did not complement the gyrA96 mutation of E. coli DH1 (iii) no detectable homology existed between these clones and the E. coli gyrA gene.

Two independent screens of the library in PA0514 (nalA2) did not yield any Nal<sup>S</sup> or Nor<sup>S</sup> isolates.

*Screening the P. aeruginosa library for  
phenylalanine prototrophy*

The phe-2 allele is 67% cotransducible with the nalA locus as shown by F116 transduction (18). It was calculated that it was possible to have cloned and packaged a large enough DNA fragment to contain both the phe-2 and the nalA genes. Therefore, the chromosomal library was mobilized from E. coli DH1 to the P. aeruginosa strain PA0660 (phe-2), and screened for phenylalanine prototrophy. Three independent Phe<sup>+</sup> isolates were obtained and found to have identical plasmid DNA restriction patterns. However when the plasmid DNA was re-transformed to PA0660, the Phe<sup>+</sup> phenotype could no longer be restored. It is likely that

the Phe<sup>+</sup> phenotype was the result of chromosomal reversion, and not due to the cloned fragment. In addition these clones were unable to complement a Nal<sup>r</sup> isolate of PA0660 (spontaneously obtained by plating PA0660 on 1000 ug nalidixic acid/ml) to the Nal<sup>s</sup> phenotype. Consequently, these clones were not pursued any further.

*Screening the P. aeruginosa library  
in E. coli DH1*

The P. aeruginosa library was screened in E. coli DH1 (gyrA96) for the Nal<sup>s</sup> phenotype. Screening was accomplished using an intermediate concentration of 50 ug nalidixic acid/ml because DH1 (Nal<sup>r</sup>) is resistant to 100 ug nalidixic acid/ml and a Nal<sup>s</sup> E. coli strain such as AB1157, is sensitive to 1-2 ug nalidixic acid/ml of medium. The library was initially plated for isolated colonies onto NB containing tetracycline (200 ug/ml). These plates were used as masters to replica plate first onto L-agar containing nalidixic acid (50 ug/ml) and then onto L-agar containing tetracycline (200 ug/ml). The library was independently screened in this manner three times with the isolation of a total of 22 Nal<sup>s</sup> clones. It was found that each of these clones contained an identical 25 kbp HindIII insert based on the observed restriction endonuclease

patterns of plasmid DNA. One such construct was designated pTML8 (Figure 4) and was used in most of the experiments that will be described. pTML8 was re-transformed into DH1 and it was shown that the Nal<sup>S</sup> phenotype was associated with pTML8 and was not a chromosomal mutation. DH1 transformed with pCP13\* did not display the Nal<sup>S</sup> phenotype.

It was also shown by Southern hybridization under high stringency conditions, that pTML8 did not share extended common sequences with any of the clones isolated in any of the P. aeruginosa genetic backgrounds previously specified (results not shown). The gene that imparted the quinolone-sensitive (Qin<sup>S</sup>) phenotype associated with pTML8 was designated qin.

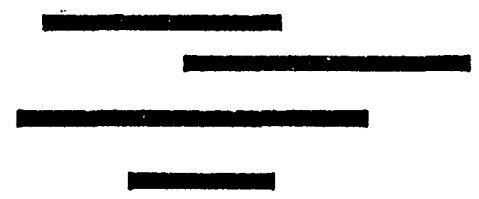
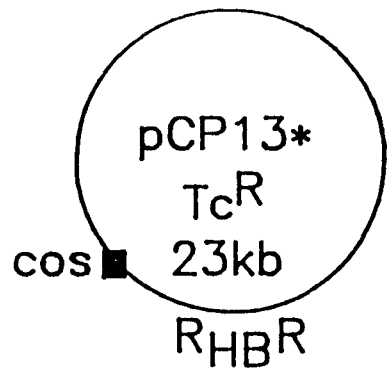
*Complementation of the E. coli gyrA96  
and P. aeruginosa nalA5 mutations by pTML8:*

*Determination of plate MICs*

The level of quinolone sensitivity imparted to E. coli DH1 was assessed by the determination of a plate MIC (Table 5). DH1 alone or containing pCP13\* had a plate MIC of 100 ug nalidixic acid/ml of medium. The E. coli wild-type (Qin<sup>S</sup>) AB1157 strain has a plate MIC of 2 ug nalidixic acid/ml. When DH1 was transformed with pTML8 (RM1220), an intermediate MIC of 50 ug nalidixic acid /ml was observed.



Figure 4. Construction of pTML8. DNA fragments ranging from 18-25 kbp in size resulting from the partial HindIII digestion of PAO303 chromosomal DNA were ligated into the unique HindIII site of the cosmid cloning vector pCP13\*. pTML8 was isolated by screening this library in an E. coli gyrA genetic background for the  $\text{Nal}^S$  phenotype. This recombinant plasmid contains a single 25 kbp HindIII chromosomal DNA insert.



HindIII Partial Digest  
PA0303 (Nor<sup>S</sup>) Chromosomal DNA

B = *Bam*HI  
R = *Eco*RI  
H = *Hind*III

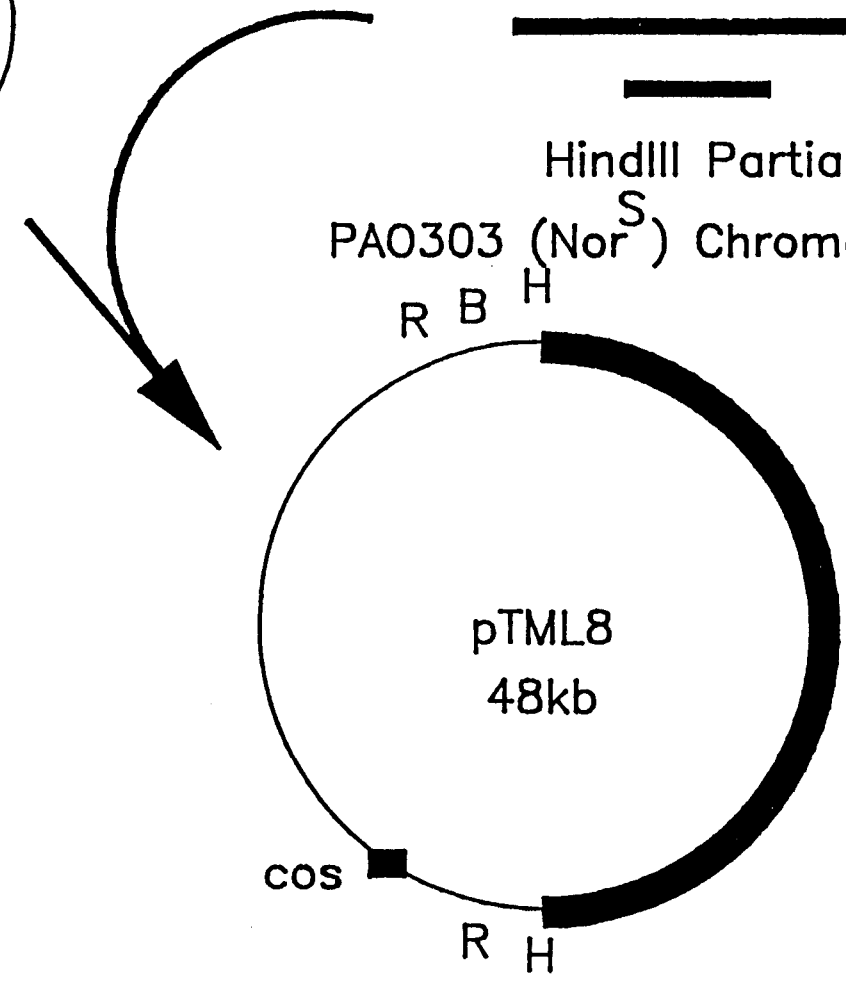


TABLE 5  
 Complementation of  $\text{Nal}^r$  in Escherichia coli

Strain	Relevant Genotype	Plasmid	Nalidixic acid Plate MIC ( $\mu\text{g/ml}$ )
DH1	<u>gyrA96</u>	None	100
RM4222	<u>gyrA96</u>	pCP13*	100
RM1220	<u>gyrA96</u>	pTML8	50
AB1157	wild-type	None	2

$\text{Nal}^r$  - Nalidixic acid resistance

RM2163 was constructed by introducing pTML8 into the nalA5 mutant of P. aeruginosa strain PA0515 by triparental mating. Plate MIC values were obtained to determine whether pTML8 was capable of complementing the Nor<sup>r</sup> phenotype of this mutant. It appears that similar to what was discovered in E. coli, pTML8 imparts an intermediate level of quinolone sensitivity to the P. aeruginosa nalA5 mutant strain PA0515 (Table 6).

*Complementation of the E. coli gyrA96 mutation by pTML8:*

*cell survival curves*

The ability of pTML8 to confer quinolone sensitivity to DH1 was further assessed by the construction of cell survival curves in the presence of nalidixic acid. Figure 5 shows two such survival curves comparing cell survival of DH1 containing pCP13\* (RM4222) to DH1 containing pTML8 (RM1220). DH1 cells containing pTML8 rapidly die off in the presence of 50 ug nalidixic acid/ml while DH1 cells harboring pCP13\* can continue to grow at this drug concentration.

An interesting observation was made in the process of repeating the drug survival curve experiments. DH1 cells that were newly transformed with pTML8 (RM1220) had a translucent appearance. In liquid culture, a

TABLE 6

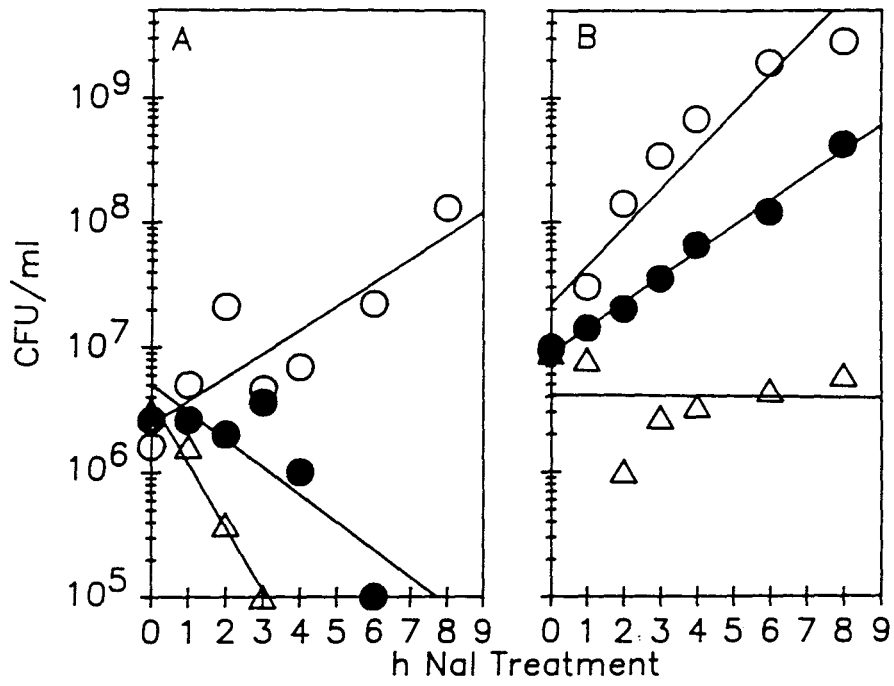
Complementation of Nor<sup>r</sup> in P. aeruginosa

Strain	Relevant Genotype	Plasmid	Norfloxacin Plate MIC ug/ml
PA0515	<u>nalA5</u>	None	4
RM2193	<u>nalA5</u>	pCP13*	5
RM2163	<u>nalA5</u>	pTML8	2
PA025	wild-type	None	1

Nor<sup>r</sup> - Norfloxacin resistance

Figure 5. Cell survival curve - Complementation of E. coli gyrA. Cultures were grown in the presence of various concentrations of nalidixic acid as described in Chapter III. The average of at least two determinations are represented by each point. Lines were determined by least-square regression analysis. A. RM1220 (E. coli DH1, pTML8) B. RM4222 (E. coli DH1, pCP13<sup>\*</sup>).

(○----○) 0  $\mu$ g nalidixic acid/ml  
(●----●) 50  $\mu$ g nalidixic acid/ml  
( $\Delta$ ---- $\Delta$ ) 100  $\mu$ g nalidixic acid/ml



subpopulation of RM1220 which differed in colony morphology emerged and increased in number with each generation. These colonies displayed an opaque appearance which was morphologically identical to DH1 and RM4222. This altered colony morphology was correlated with an increased resistance to nalidixic acid, similar to the level observed in DH1 alone. Plasmid DNA was isolated from several of the opaque isolates to determine whether pTML8 was being lost or had suffered a deletion. In each case, the restriction pattern of pTML8 was intact and only in one instance was a possible deletion observed. Consequently, analysis of the *Qin*<sup>s</sup> phenotype became inconsistent and unreliable by the method of drug-survival-curve analysis due to the apparent instability of pTML8 in DH1 after several generations in liquid culture.

*Subcloning the minimal DNA fragment defining qin from pTML8*

The basic rationale for pinpointing the location of qin within the pTML8 insert was to subclone various pTML8 restriction fragments into a suitable vector, transform DH1 with these subclones, and screen for retention of the *Nal*<sup>s</sup> phenotype. The minimal DNA fragment containing qin was successfully subcloned by the following procedure. pTML8 was digested with the PstI and HindIII restriction enzymes,

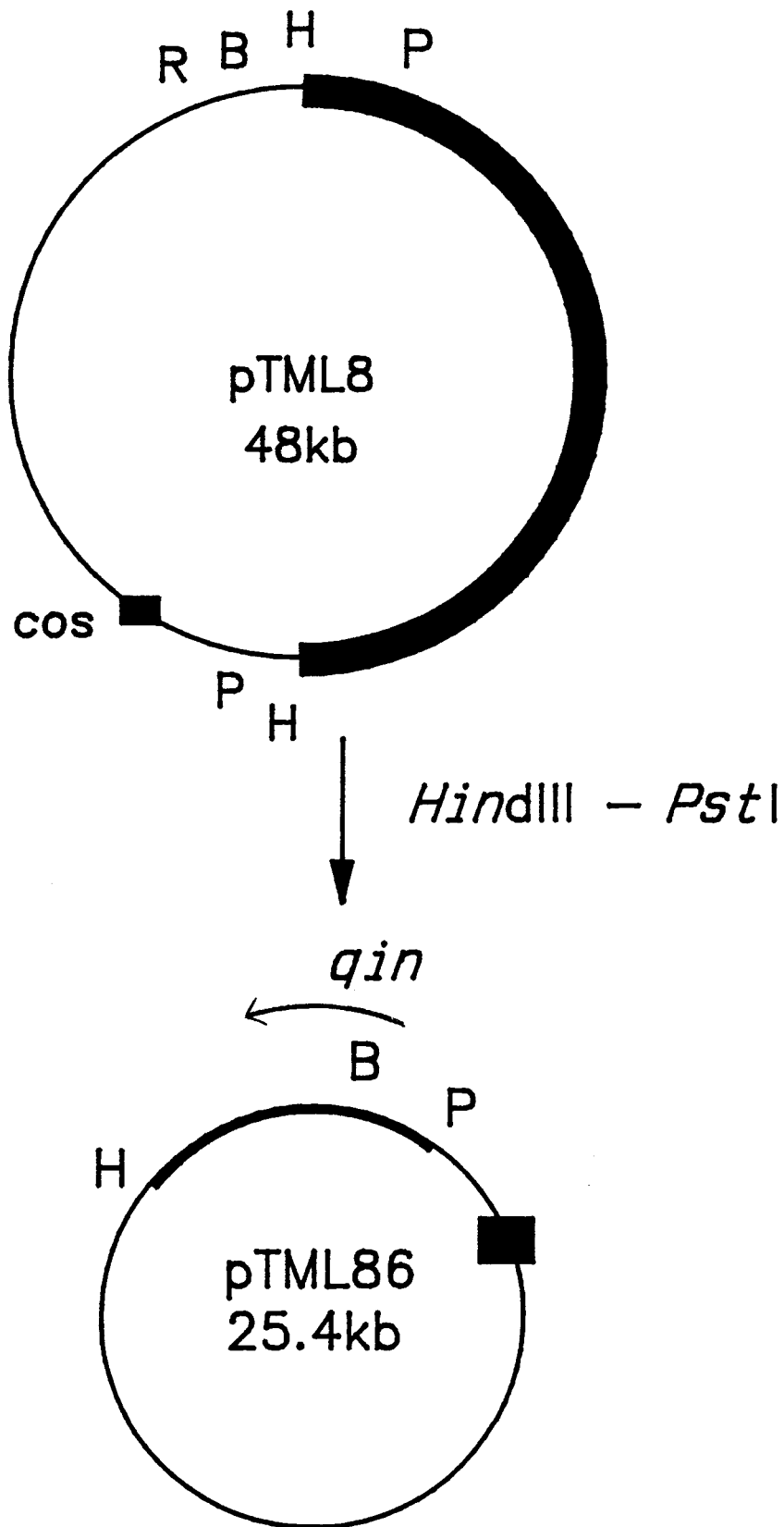


religated and transformed into DH1. Thirteen transformants were obtained and replica plated onto nalidixic acid (50 ug/ml) to screen for the  $\text{Nal}^s$  phenotype. Four of these were found to be  $\text{Nal}^s$  and the other nine were  $\text{Nal}^r$ . Plasmid DNA was prepared by the mini-plasmid preparation technique from all of the transformants. It was discovered that all four  $\text{Nal}^s$  isolates shared a common 2.4 kbp PstI-HindIII inserted fragment. None of the  $\text{Nal}^r$  isolates contained this particular subcloned fragment. It was also noted that all four  $\text{Nal}^s$  subclones had the characteristic translucent colony morphology previously observed in RM1220 cells. One subclone, RM4225, contained a plasmid with a single 2.4 kbp PstI-HindIII fragment. This construct was designated pTML86 and represented the minimal DNA fragment delineating the gin gene (Figure 6).

*Delineation of gin by Tn3 mutagenesis*

The gin-containing DNA fragment was also defined by Tn3-insertional mutagenesis. The rationale for this was that an insertion of the Tn3 element within the gin gene would cause RM1220 and RM4225 to become  $\text{Nal}^r$ . Cells containing insertions outside the gene would retain the  $\text{Nal}^s$  phenotype. Transposon Tn3 was delivered to E. coli DH1 containing either pTML8 or pTML86. The plasmid and the

Figure 6. pTML86 subclone. Subclone pTML86 was derived from pTML8 by restriction with PstI and HindIII followed by religation. pTML86 contains a single 2.4 kbp PstI-HindIII insert and retains the  $\text{Nal}^s$  phenotype originally defined by the pTML8 plasmid. B - BamHI; H - HindIII; P - PstI; R - EcoRI.



Tn3 element were coselected by plating onto L-agar containing tetracycline (12.5 ug/ml) and ampicillin (50 ug/ml) respectively. The resulting Tn3-containing isolates were tested for the  $\text{Nal}^s$  phenotype by replica plating and the transposon insertions were mapped by restriction enzyme analysis.

Twenty-seven RM1220 isolates containing Tn3 insertions were obtained following this procedure and all were found to be  $\text{Nal}^s$ . Plasmid DNA was isolated and digested with BamHI and HindIII to determine the position of the Tn3 insertions. All twenty-seven insertions in pTML8 were shown to lie outside of the 2.2 kbp BamHI-HindIII fragment of gin as defined by the pTML86 subclone (Figure 6). The inability to recover Tn3 insertions within the gin fragment is not unexpected if one considers random insertion into the entire pTML8 plasmid (48 kbp), and the size of the gin fragment examined (2.2 kbp). The Tn3 insertions occurred outside of the 2.2 kbp BamHI-HindIII gin fragment, however, the exact positions were not mapped in pTML8.

Three-hundred RM4225 isolates containing Tn3 insertions within pTML86 were also screened for nalidixic acid sensitivity. Of these, five were  $\text{Nal}^r$ , and a single Tn3 element was mapped to within the PstI-HindIII gin

fragment of pTML86 in each case. Two insertions from Nal<sup>r</sup> isolates and three from Nal<sup>s</sup> isolates were precisely mapped (Figure 7).

*Southern hybridization of the E. coli gyrA gene with pTML8*

Plasmid pMK90 contains the cloned E. coli gyrA gene and was generously provided by Dr. Martin Gellert, NIH, Bethesda, MD. pMK90 contains an 11 kbp BamHI E. coli chromosomal fragment ligated into the unique BamHI site of the cloning vector pKC16 (91). The pKC16 vector contains sequences from the N-cI-O-P region of phage lambda and a fragment of pBR322 (110). Figure 8 depicts the restriction map of pMK90 and the location of the gyrA gene. pMK90 was restricted with the BamHI and HindIII enzymes and run on a 0.7% agarose gel. The DNA was Southern transferred to nitrocellulose and probed with a [ $\alpha$ ]-<sup>32</sup>P-labeled 2.4 kbp HindIII-PstI qin fragment which was isolated by electroelution. Figure 9 shows the EtBr stained agarose gel and the corresponding autoradiograph. Under low stringency conditions, only nonspecific hybridization was observed. This conclusion is based on the detection of autoradiographic bands corresponding not only to gyrA-specific fragments, but also to sequences upstream from the gyrA gene, as well as vector-derived sequences.

Figure 7. Tn<sub>3</sub>-insertional mutagenesis of pTML86. The mapped positions of five single Tn<sub>3</sub> insertions within pTML86 are indicated by arrows. The "S" and "R" indicate the Nal<sup>s</sup> and Nal<sup>r</sup> phenotypes, respectively, associated with each RM4225 Tn<sub>3</sub>-containing isolate. B - BamHI; H - HindIII; P - PstI; Sa - SalI.

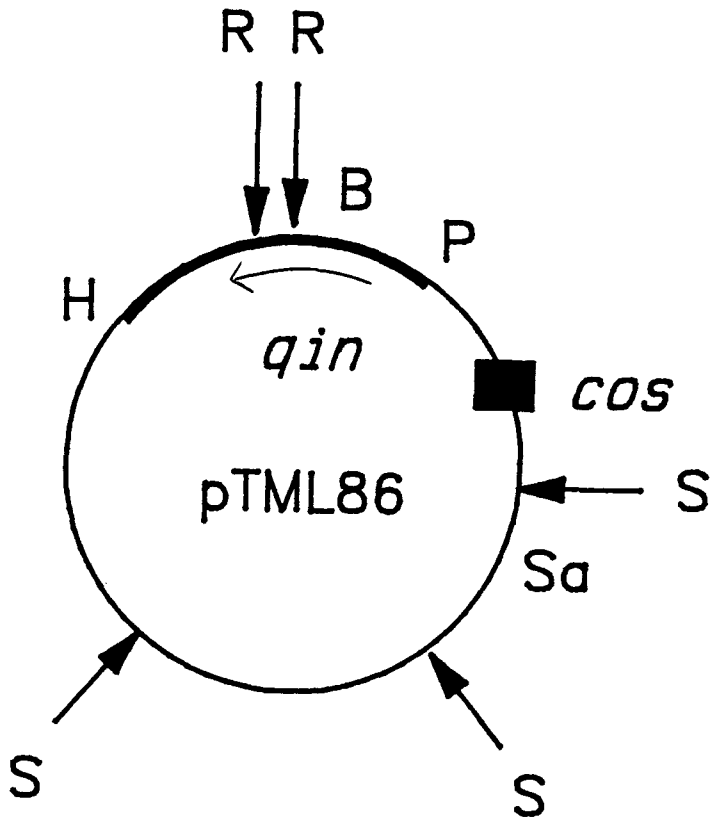


Figure 8. Restriction map of pMK90 (91). The approximate location of the gyrA gene is indicated. Lettered fragments correspond to EtBr-stained bands in Figure 9. Lower-case letters indicate vector derived fragments, upper-case letters represent DNA fragments which correspond to inserted E. coli DNA. Bm - BamHI; H - HindIII.



pMK90

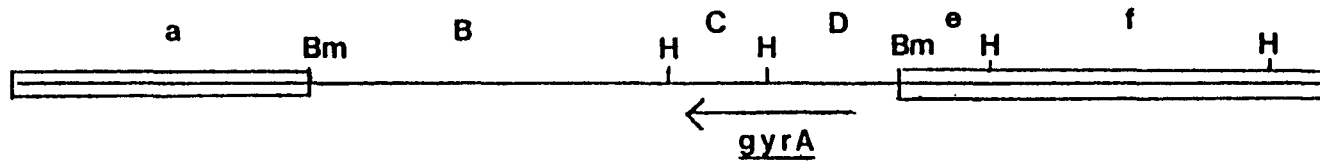
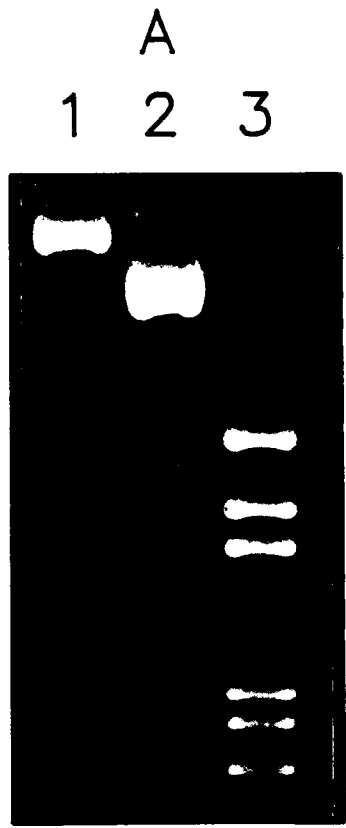


Figure 9. Southern Hybridization - E. coli gyrA probed with the qin gene. A. EtBr stained agarose gel. B. Autoradiograph. Lane 1: pTML86 digested with PstI and HindIII. Lane 2: pMK90 digested with BamHI. Lane 3. pMK90 digested with BamHI and HindIII. A  $^{32}\text{P}$ -labeled PstI-HindIII qin-containing DNA fragment was used to probe the filter under low stringency conditions. Lettered bands correspond to the map of pMK90 shown in Figure 8.



B  
f  
a  
D  
C  
e



B  
f  
C

*Complementation of a P. aeruginosa nalA mutant*

*by the E. coli gyrA gene*

It was of interest to determine whether the E. coli gyrA gene was capable of complementing a P. aeruginosa nalA mutant in a manner similar to qin.

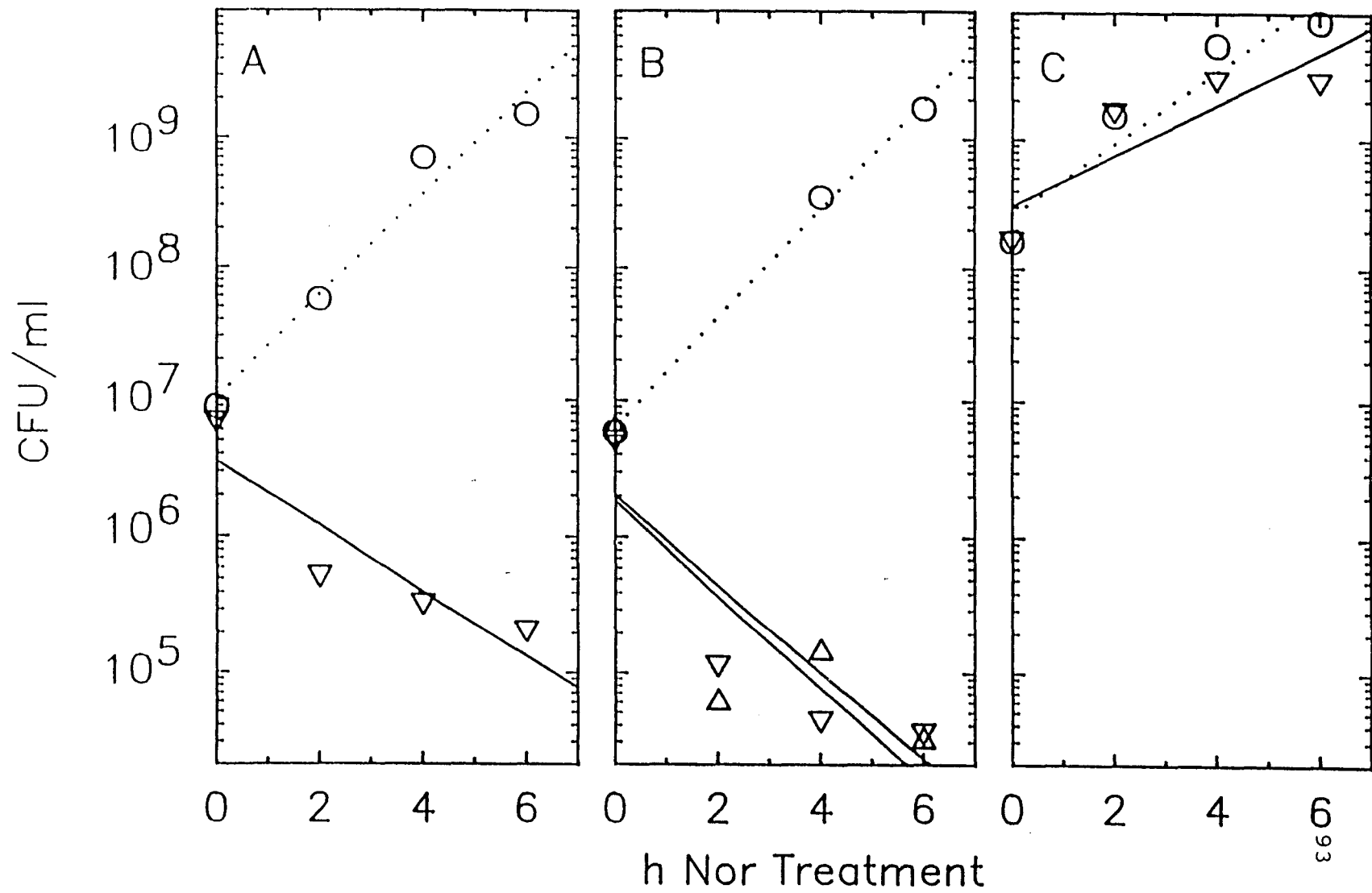
The gyrA gene was subcloned into the pCP13\* vector which can be maintained in P. aeruginosa. pMK90 was BamHI digested and cloned into the unique BamHI site of pCP13\*. E. coli DH1 was transformed with the ligation mixture and Tc<sup>r</sup> transformants were screened for nalidixic acid sensitivity. Three such isolates were found and the correct insertion of the gyrA fragment was confirmed by plasmid isolation and restriction pattern analysis. This construct, designated pTML90, was mobilized from DH1 into P. aeruginosa PA0515 (nalA5) by triparental mating to create RM2197. The ability of pTML90 to complement the nalA5 mutation was assessed by a cell survival experiment in the presence of norfloxacin. The results of this experiment demonstrate that the E. coli gyrA gene can completely complement the nalA5 mutation to the wild-type level of norfloxacin sensitivity (Figure 10). RM2197 did not achieve the level of norfloxacin sensitivity that is characteristic of E. coli, but displayed a level of

Figure 10. Cell survival curve - Complementation of P. aeruginosa nalA by the E. coli gyrA gene. Cultures were grown in the presence of various concentrations of norfloxacin as described in Chapter III. Points represent averages of at least two determinations. Lines were computed by least-square regression analysis. A. PA025 (wild-type) B. RM2197 (nalA5/pTML90) C. PA0515 (nalA5).

○ - 0  $\mu$ g norfloxacin/ml

▽ - 2  $\mu$ g norfloxacin/ml

Δ - 6  $\mu$ g norfloxacin/ml



norfloxacin sensitivity which is characteristic of a wild-type P. aeruginosa strain.

*Complementation of the E. coli gyrA96  
and the P. aeruginosa nalA, nalB,  
and pip mutations by pTML86*

In addition to the E. coli DH1 strain, pTML86 was also introduced into P. aeruginosa nalA, nalB, and pip mutants by triparental mating. The pip-6003 mutation confers resistance to pipemidic acid, a nalidixic acid analog that is active against P. aeruginosa, and maps very close to nalA (111). Plate MICs were determined for two alleles of nalA, three alleles of nalB and one pip allele (Table 7). Although an intermediate level of sensitivity was imparted to the nalA5 mutation of PA0515, pTML86 did not complement the nalA2 mutation of PA0514. The nalB4 (PA06002), nalB8 (PA06005) and pip (PA06004) mutations were also not complemented by pTML86. However, pTML86 does confer an intermediate level of sensitivity to PA06006 which contains the nalB9 mutation. This intermediate sensitivity was demonstrated by the plate MIC method. Cell survival experiments conducted in the presence of norfloxacin showed no difference in norfloxacin sensitivity between PA06006 and RM4280 (results not shown).

TABLE 7  
Complementation of Nor<sup>r</sup> in P. aeruginosa

Strain	Relevant Genotype	Plasmid	Norfloxacin Plate MIC $\mu\text{g/ml}$
PAO25	wild-type	None	2
PAO515	<u>nalA5</u>	None	5
*RM4276		pTML86	3
PAO514	<u>nalA2</u>	None	10
RM4275		pTML86	10
PAO6002	<u>nalB4</u>	None	2
RM4227		pTML86	2
PAO6005	<u>nalB8</u>	None	2
RM4279		pTML86	2
PAO6006	<u>nalB9</u>	None	10
*RM4280		pTML86	6
PAO6004	<u>pip6003</u>	None	0.5
RM4278		pTML86	0.5

\*Indicates strains showing complementation of the norfloxacin resistant (Nor<sup>r</sup>) phenotype by pTML86.



*Integration of gin back into the P. aeruginosa chromosome*

In light of the inconclusive complementation data presented in the previous section which show an intermediate level of sensitivity imparted to both the nalA5 and the nalB9 mutations by pTML86, it became necessary to determine the map position of gin. In order to map the gin gene on the P. aeruginosa chromosome, it was essential to construct a chromosomal co-integrate of gin. This was accomplished by cloning gin into a plasmid which is unable to replicate in P. aeruginosa. Using this approach, a single-crossover event at the position homologous to gin could be identified by selection of the plasmid antibiotic-resistance gene (21). A PstI DNA fragment containing gin, along with some adjacent sequences, was subcloned into the unique PstI site of pBR325. This construct was designated pTML822 and was mobilized from DH1 into PA01 by triparental mating. The pBR325 plasmid is unable to replicate in P. aeruginosa (43), therefore only those cells which had integrated the pTML822 plasmid by homologous recombination at the gin locus would be expected to become Tc<sup>r</sup>. Twenty-two Tc<sup>r</sup> isolates of PA01 were obtained by this protocol. The frequency of integration was very low, approximately 10<sup>-9</sup>

transformant per recipient cell was obtained. Plasmid DNA could not be isolated from these clones indicating that the tet gene was not extrachromosomally located.

*Cotransduction of phe-2 with the  $Tc^r$  gene*

A  $Tc^r$  chromosomal integrate of PA01 (RM4281), was used to determine whether the  $Tc^r$  gene cotransduces with the phe-2 allele. Since it is known that phe-2 is 67% cotransducible with nalA (18), it is reasonable to postulate that if gin is in fact the nalA gene, the  $Tc^r$  gene of pBR325 should frequently cotransduce with the phe-2 prototrophic marker.

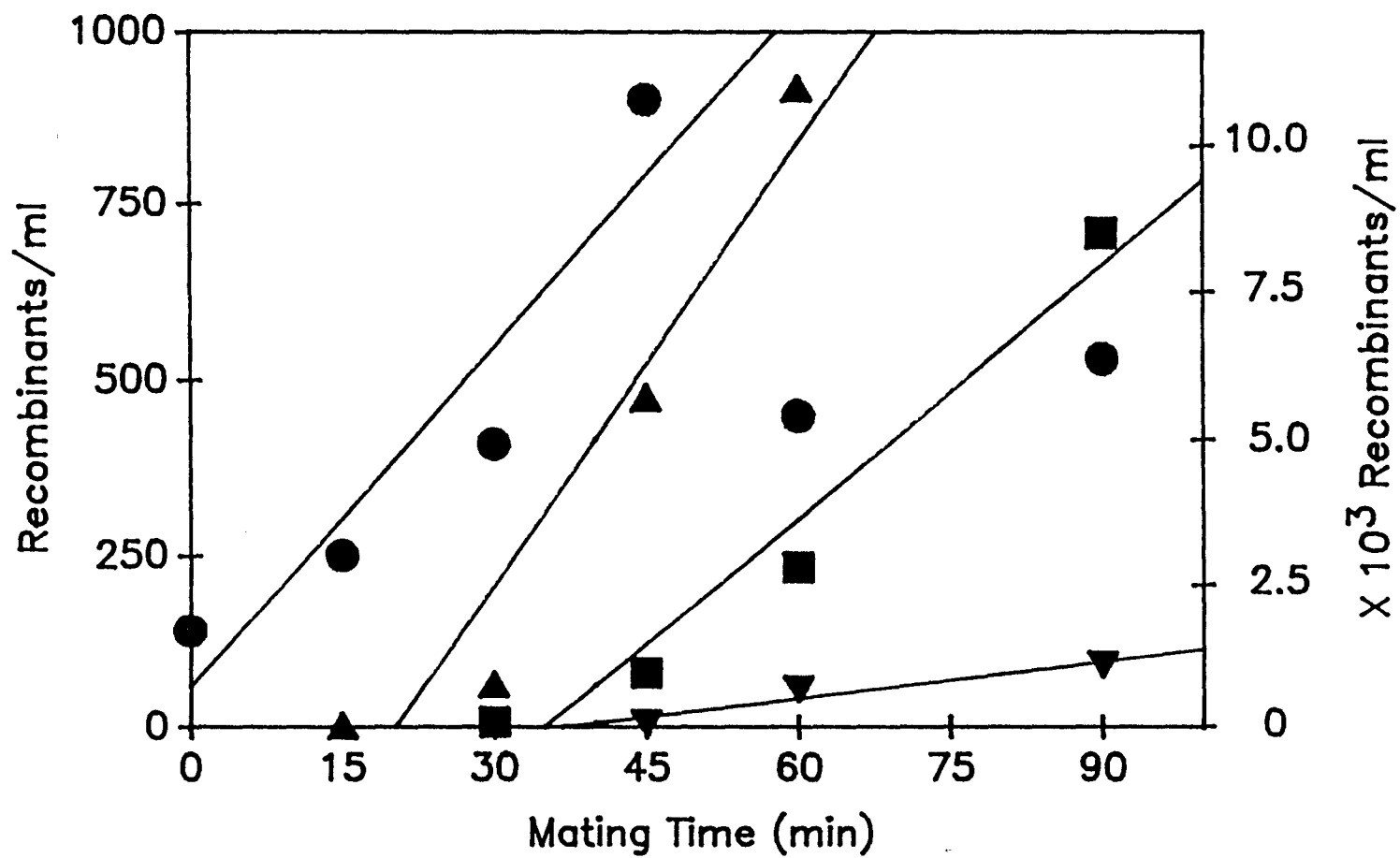
Bacteriophage F116L was grown on RM4281 and a transducing phage lysate was prepared. This lysate was used to transduce PA0660 (phe-2) to phenylalanine prototrophy ( $Phe^+$ ). All of the 254  $Phe^+$  transductants screened were also tetracycline sensitive ( $Tc^s$ ). When tetracycline resistance was used as the primary selection, only nineteen isolates were recovered, and all were  $Phe^-$ . These results showed that the  $Tc^r$  gene does not cotransduce with the phe-2 allele and suggested that gin is in fact not identical to nalA.

*Time of entry conjugations*

The time of entry of the integrated  $Tc^r$  gene was determined using the conjugative plasmid FP5 in an interrupted mating. FP5 was transferred from RM2116 to RM4281 by a liquid mating. RM4281 transconjugates containing FP5 (RM4282) were selected on PMM containing mercury hydrochloride (5 ug/ml) and tetracycline (200 ug/ml) to select for FP5 and the tet gene of pBR325 respectively. RM4282 and JC3600 were mated, and at selected time intervals following the initiation of mating, the conjugation was terminated as described in Materials and Methods. Prototrophic transconjugates of three mutations in JC3600, lys-12, met-28, and trp-C6 (located at 20, 31 and 34 minutes respectively on the P. aeruginosa chromosome) were independently selected and screened for the acquisition of  $Tc^r$ . Streptomycin (750 ug/ml) was included in all the selective medium to select against RM4282. The order and extrapolated time of entry of these markers was  $Tc^r$  (0 min), lys-12 (20 min), met-28 (33 min), and trpC6 (35 min) (Figure 11). These results indicate that gin maps very close to the FP5 origin of transfer.

To verify the map position of gin, plasmid pTML86 was sent to the laboratory of G. Tümmler (Medizinische Hochschule Hannover, Federal Republic of Germany) for

Figure 11. Chromosomal mapping by FP5 conjugation. At various times following the initiation of mating of RM4282 and JC3600, samples were removed from the mating mixture, mating pairs were disrupted, and the cells were plated onto selective media. Recombinants/ml were plotted versus mating time and the line of best fit was drawn. Time of entry of each allele corresponds to the intercept of the line at the x-axis. Recombinants/ml: ●  $Tc^r$ ;  $10^3 \times$  Recombinants/ml: ▲  $Lys^+$ ; ■  $Met^+$ ; ▼  $Trp^+$ .



pulse field gel electrophoresis and Southern hybridization analysis. They demonstrated that qin DNA used as a probe hybridizes to a P. aeruginosa fragment that contains the proB allele. This corresponds to a map position of qin at 0 +/- 2.1 min which flanks the FP2-FP5 origin of transfer on the old map and (57) and 69 +/- 2.6 min on the new map (101) (personal communication). The independent results of time of entry reported in this dissertation and Southern hybridization data (G. Tümmeler, personal communication) confirm that qin is located near the FP5 origin of transfer on the P. aeruginosa chromosome.

*Nucleotide sequence determination of the*

*PstI-HindIII qin containing DNA fragment*

The nucleotide sequence of the entire 2.4 kbp PstI-HindIII DNA fragment defining qin, as previously described, was determined. Both strands of the DNA were analyzed for a 1.5 kbp stretch beginning at the PstI restriction site, only one strand was analyzed for the remainder of the sequence. Plasmids pTML69 and pTML96 represent the PstI-HindIII DNA fragment cloned in inverse orientation in the Bluescript SK and KS vectors respectively. These constructs permitted the determination of the nucleotide sequence of both strands of the DNA.

The PstI-HindIII DNA fragment was found to contain 2,421 nucleotides. Both DNA strands of this sequence were used to search the GenBank and EMBL databases for twenty bacterial genes showing the highest sequence similarity. Neither strand of DNA picked up any similarity to the gyrase genes in these databases. The highest percent identities recognized were to an E. coli sequence containing the 5' region of the tufB gene and genes for tRNA-Threonine (49%) and to the NR1 plasmid encoded mercury resistance operon (47%). The sequences identified from these searches were not considered to display a high enough degree of similarity to be significant. When each DNA strand of the gin sequence was directly compared to the E. coli gyrA nucleotide sequence (allowing the introduction of spaces), 50% overall matches were found, however no specific regions of similarity could be identified. These results demonstrated that the gin sequence of P. aeruginosa and the E. coli gyrA gene are dissimilar, suggesting that gin does not encode the P. aeruginosa DNA gyrase subunit "A" protein.

The gin sequence was translated in all six possible reading frames. Figure 12 represents the open reading frame determined to be the most likely candidate to code for gin. This open reading frame was the largest

Figure 12. Nucleotide sequence of the gin gene. The four possible start codons are bold-faced and the predicted amino acid sequence appears below the nucleotide sequence. The open-reading frame begins at the second GTG codon, which is the most logical translational start site. Stop codons are indicated by stars. Sequences similar to the E. coli Shine-Delgarno box are underlined, and to the P. aeruginosa Shine-Dalgarno box are double-underlined. A transcriptional termination site is indicated by converging arrows followed by a consecutive run of T's which is overlined.





510 520 530 540 550 560  
 CCTTCCTCCGCCCCGAGCGCAGCAGCCGCTCAACCCGATCTACCGCCCCCAGCCCAACGG  
 F L R P Q R S S R S T R S T A P S P T A

570 580 590 600 610 620  
 CGCCACGGTCCCCCTCGCCGCCCTCGCCAGCTAACCGCCACCCGCACGGGGAGCCCCGCT  
 P R S P S P P S P A N R H P H G E P R S

630 640 650 660 670 680  
 CCCCTGGCGGAAAAACCCAGCGAAATCAACAAAAACCCCGCCCCGGGCTTCACCTGCCGC  
 P G G K T Q R N Q Q K P R P G L H L P P

690 700 710 720 730 740  
 CGCGCATCTGGTAAAAAGGCGCCCTGGCCGCAAAAGCCGTCCGAGCGGGCGTCGTATAAT  
 R I W \*

750 760 770 780 790 800  
 GGCATTACCTGAGCTTCCCAAGCTCATGACAGAGGTTTCGATTCCCTTCGCCCGCTCCAAA

810 820 830 840 850 860  
 TCACCCTGCCAAGCCCCTGAAATGCCTAGCGTTTCGGGGGCTTTGCTTTTTGGGGGAAGG  
 ▶▶▶▶▶▶ ◀◀◀◀◀◀

870 880 890 900 910 920  
 AAAGTGTCGAAGAAGTGTCGAGGCGCATTCAGCGCTACATGCTCAGGAGTAGAAAGCCA

930 940 950 960 970 980  
 TCACTCGATTGGCCCCACCAAGCAGTAGCGGATCAATTGCTGCTCAACGCTCTGCTTCTG

990 1000 1010 1020 1030 1040  
 TCGCAAAACCAAGATCACTGCCACCTCGCCTTGCTCTTCGTGGAATTCGTAAAAAACAG

1050 1060 1070 1080 1090 1100  
 ATAGGGGCCGGTATTAAGCTCGCGATAGCTCAGCACCCCCAGAAGACTCGCCTGCTGGCT

1110 1120 1130 1140 1150 1160  
 GACTGGGTAACCTTTAGGTGCAAGTCAAATCTTCTCTTCAATCTCATCCAAAAGGCTCAG

1170 1180 1190 1200 1210 1220  
 TACTGACTGGAGTGCAGCCTGTTACCTTGGAAATGGAGCCAAGTGGTGGACTTGGTCTTC

1230 1240 1250 1260 1270 1280  
GATGCTTTGCTCTGCGGTATCAGTAAAACGAATGACGACCGGGGACATTAAACCTCCTTA

1290 1300 1310 1320 1330 1340  
TTCTGGCTGAGCGAACCTCCTGGAAAGGCGAGCTTTCAAATCATCAACAGAGCGATGCTT

3'  
GCC

identified of all the six possible reading frames. This frame displayed the highest frequency of "G" or "C" in the third position of each codon. This high percentage of GC is characteristic of the codon usage described for P. aeruginosa (145). The overall percentage of GC, including 252 base pairs upstream of the initiation codon, was calculated to be 64%. This is in agreement with an average of 67% GC content determined for a number of P. aeruginosa genes (104). In addition to the ATG possible initiation site located at position 97 as shown in Figure 12, three GTG codons appear at positions -15, 1, and 61. GTG is known to be a less frequent initiator (100). Using only this open reading frame to search for similar nucleotide sequences in the databases, several P. aeruginosa genes were found such as the phosphomannose isomerase gene, exotoxin A gene, and the alginate D gene. However, no significant regions of homology (<50%) exist between gin and these genes.

Upstream of the gin gene is another open reading frame which leads in from the 5' border of the P. aeruginosa DNA insert that is interrupted by the PstI cloning site. This open reading frame terminates at two consecutive stop codons that are superimposed with the first GTG initiation codon, immediately upstream of the second GTG initiation codon (Figure 12). This open reading frame is most likely the 3'

end of a gene directly upstream from qin. The very short intergenic space found between this gene and qin suggests that qin may be part of an operon. The TFASTA program (107) of the University of Wisconsin GCG software was used to search the database with the deduced 47 amino acid sequence of the 3' end of this putative gene. This program translates the nucleotide sequence database in all six reading frames to which the query peptide of interest is compared. The highest score revealed 33% identity and 75% similarity to a 45 amino acid region of the E. coli trpC gene of the trp operon. The next highest score was with the human glucose transport membrane protein which showed 28% identity and 74% similarity to a 39 amino acid region.

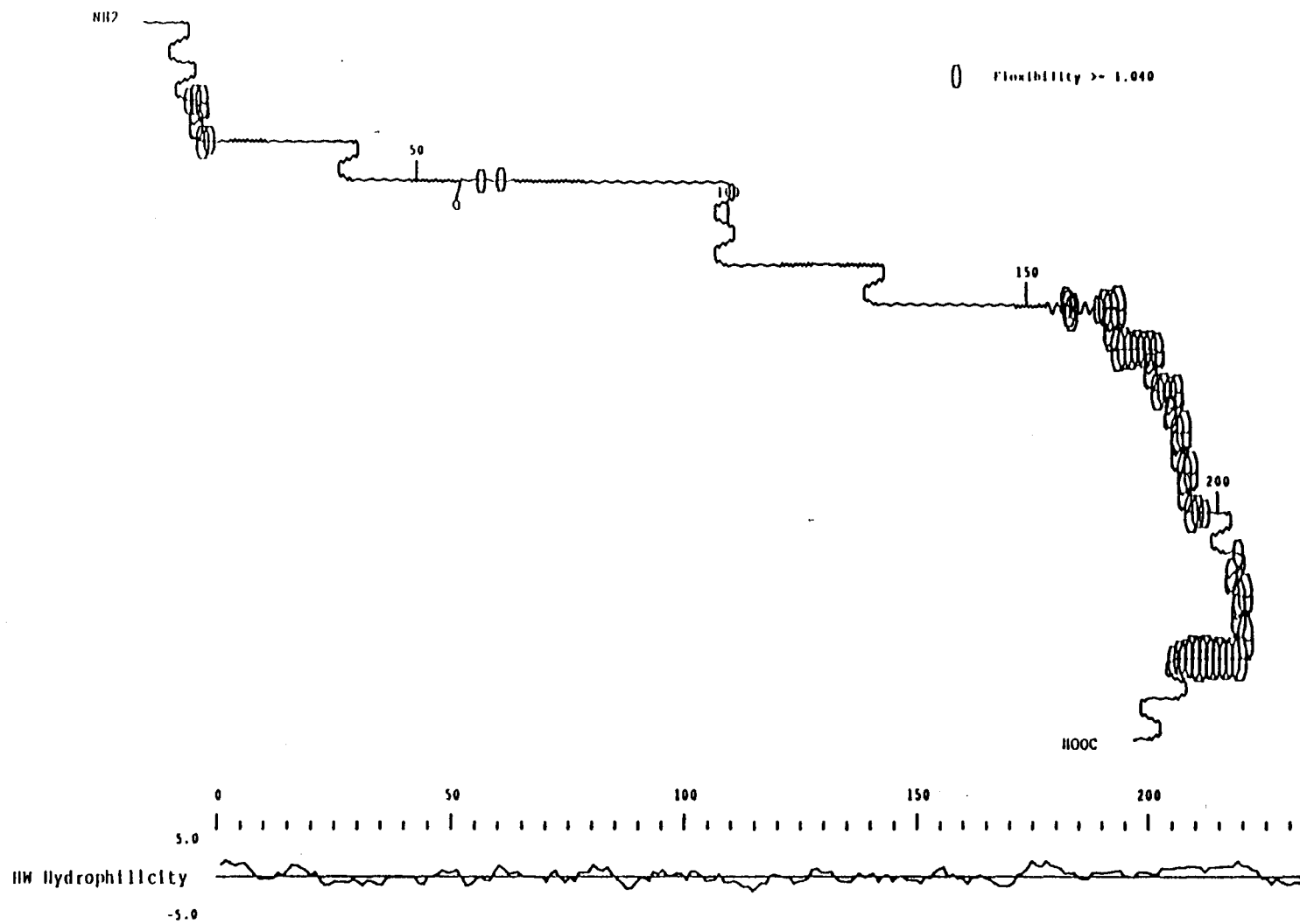
The sizes of the protein translated from each possible initiation codon have molecular weights of 26,771, 26,248, 24,137 and 22,883. The region upstream of the ATG initiation codon was searched for sequence identity with the consensus sequences of the sigma 70 (TTGACAN<sub>17</sub>TATAAT) and sigma 54 (CTGGCACN<sub>5</sub>TTGCA) binding sites (112). Seven putative sigma 70 binding sites were found which showed 79% identity (23/29 total matches; 6/12 not including "N") in this region. A single sequence showing 76% identity (13/17 total matches; 8/12 not including "N") to the sigma 54 consensus sequence was identified. A consensus sequence derived from the

analysis of Pseudomonas recA promoters (B. A. Lanzov, personal communication) (TAGGCCN<sub>17</sub>AATAATA) was also used to search the upstream sequences of qin. Two sequences were identified which displayed 83% total matches (8/13 not including "N"). Since the percentage of homology to these various promoter consensus sequences include several "N" bases, it can be concluded that a distinct promoter region cannot be identified.

Several regions showing some identity with the Shine-Dalgarno consensus sequences of E. coli (AGGAGG) and P. aeruginosa (AGGA or AGGAG) (126) were identified upstream of the possible initiation codons (Figure 12). A putative transcription termination signal was identified downstream of the qin gene located between nucleotides 813-853 which includes two GC-rich inverted repeats separated by 18 nucleotides and followed by several "T" bases (Figure 12). These features are typical termination sequences which are believed to form stem and loop structures capable of slowing down RNA polymerase and facilitating its release (115).

The Chou-Fasman (14) prediction of the Qin protein secondary structure, regions of flexibility and hydrophilicity (61) are illustrated in Figure 13. From these analyses it appears that Qin has a highly hydrophilic and flexible carboxyl terminus. Using the deduced amino acid

Figure 13. Chou-Fasman predicted secondary structure of Qin. The predicted secondary structure (14) of the Qin amino acid sequence translated from the second GTG codon is shown. Regions of flexibility are indicated. The Hopp-Woods (61) hydrophilicity plot appears below the secondary structure prediction. Hydrophilic amino acid regions are plotted above the zero line.



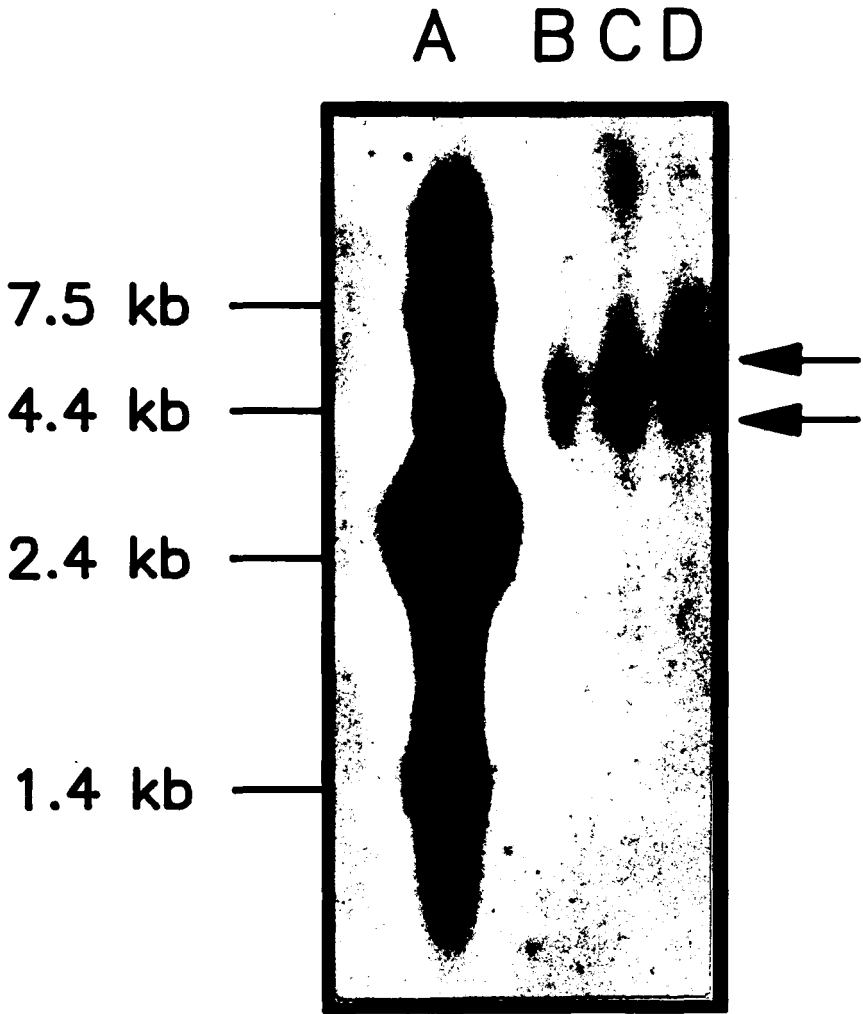


sequence of gin, a homology search of the database revealed similarities to several collagen-like proteins and proline-rich peptides. The TFASTA program (107) of the University of Wisconsin GCG software was used to search the databases with the deduced amino acid sequence of Qin. None of the forty genes selected using the gin amino acid sequence as the query peptide belonged to the topoisomerase family of enzymes.

*Examination of the gin transcript by Northern hybridization*

Total RNA was isolated from strains PA0303 (wild-type), PA0515 (nalA2), and PA06004 (pip-6003), and separated on a formaldehyde-agarose gel. The RNA was Northern transferred to nitrocellulose and probed with the radioactively labeled pTML86 plasmid. Two transcripts were present following autoradiography in all three P. aeruginosa strains examined (Figure 14). The size of the messages appeared to be approximately 4 and 5 kbp. These messages are significantly larger than the size that would be predicted from the open reading frame identified for gin. It is possible that the gin gene is part of an operon. This conclusion is supported by the sequence data which reveal the presence of another gene directly upstream

Figure 14. Northern hybridization. Autoradiograph of total RNA isolated from three strains of P. aeruginosa and probed with  $^{32}\text{P}$ -labeled plasmid pTML86. An RNA ladder is shown in lane A along with the fragment sizes in kb (sequences in the RNA ladder are homologous with the pCP13\* vector). Lane B: PA0303 RNA; Lane C: PA0515 RNA; Lane D: PA06006 RNA. Arrows indicate the presence of two gin transcripts in each lane.



of gin, and the absence of obvious promoter and Shine-Dalgarno sequences.

*Expression of the gin gene product in E. coli mini-cells*

Plasmids pTML86 and pCP13\* were transformed into the E. coli mini-cell producing strain  $\chi$ 1488. Mini-cells were isolated from strains RM4268 and RM4269 and the proteins were labeled with  $^{35}\text{S}$ -methionine. The proteins were separated by polyacrylamide gel electrophoresis and the radioactively labeled proteins were visualized by autoradiography. A protein was consistently identified in mini-cells containing pTML86 which was not produced in mini-cells containing pCP13\* (Figure 15). The molecular mass of this protein was extrapolated from a plot of the migration of the protein standards of known molecular mass and was determined to be approximately 23,000 Daltons (Figure 16).

Figure 15. Expression of qin in E. coli mini-cells. Mini-cells were prepared as described from the mini-cell producing strain  $\chi$ 1488 transformed either with pCP13\* (RM4269) or pTML86 (RM4268). A. Autoradiograph of  $^{35}\text{S}$ -labeled proteins from RM4269. B. Autoradiograph of  $^{35}\text{S}$ -labeled proteins from RM4268. The molecular weights of protein standards are shown. The Qin protein that is present in lane B is indicated at the arrow.

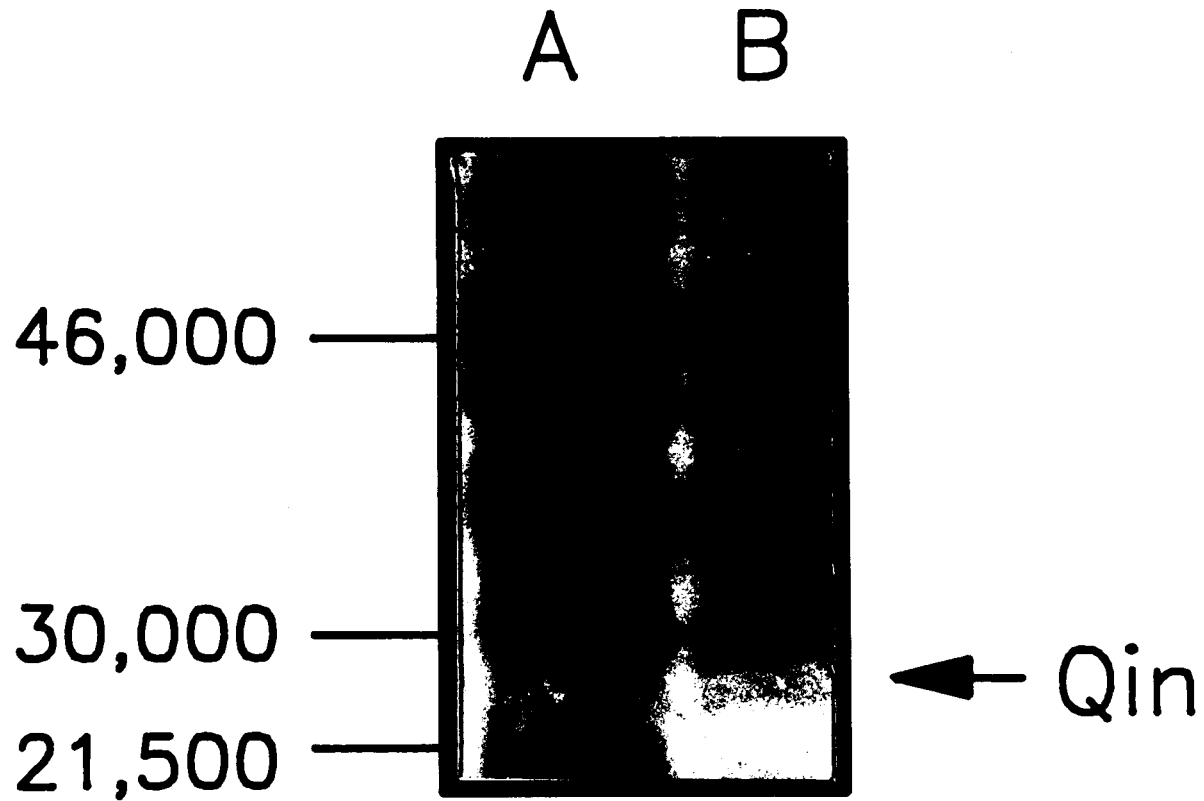
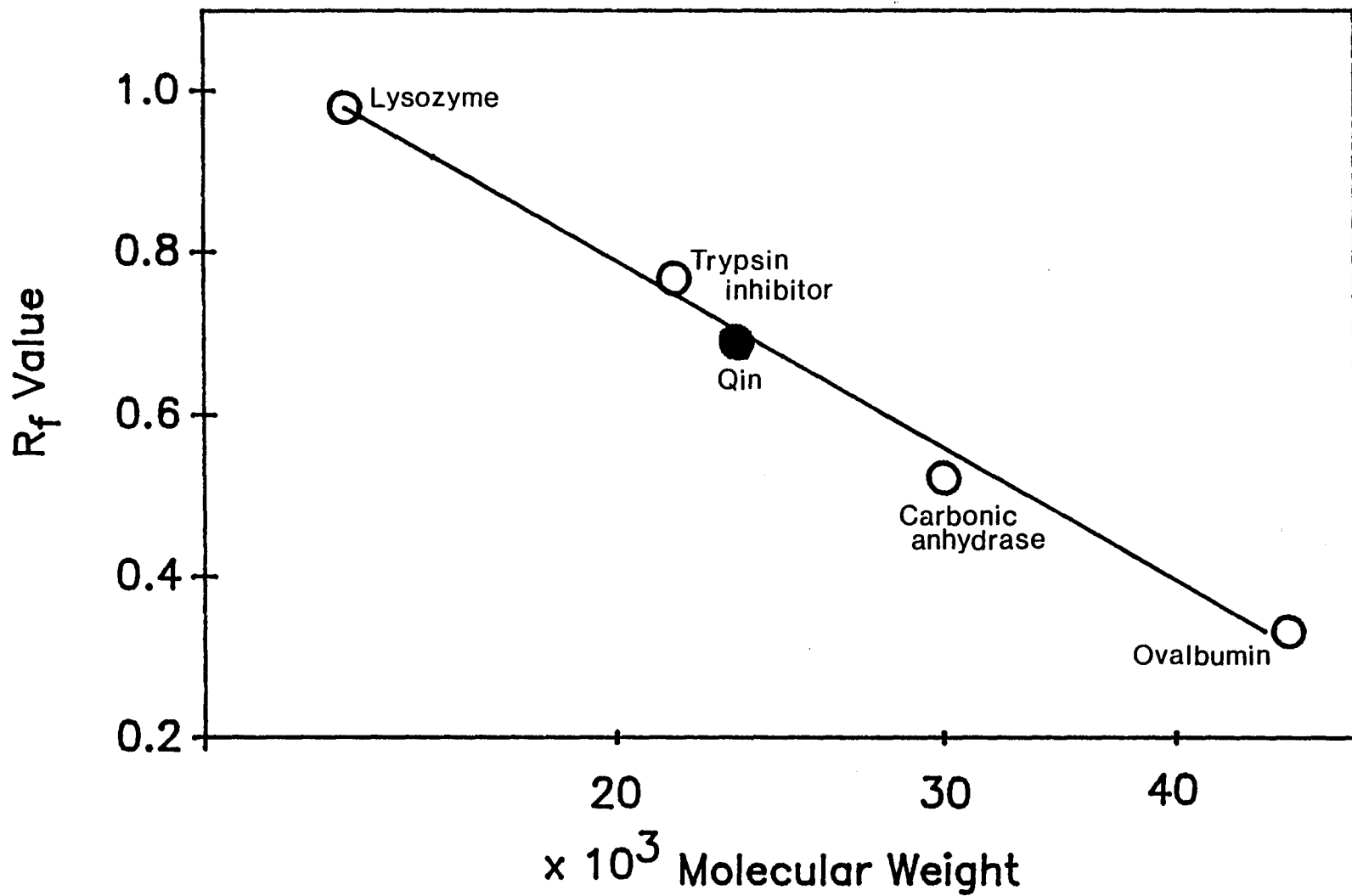


Figure 16. Protein standard molecular weight curve.  $R_f$  values were determined for known protein molecular weight markers that were electrophoresed along with  $^{35}\text{S}$ -labeled mini-cell proteins. The  $R_f$  values were plotted against the log molecular weight of the protein standards. The measured  $R_f$  value of Qin corresponded to a molecular weight of approximately 23,000.





## CHAPTER V

### DISCUSSION

The results presented in this dissertation describe the cloning of a gene from P. aeruginosa which is capable of imparting intermediate quinolone sensitivity to  $Qin^r$  mutants of both E. coli and P. aeruginosa. This gene has been designated qin. It is clear that the qin gene does not encode the "A" subunit of DNA gyrase, but rather represents a separate gene affecting the sensitivity of the cell to quinolone antibiotics. This conclusion was based on both physiological and molecular characterization of the qin gene.

#### *Complementation of $Qin^r$ mutants of E. coli and P. aeruginosa*

The qin gene was isolated by screening a P. aeruginosa chromosomal library for complementation of the gyrA mutation (confers  $Qin^r$ ) of E. coli to the wild-type

(Qin<sup>S</sup>) phenotype. This method of screening was a legitimate approach for the isolation of the gyrA gene since in merodiploids it has been shown that the wild-type phenotype (Qin<sup>S</sup>) is dominant to the gyrA mutant phenotype (Qin<sup>r</sup>) (51). This screen would also select any gene encoding a protein affecting entry of quinolone antibiotics into the cell since drug uptake is partially responsible for quinolone resistance (56,111). The DNA fragment containing the qin gene as originally isolated on plasmid pTML8 was repeatedly selected using this criterion. The qin clone, however, did not completely restore the Qin<sup>S</sup> phenotype to the E. coli wild-type level, but exhibited an intermediate level of quinolone sensitivity at 50 ug nalidixic acid/ml. Although the library was similarly screened in two P. aeruginosa nala genetic backgrounds, the clones that were isolated did not stably display the Qin<sup>S</sup> phenotype, making these clones unlikely gyrA candidates. It is reasonable to speculate that the P. aeruginosa gyrA gene contains one or more HindIII restriction sites making it very difficult to isolate the full length gyrA gene from this library.

Physiological characterization of qin was first explored by measuring the level of quinolone sensitivity imparted to gyrA and nala mutations of E. coli and P.

aeruginosa respectively. Both the gin clone pTML8 and the subclone pTML86 imparted intermediate quinolone sensitivity to E. coli strains carrying the gyrA96 mutation and P. aeruginosa strains carrying the nalA2 mutation (Tables 5, 6, and 7). These results are in contrast to the complete complementation observed by the introduction of the E. coli gyrA gene into the P. aeruginosa nalA genetic background (Figure 11). This inconsistency supported the notion that the gin gene was not a gyrA analog of P. aeruginosa and lead to the development of several hypotheses. However intermediate sensitivity observed in the E. coli gyrA genetic background might be expected considering the known elevated resistance of the P. aeruginosa DNA gyrase "A" subunit (89). Due to this intrinsic high level of resistance, an intermediate level of quinolone sensitivity is a predictable result in E. coli. The intermediate level of sensitivity observed in the P. aeruginosa nalA mutant, however, was unexpected. Two possibilities exist which could explain these observations. (i) The intermediate phenotype is a result of phenotypic mixing with both the wild-type and mutant DNA gyrase "A" subunits exerting activity within the cell. (ii) The P. aeruginosa DNA gyrase enzyme is not similar to the E. coli enzyme therefore the dominant/recessive relationship that is known

for the E. coli DNA gyrase "A" subunit may not be expected to prevail in P. aeruginosa. The only other organism in which the dominant/recessive relationship of the wild-type and resistant gyrase "A" subunits has been tested is in B. subtilis (73). However, due to a gene conversion event, the merodiploid state was unattainable, therefore this phenomenon could not be verified in B. subtilis. While the physiological data are in accord with the hypothesis that gin and gyrA are one and the same gene, they are equally consistent with the theory that gin is not the gyrA gene, but in fact represents a different gene which can affect the sensitivity of P. aeruginosa to the quinolone antibiotics.

Interestingly, gin was capable of restoring intermediate quinolone sensitivity to the nalA5 mutation, but did not alter the  $Qin^r$  phenotype exhibited by the nalA2 mutation. These results indicate that the nalA2 mutation of the DNA gyrase A subunit cannot be alleviated by the gin gene product, while the nalA5 mutation can be compensated at least in part by the gin gene product. Depending upon the nature of the altered protein product, differential interactions of that protein with other proteins and cofactors may be expected. An example of this is the mutations localized to the gyrB gene of E. coli which

affect the interaction of the "A" subunit of DNA gyrase with nalidixic acid (152,153).

The complementation studies also revealed the intermediate restoration of quinolone sensitivity to PAO6005 which contains the nalB9 mutation. Neither the nalB4, nalB8, nor pip mutations were affected in this manner. Clearly, the Qin protein is able to differentiate among these mutations even though all affect the quinolone sensitivity of P. aeruginosa. It is possible that concurrent with the nalB9 mutation, a mutation in the qin gene also occurred. Therefore by providing the qin gene, quinolone sensitivity is partially restored to these mutant cells.

Several experiments were designed to answer the following questions:

1. Does qin map to the nalA locus of the P. aeruginosa chromosome?
2. Is the qin gene of P. aeruginosa an E. coli gyrA analog?
3. What are the physical characteristics of the Qin protein?

*Placement of the qin gene on the P. aeruginosa chromosome*

It was found that qin does not cotransduce with phe2 using F116L. phe2 and nalA have been shown to be 67% cotransducible (18). Therefore, it seems unlikely that qin is analagous to the nalA gene. The precise location of qin on the P. aeruginosa chromosome was then pursued by conjugation experiments. The time of entry as determined by FP5-mediated conjugation placed qin within the first five minutes of the Holloway and Morgan chromosomal genetic map (57), indicating a map location near the origin of FP5 transfer. This position was verified by B. Tümmler (Medizinische Hochschule Hannover, Federal Republic of Germany) by hybridization of the qin fragment to fragments of the P. aeruginosa chromosome using pulse field gel electrophoresis (personal communication).

*Physical examination of the qin gene  
and Qin protein*

Although qin was shown to map to a different chromosomal locus than the nalA allele, the possibility existed that qin represents the gyrB gene or the structural gene encoding a topoisomerase I enzyme, neither of which has been mapped in P. aeruginosa. It was also possible

that gin is a structural gene coding for an additional subunit of DNA gyrase not yet identified. These alternatives were explored.

The structural gene of the DNA gyrase "A" subunit has been cloned and sequenced from two bacterial species, E. coli (91,132) and B. subtilis (72,93). In addition, sequence data are available on the topoisomerase II genes of yeast (79), Drosophila melanogaster (97,151) and humans (136). Sequence similarities have been demonstrated for each of these genes (151). Southern hybridization analysis indicate that the P. aeruginosa gin and the E. coli gyra genes do not display homology under low stringency conditions. To provide information concerning the identity of the gin gene, as well as the exact degree of similarity to known DNA gyrase and topoisomerase II genes, the minimal DNA fragment containing the gin gene was defined and the nucleotide sequence was obtained. Additionally, the apparent molecular weight of the Qin protein observed in E. coli mini-cells was compared to the predicted molecular weight of Qin as deduced from the nucleotide sequence.

Subcloning and Tn<sub>3</sub> mutagenesis demonstrated that a 2.4 kbp PstI-HindIII fragment encompasses the gin gene as was defined by the retention of the Qin<sup>s</sup> phenotype. The nucleotide sequence revealed an open reading frame with

four possible start codons, three GTG codons which can be used as initiators (100) and a single ATG initiator. From the deduced amino acid sequence of this open reading frame, the molecular weight of the translated protein may range from 22,883 to 26,771, depending upon which start codon is used.

The molecular weight range of the Qin protein is much smaller than would be expected for the subunits of DNA gyrase. The P. aeruginosa "A" subunit molecular weight was previously measured at 77,000 (65). Based on the tremendous size differential between Qin and the "A" subunit of DNA gyrase and the different map locations of their respective genes, gin cannot possibly encode the DNA gyrase A subunit. The map position for the gene encoding the "B" subunit of P. aeruginosa DNA gyrase has not been identified, therefore it was possible that the gin gene codes for this protein. However, the physical characteristics of the "B" subunit have been described, and it was reported that the "B" subunit molecular weight is approximately 110,000 (65). Topoisomerase I has not been purified from P. aeruginosa, however the molecular weight of the E. coli enzyme is also 110,000 (139). Therefore it is unlikely that the Qin protein, with a molecular weight of approximately 22,000-26,000, is the "B" subunit of DNA



gyrase or a topoisomerase I enzyme.

To explore the possibility that the gin sequence or a similar sequence had been previously identified, a search of all nucleotide sequences in the GenBank and EMBL databases was carried out. Twenty nucleotide sequences exhibiting the highest degree of sequence similarity were selected. This search did not uncover any of the known DNA gyrase or topoisomerase genes. The sequences which were found did not show any significant sequence similarity to gin. Since it is known that the amino acid sequence of the topoisomerases is more tightly conserved than the nucleotide sequence, the TFASTA program (107) of the University of Wisconsin GCG software was used to search the database with the deduced amino acid sequence of Qin. Once again, none of the proteins found in the search belonged to the topoisomerase family of enzymes.

It can be concluded from these database searches, and the deduced molecular weight of the protein, that Qin is not structurally or functionally related to the topoisomerase class of enzymes.

It is difficult to determine which translational start site is correct because of the absence of an identifiable promoter sequence and Shine-Dalgarno consensus sequence. A protein of a molecular weight of approximately

23,000 was expressed in E. coli mini-cells only when the gin gene fragment was present. From these data, it appears that the likely start codon is either the ATG (coding for a protein of molecular weight 22,883) or the GTG which lies 36 nucleotides upstream of the ATG (codes for a protein of molecular weight 24,137). If one considers that the usual placement of the Shine-Dalgarno sequence is reported to occur within 4 to 7 nucleotides of the translational start site (126), both the first and third GTG codons are likely candidates (Figure 12).

Information obtained from Northern hybridization provided the most likely explanation for the lack of a distinctive promoter and ribosome binding site. These results demonstrated that two gin transcripts are produced in P. aeruginosa. The sizes of these transcripts, approximately 4 and 5 kb, are much larger than would be expected from the largest open reading frame (709 nucleotides in length). Both transcripts were present in the wild-type as well as the nalA, pip, (Figure 14) and nalB (result not shown) mutants, and may be the result of transcription from alternative promoters. The large size of the transcripts, and the presence of an open reading frame directly upstream of gin, are data consistent with gin belonging to part of an operon. It is likely that gin

represents the last gene of the operon since a typical transcription termination signal was identified at the 3' end of the gene. Therefore, expression of qin from pTML8 and pTML86 may be driven from an external promoter located on the pCP13\* vector. It is also possible that a cryptic promoter exists upstream of qin, as was discussed in the results, which can weakly interact with RNA polymerase. This would explain the sparse amount of protein expressed in E. coli mini-cells (Figure 15).

*Speculations regarding the identity and function  
of the Qin protein*

Quinolone resistance in several bacteria has been shown to be due to either an alteration of the DNA gyrase "A" subunit or the permeability of the membrane. The data in this dissertation indicate that the qin gene product causes certain quinolone-resistant mutants to become more sensitive to the drug. qin does not represent the gyrA gene of P. aeruginosa as was discussed above, nor does qin appear to encode any known topoisomerases.

The alternative hypothesis that the Qin protein resides in the bacterial outer membrane where it may regulate the entry of quinolone antibiotics is worth

considering. The hallmark feature of proteins transported to or through membranes is the presence of a signal sequence located at the amino terminus of the protein (8,144). This sequence is usually 20-40 amino acids in length and is cleaved off by a signal peptidase as the protein is translocated through the membrane. The most common elements of the signal sequence are (144) (i) a charged amino acid residue within the first five residues; (ii) a core of at least eight hydrophobic and neutral residues; (iii) a helix-breaking residue such as proline or glycine or a large polar amino acid, such as glutamine, 5-8 residues preceding the cleavage site; (iv) a cleavage site consisting of amino acids with small uncharged side chains. The amino termini beginning from each putative start codon of the Qin deduced amino acid sequence was examined for the presence of a signal sequence consensus (Figure 17). This analysis reveals that the amino acid sequence translated from the second GTG start codon exhibits most of the features of a typical signal peptide. As depicted in Figure 17, several P. aeruginosa protein signal sequences deviate to some extent from the above characteristics. For example, the P. aeruginosa PAK pilin signal sequence is only six amino acids long and lacks the characteristic hydrophobic core. It is thought that the hydrophobic nature

Figure 17. Putative signal sequences of gin. The amino acid sequence from each possible start codon of gin are shown. Sequences 1-4 are ordered in the occurrence of each putative initiation codon from the 5' end of the gin gene. Amino acid sequences 5-6 represent selected P. aeruginosa signal sequences. These sequences are from: 5 - outer membrane porin protein F (28); 6 - outer membrane protein H1 (4); 7 - cytochrome *C<sub>551</sub>* (98); 8 - PAK pilin (106). Positively charged amino acids located within the first five N-terminal residues are underlined. Stretches of at least three hydrophobic and neutral amino acids are bold-faced. Glutamines, glycines and prolines are starred. Known cleavage sites are indicated by a space, putative cleavage sites are indicated by an arrow.

A = ala; C = cys; D = asp; E = glu; F = phe; G = gly; H = his; I = ile; K = lys; L = leu; M = met; N = asn; P = pro; Q = gln; R = arg; S = ser; T = thr; V = val; W = trp; Y = tyr.



of the N-terminal region of the mature pilin protein may substitute for the missing hydrophobic core of the signal sequence (106). Based on the presence of a putative signal sequence located at the amino terminus of the Qin protein, it is conceivable that this protein is targeted to the membrane.

The predicted molecular weight of Qin beginning translation from the second GTG initiator, which encodes the amino acid sequence that most closely resembles a signal sequence, is 26,248. The size of the Qin protein as determined by expression in E. coli mini-cells is approximately 23,000 Daltons. The size of the mature protein would be expected to be smaller than the predicted molecular weight assuming the removal of an amino terminal signal sequence. This particular GTG start codon is directly downstream from the double stop codon which terminates the open reading frame preceding qin. Therefore, this is the logical translational start site if one considers that qin is likely a part of an operon. Based on the above data, it was concluded that the second GTG codon is the most reasonable start codon as is depicted in Figure 12.

As was discussed in the literature review, several quinolone-resistant mutants of E. coli, P. aeruginosa, S.

marcescens, and K. pneumoniae were found to have mutations affecting outer membrane components (56). Most of these mutants lack key outer membrane proteins such as the OmpF porin protein of E. coli (6). This porin forms a channel which provides the means of entry for many antimicrobial agents. Therefore these mutants are often cross-resistant to beta-lactams, tetracycline and chloramphenicol. In contrast, however, several quinolone-resistant mutants of P. aeruginosa are associated with the appearance of additional proteins in the outer membrane as opposed to the loss of membrane proteins. The nalB mutant of PA06006 and the nfxB mutant of KH413E were found to have acquired new outer membrane proteins of apparent molecular weights of 49,000 and 54,000 respectively (56). The nfxB mutation maps between proB and ilvBC which flanks the FP5 origin of transfer, while the nalB locus is at 32 min on the P. aeruginosa chromosome. In addition, a protein of molecular weight of approximately 25,000 is apparently decreased in both the nalB and nfxB mutants as compared to the wild-type parental strains. An outer membrane protein of the same molecular weight identified as protein G by a monoclonal antibody was also reported to be reduced in quinolone-resistant clinical isolates of P. aeruginosa (13). The diminution of a 31,000-32,000 Dalton outer membrane protein



was noted in three ciprofloxacin-resistant clinical isolates of P. aeruginosa (19). Clearly both the appearance of new outer membrane proteins and the disappearance of known outer membrane proteins are important resistance mechanisms to quinolones in P. aeruginosa.

The nfxB mutation maps to the same chromosomal region as does gin, near the origin of FP5-mediated transfer (56). It is also interesting that a protein of the same size as that of Qin is decreased in both the quinolone-resistant mutants, nfxB and nalB. It is not known whether gin is capable of complementing the nfxB mutation, however complementation was shown for the nalB9 allele (Table 7) the same mutant shown to be depleted of a 25,000 Dalton protein. It is tempting to speculate that the gin gene resides at the nfxB locus. This question can be addressed by a Western blot experiment in which the antibody to the 25,000 Dalton outer membrane G protein is reacted with the gin protein. If gin does encode the G protein, this may explain the instability of the  $\text{Nal}^S$  phenotype in RM1220 cells observed during cell survival curve experiments. Following several generations of growth in liquid culture, nutrient availability may be changing. Since it is known that the expression of many outer

membrane proteins is affected by alterations in nutrient conditions (121,156), the expression of qin may have been decreased in response to slight changes in the nutrient supply following several generations of growth.

From the information known at this time about the Qin protein, the most reasonable function is as an outer membrane porin protein, affecting the entry of quinolone antibiotics into the cell. It is equally possible that Qin is a component of an efflux system for quinolone drugs, similar to the active efflux recently reported for norfloxacin in susceptible E. coli cells (16). This effluxer is an energy-dependent system that is located at the inner bacterial membrane and is specific for several fluoroquinolones. It is postulated that decreased outer membrane permeability in conjunction with active efflux across the inner membrane, result in lowered drug susceptibility (60). Specific proteins of this efflux system have not yet been isolated.

Determining the function of the Qin gene product was not facilitated by searching the various databases for similarities to known sequences. The only consistent theme found in searching the protein database was the similarity to several collagen-like proteins and proline-rich peptides. Therefore, it is possible that qin may mediate

quinolone sensitivity of P. aeruginosa by mechanisms other than drug entry. Although there is no evidence of a protein which regulates DNA gyrase activity or expression, it is possible that gin may function in this capacity. As to how the intermediate phenotype shown to be associated with gin can be reconciled with a regulatory function is unknown at this time.

The incomplete complementation of the nalA as well as the nalB9 mutations suggest that the many quinolone-resistant mutants may have acquired multiple membrane and/or DNA gyrase mutations. Therefore in these various mutants, the gin gene is capable of partially restoring quinolone sensitivity to only those mutants suffering a mutation in the gin locus. It is possible that single mutations in gin have not been isolated because such a mutation may only attenuate the level of quinolone resistance, and mutants do not survive selective conditions. Alternatively, the Qin protein may facilitate the entry of quinolone drugs into the cell and consequently can overcome the resistance of the mutant gyrase "A" subunit simply by increasing the concentration of drug.

### *Conclusions*

The data presented in this dissertation indicate that the gin gene encodes an outer membrane protein of a mature molecular weight of approximately 23,000 which effects the entry of quinolone antibiotics into the cell. The results suggest gin is the last gene in an operon and maps very close to the FP5 origin of transfer on the P. aeruginosa chromosomal genetic map. It has been demonstrated that the gin gene can partially restore quinolone sensitivity to the E. coli gyrA96 mutation and the P. aeruginosa nalA5 and nalB9 mutations, all of which confer quinolone resistance to the cell.

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APPROVAL SHEET

The dissertation submitted by Debra A. Tonetti has been read and approved by the following committee:

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

12 April, 1990  
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