CHAPTER III

LITERATURE REVIEW

Overview on the genus *Vibrio*

The genus *Vibrio* is classified in the facultative anaerobic gram negative rod-group, which comprise with the family *Enterobacteraceae*, *Vibrionaceae* and *Pseudomonaceae*. It belongs to the family *Vibrionaceae*, which also includes the genera *Aeromonas*, *Plesiomonas* and *Photobacterium*, as the family, itself, was initially proposed by Veron in 1965.The genus *Vibrio* was originally described in 1854 by Pacini, who described an organism associated with Asiatic cholera. He named the organism *Vibrio cholera*, subsequently changed to *Vibrio cholerae*, which is now the type species of the genus. The genus *Vibrio* has historically included other curved, gram negative bacilli such as the microaerophillic species, which are now classified in the genus *Campylobacter*. A number of marine species previously assigned to the genus *Beneckea* have recently been assigned to the genus *Vibrio* in a reevaluation of the taxonomy of this group (Baumann et al., 1980). This included the reassignment of the pathogenic species originally described as *Beneckea vulnifica* to the genus *Vibrio* as *Vibrio vulnificus*. Members of the genus *Vibrio* are commonly found in marine and estuarine environments both in water and associated with marine animals. It is composed of straight or

curve gram-negative bacilli 0.5 to 0.8 um in diameter and 1.4 to 2.6 um in length. All species in the genus are motile, usually by a single polar flagellum when grow in liquid media. In old cultures or under adverse all morphology becomes very irregular or coccoid. *Vibrio* species are facultative anaerobes capable of both fermentative and respiratory metabolism. Growth of all species is stimulated by Na^+ and is an absolute requirement of most. Most species are oxidase-positive. All species grow at 20^0C, while those species that are pathogenic to humans will grow at 40^0C (Tison et al., 1984). Sensitivity to the vibriostatic compound 0/129 (2-4- diamino 6,7-diisopropylpteridine) varies but most strains are sensitive to 0/129 vibriostatic compound. The DNA content of *Vibrio* species ranges from 38 to 51 mol% guanine + cytosine (%G+C).

The genus *Vibrio* is composed of 34 Species, 12 of these are human pathogens or have been isolated from human clinical specimens (Table1). Species most commonly associated with cases of gastroenteritis include *Vibrio cholerae* and *Vibrio parahaemolyticus*, *Vibrio fluvialis*, *Vibrio. furnissii*, *Vibrio hollisae* and *Vibrio mimicus*. *Vibrio vulnificus* is an agent of septicemia and wound infection associated with consumption of raw seafood or exposure to seawater. *Vibrio alginolyticus* and *Vibrio damsela* cause soft tissue infection. *Vibrio metschnikovii* has been implicated in a case of cholecytitis with bacterimia. *Vibrio cincinnatiensis* was isolated from the blood and celebrospinal fluid of a patient with meningitis, and *Vibrio carchariae* was isolated from the bite wound of a child following a shark attack.

Table 1 : Disease caused by pathogenic *Vibrio* species . (Pavia et al.,1989 and Kelly et al., 1991)

Organism	Primary disease	Other diseases
V. cholera O1	Cholera	Wound infections
V. cholera non-O1	Gastroenteritis	Wound infections
V. mimicus	Gastroenteritis	Wound infections
V. parahaemolyticus	Gastroenteritis	Wound infections and eye infections
V. fluvialis	Gastroenteritis	None reported
V. furnissii	Gastroenteritis	None reported
V. hollisae	Gastroenteritis and septicemia	None reported
V. vulnificus	Septicemia or wound infections	None reported
V. alginolyticus	Wound infections and septicemia	External otitis
V. damsela	Wound infections and septicemia	None reported
V. metschnikovii	Cholecystitis with septicemia	None reported
V. cincinnatiensis	Septicemia	None reported
V. carchariae	Wound infections	None reported

Initial separation of members of the genus *Vibrio* from other groups of medically important bacteria in the family *Enterobacteraceae* and *Pseudomonaceae* can be established by demonstration of fermentative metabolism, indophenol oxidase production, requirement for or growth stimulation by NaCl and susceptibility to 0/129 (Table2).

Table 2 : Some major characteristics of the families *Enterobacteraceae*, *Vibrionaceae*, and *Pseudomonaceae*. (Tison et al., 1984)

Characteristics	*Enterobacteraceae*	*Vibrionacecae*	*Pseudomonaceae*
Typical genus	*Escherichia*	*Vibrio*	*Pseudomonas*
Fermentative metabolism	+	+	-
Oxidase production	-	+	+
Growth stimulated By NaCl	-	+	-
Sensitivity to 0/129 vibrio static compound	-	+	-

Symbol : +, most strains positive (generally about 90%)

Symbol : -, most strains negative (generally about 0 to 10 positive)

Fermentative metabolism distinguishes members of the genus *Vibrio* from oxidase-positive *Psuedomonas* species. The production of oxidase and/or the salt requirement of *Vibrio* species differentiate them from members

of the *Enterobacteriaceae*. Sensitivity to the vibriostatic compound 0/129 is also useful in identificating members of the genus *Vibrio Aeromonas* and *Plesiomonas* species vary in sensitivity to 0/129 and neither genera requires NaCl for growth (Table 3).

Table3 : Chracteristics generally useful in differentiating the pathogenic *Vibrio* species from pathogenic *Aeromonas* and *Plesiomonas*. (Farmer et al., 1985)

Characteristics	*Vibrio*	*Aeromonas*	*Plesiomonas*
Growth stimulated by NaCl	+	-	-
Sensitivity to 0/129 Vibrio static compound	+	-	V [d]
Arginine dihydrolase	- [a]	+	+
Lysine decarboxylase	+ [b]	-	+
Gelatinase	+ [c]	+	-

Symbol : +, most strains positive (generally about 90%)

Symbol : -, most strains negative (generally about 0 to 10 positive)

[a] *Except V. fluvialis, V. furnissii, and V. damsela.*

[b] *Except V. hollisae and those species in footnote a.*

[c] *Except V. hollisae and V. damsela.*

[d] *Variable.*

Review on *Vibrio parahaemolyticus*

1). History

Vibrio parahaemolyticus is one of 12 members of genus *Vibrio* which have been associated with human disease. It was first recognized in 1950 as a cause of food poisoning outbreak in Japan that had 272 individuals involving, of whom 20 died (Joseph et al., 1983). After the properties of this organism were studied, it was firstly named as a new bacteria species, *Pasteurella parahaemolytica.* But it was reclassified later as a halophilic Vibrio species, *Vibrio parahaemolyticus* when the halophilic nature was discovered in 1955 (Miwatani et al., 1976).

2). Ecology and Epidermiology

Vibrio parahaemolyticus is a common inhabitant of coastal water and estuaries in tropical and temperate regions (Sakazaki et al., 1968). It has been isolated from sea water, sea mud, or seafood in Asia, North America, Australia, New Zealand, Africa, Hawaii and Europe. It has also been isolated from fresh water and fresh water fish in India (De et al., 1977). It absorbs onto chitin and onto copepod (minute crustacean animals) and most of the organisms in water are associated with zooplankton. The organism is present in sediment in the winter but increases in numbers and appears in water

column by reattaching to zooplankton as the water temperature rises (Blake et al., 1980).

Occuring of food-borne outbreak and sporadic cases by *Vibrio parahaemolyticus* are worldwide (Kaneko et al., 1973). Although it is not a usual cause of diarrhea in North America, it is a common cause of food-borne gastroenteritis in other countries (Kaneko et al., 1975). In Japan, *Vibrio parahaemolyticus* causes 50 to 70% of food-borne enteritis cases and even deaths were recorded (Farmer et al., 1985). The infections by this organism in all outbreaks are associated directly or indirectly with seafood. Direct infection results from ingestion of raw seafood contaminated with *Vibrio parahaemolyticus*, while indirect infection results from ingestion of *Vibrio parahaemolyticus* - recontaminating - cooked food (Kelly et al., 1995). *Vibrio parahaemolyticus* also causes extraintesternal infection such as ear and eye infection, wound infection and septicemia. All of the infections were acquired after exposure to marine environments harboring the organism (Tison et al., 1984).

3). Clinical manifestation and Pathogenicity

The symptoms of *Vibrio parahaemolyticus* gastroenteritis commonly include watery and sometimes bloody diarrhea, mild to moderately severe abdominal cramping pain, nausea, vomiting, headache, low-grade fever, and drills with an incubation period from 4 to 96 hours. The illness is usually mild and self-limited with a median duration of 3 days but severe or even fatal illness may also occur (Farmer et al., 1985).

Although the pathogenicity of *Vibrio parahaemolyticus* has not been established (Doyle M.P., 1990). The pathogenicity of this organism has been proposed that relating to several virulence factors or properties which are a heat stable hemolysin, other cellular or cell-associated toxin or enzyme, adherence and invasiveness (Nishibushi et al., 1992). Of these potential virulence factors, a thermostable direct hemolysin (TDH) has been considered an important virulence factor and most extensively studied (Joseph et al., 1983), at least four types of hemolysin in TDH family have been described (Honda et al., 1991).

The thermostable direct hemolysin is a homodimer protein with the molecular weight of 46,000 (Tang et al., 1994). It was named on the basis of its characteristic which is not inactivated by heating 100 ^{0}C for 10 min and the hemolytic activity was not enhanced by the addition of lecithin, indicating a direct action on erythrocytes (Sukurai et al., 1973). The biological activities of TDH include hemolysis of various species of erythrocytes, cytotoxicity, lethal toxicity for small experimental animals, stimulation of fiuid accumulation in the rabbit ileal loop (RIL), and production of vascular permeability in rabbit skin (Ljungh and Wadstrom, 1983).

There is a strong correlation between the thermostable direct hemolysin and Kanagawa Phenomenon (KP), which is beta-hemolysis on a high salt-manitol agar containing human erythrocyte, Wagutsuma agar. This association based on the observation that over 95% of clinical isolates are kanagawa–positive as compared with 1% or less of environmental isolates (Miyamoto et al., 1969).

4). Collection, Transport and Storage

A: Collection

1) Clinical specimens

1.1) Extraintestinal specimens (Farmer et al.,1985)

Specimens from extraintestinal pathological material such as wound, blood, and other body fluids and sites should be collected and processed by routine procedure due to there is no special procedures for *Vibrios*. Pus, body fluids, or tissues rather than swabs should be submitted for culture wherever possible. When swabs must be used, they should be transported to the laboratory in suitable holding media such as Cary-Blair transport medium to avoid desiccation.

1.2) Stool specimens (Farmer et al., 1985)

Stool specimens should be collected in the acute stage of illness, preferably within the first 24 h of illness, and before the patient has received any antimicrobial agents. Fluid stool may be collected by inserting a petrolatum-lubricated soft rubber catheter into rectum. Rectal swabs may also be used, but care must be exercised in collection of rectal swab specimens to

ensure that mucus inside the rectal vault, not merely the anal surface, is sampled. Although highly efficient in the acute phase of illness, rectal swabs probably are less satisfactory for convalescent patients or transiently infected asymtomatic persons. Vomitus, if available, may also be collected for culture.

2) Environmental samples

A different technique of collection is needed for each type of sample. Samples to be examined include water, sewage, sediment, plankton, fish, and shellfish. Since the concentration of *Vibrios* may be low in water and sewage samples, some type of concentration procedure is generally required. Filtration through gauze or a membrane filter, or use of a Moore swab, has been successfully by various investigators (Berrett et al., 1980 and Spira et al., 1981). Shellfish samples not examined immediately should be held refrigerated as living –shellstock (Janda et. al., 1988).

B: Transport and storage

All kinds of specimens should be inoculated on isolation plates with minimal delay. When there will be a delay in plating, a specimen should be placed in Cary-Blair transport medium, which maintain viability of the cultures for up to 4 weeks. Buffered glycerol saline,

often used in enteric bacteriology, is an unsatisfactory because glycerol is toxic to *Vibrios*. Specimens in transport medium should be shipped to the laboratory without refrigeration (Tison et al., 1984). In the absence of available suitable transport media, strips of blotting paper may be soaked in liquid stool and inserted into airtight plastic bags. Specimens collected in this way may remain viable for up to 5 weeks. For environmental sample storage, samples may be kept at cold temperatures when transport media are not available and transportation is delayed because the changes of overall microbial are minimized at cold temperature (Janda et al., 1988).

5). Culture and Isolation

Extraintestinal specimens are usually processed with no particular attention to *Vibrios*. However, when clinical history indicates that there has been exposure to seawater or seafood, vibrios should be considered (Farmer et al., 1985). Direct microscopic examination of stool specimen is not recommended since it may not be possible to distinguish *Vibrio* sp. from other motile, straight, or curved rod shaped bacteria . For environmental samples, direct isolation techniques have been most successfully (Janda et. al., 1988). Reliance on blood or MacConkey agar for detection of *Vibrio parahaemolyticus* from stool specimens may be sufficient for many laboratories in land location. In coastal regions, or when vibrios infection is suspected, addition of a TCBS plate to the routine battery may be indicated (Kelly et al., 1995). Alkaline peptone broth (1% peptone, 1% NaCl , pH 8.5)

can be used for increasing the yield of *Vibrio parahaemolyticus* from stool specimens and environmental samples. After inoculation, alkaline peptone broth should be incubated 6-8 hr at 35 ^{0}C and then subcultured to a nonselective medium (blood or gelatin agar), MacConkey agar, and TCBS agar (World Heath Organization Scientific Working Group). Oxidase testing of colonies on a nonselective medium such as blood agar can also provide a high isolation rate. To perform such a test, the suspect colonies should be transferred from TCBS or MacConkey agar to a nonselective medium. Since an acidic medium will cause a false-negative reaction (Tison and Kelly, 1984).

6). Laboratory Identification

Gram-stained smear of broth cultures inoculated with clinical specimens often reveal pleomorphic gram-negative bacilli, including typical curved forms as well as straight rods and large bulbous element. After 18-24 h incubation at 37 ^{0}C, colonies on sheep blood agar are generally nonhemolytic, white, circular, convex, entire colonies, 2 to 4 mm in diameter. The organism produces typical colorless to pale pink colonies with 1 to 4 mm in diameter on MacConkey agar. On TCBS, *Vibrio parahaemolyticus* shows green colonies with 2 to 3 mm in diameter (Kelly et al., 1992).

Vibrio parahaemolyticus is halophilic and belongs to the lysine decarboxylase positive, arginine dihydrolase negative group (Table 4). It is distinguished from other members of the group by negative reaction for sucrose, salicin, and cellibose fermentation. The other useful characteristics

in the separation of *Vibrio parahaemolyticus* are lactose and arabinose fermentation, Voges Proskauer test and grows in 10% NaCl. Additional biochemical characteristics of *Vibrio parahaemolyticus* are shown in Kelly et al. (1995).

7). Antimicrobial susceptibility testing

Antimicrobial susceptibility testing should be performed with Muller-Hinton broth or agar. Although Mueller-Hinton agar contains no added NaCl, it contains a hydrochloric acid, hydrolysate of casein, apparently has given enough NaCl to allow good growth of the halophilic vibrio species (Hollis et al., 1989). Several techniques of antimicrobial susceptibility test such as disk diffusion, broth or agar dilution, or automated methods may be performed and the disk diffusion is applied in most routine laboratories (Tison et al., 1984). *Vibrio parahaemolyticus* is usually resistant to amplicillin and carbenicillin but susceptible to colistin chlramphenicol, gentamicin and nalidixic acid. These properties are shared with *Vibrio alginolyticus*. More details on antimicrobial susceptibility of *Vibrio parahaemolyticus* has showed by Farmer et al.(1985).

8). Treatment

The limited severity and duration of the illness militates against prescribing specific antimicrobial therapy or any other medication in most cases. Rehydration usually the only treatment needed, although severe cases may require hospitalization. Antimicrobial therapy may be beneficial in more severe or prolonged infection (Barker et al., 1974).

Table 4 : Differentation of arginine dihydrolase-negative, lysine decarboxylase- positive *Vibrio* species. (Kelly et al., 1992)

Test or property	*V. Alginolyticus*	*V. parahaemolyticus*	*V. vulnificus*	*V. Carchariae*
Voges-Proskauer	+	-	-	-
Growth in broth with:				
8% NaCl	+	(+)	-	-
10% NaCl	V	-	-	-
Fermentation of:				
Sucrose	+	-	(-)	+
Salicin	-	-	+	-
Cellobiose	-	-	+	V
Lactose	-	-	(+)	-
L-arabinose	-	(+)	-	-
Awarming (marine agar,25C)	+	+	-	+
Zone of inhibition around:				
Colistin	Large	Large	Small	Small
Amplicillin	Small	Small	Large	Small
Carbinicillin	Small	Small	Large	Small

Symbol : +, most strains positive (generally about 90%)

Symbol : -, most strains negative (generally about 0 to 10 positive)

Symbol : (+) , positive(generally about 75 to90%)

Saymbol : (-), negative (generally about 10 to 25% positive)

Review on fluoroquinolone resistant mechanisms

1. Mechanism of fluoroquinolone resistance.

The resistance of bacteria to fluoroquinolone is mediated by two types of mechanism, alteration in drug target enzymes and alteration that decrease drug accumulation (Piddock et al., 1999).

1.1 Alteration in drug target enzyme.

Fluoroquinolone resistance that alter drug target causes by mutation in DNA gyrase and Topoisomerase IV, these type II topoisomrase are essential for cell survival. Type II topoisomerase function by breaking both strands of duplex DNA, passing another DNA strand through the break and resealing the initial broken strand. This activity occurs at the expense of ATP, with ATP hydrolysis serving to reset the enzyme for another cycle of strand passage (Bates et al., 1996). DNA gyrase is composed of two subunits of GyrA and two subunits of GyrB , encoded; by the *gyrA* and *gyrB* genes, respectively. It can introduce negative supercoiling into closed circular DNA, as well as ATP-independent relaxation of supercoiling DNA (Bernard F.M., 2001)(Fig 1).

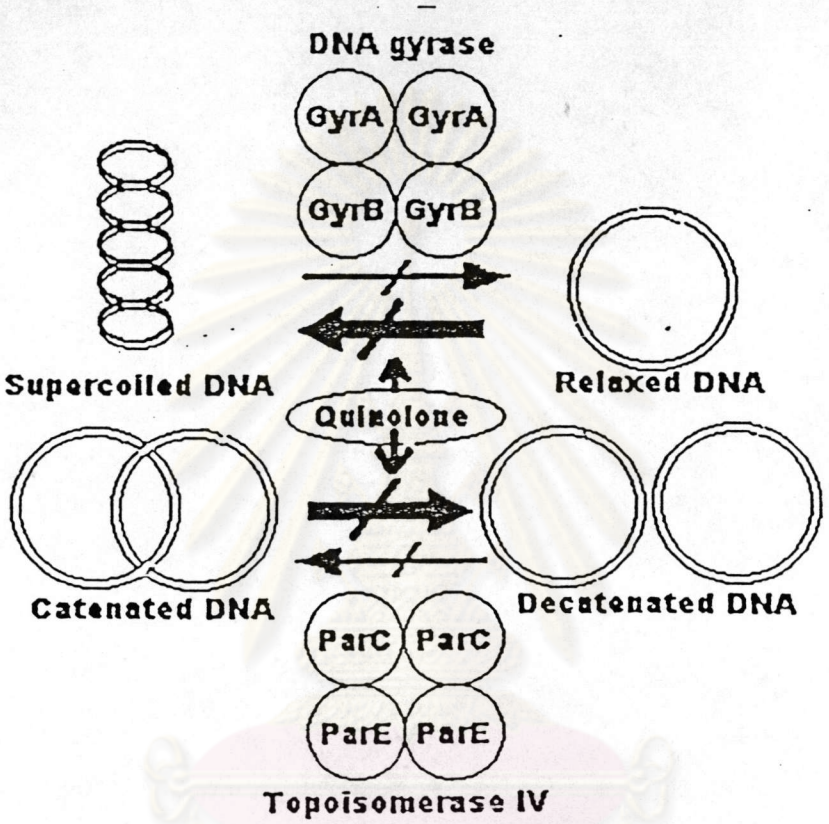

Fig.1 Major activities of DNA gyrase and topoisomerase IV (Hooper, 1999).

Quinolones block these activities by stabilizing a enzyme DNA complex, which also functions as a barrier to the movement of other proteins such as DNA polymerase and RNA polymerase along the DNA.

1.1.1 DNA gyrase

DNA gyrase is essential for DNA replication and is involved in both initiation and elongation. Negative supercoiling itselfs facilitate binding of initiation protein to DNA and enhances DNA strand unwinding during fork propagation. The enzyme may also be involved in the determination phase of DNA replication by decatenating interlocked daughter DNA molecules resulted from completion of a cycle of DNA replication.

In addition, DNA gyrase is directly involved in transcription processes by the removal of positive supercoils accumulating ahead of RNA polymerase as it progresses along constrained DNA templates. This effect is suggested by the accumulation of positive supercoils in *p*BR322 DNA in cells treated with gyrase inhibitors (Lockshon et al., 1983 and Aleixandre et al., 1991). It is also involved in aspects of DNA rccombination (Von Wright et. al., 1981) DNA repair (Hays et al., 1978) and transposition (Isberg et al., 1982).

1.1.2 Topoisomerase IV

Topoisomerase IV is composed of two subunits homologues to those of DNA gyrase. ParC, encoded by the *parC* gene has some homologues to GyrA, and ParE, encoded by the

parE gene has some homologous to GyrB (Ferrero et al., 1994). Despite the similarities between the two topoisomerases, *par* genes cannot compensate for the temperature-sensitive phenotype of *gyr* mutation, whereas simultaneous expression of the *gyrA* and *gyrB* genes can partially complement the defect in *par* mutation (Kato et al., 1992 and Peng et al., 1993).

The function of topoisomerase IV is suggested to be involved in decatenating or removal of the interlinking of daughter chromosomes at the completion of a round of DNA replication which allows their segregation into daughter cells (Zechiedrich et al., 1995). This activity has been proved by the accumulation of plasmid catenanes in *parC* and *parE* thermosensitive mutant at nonpermissive temperature, while none of accumulation has been found in *gyrA* and *gyrB* thermosensitive mutants under similar conditions (Kato et al., 1990). Thus, it seems that both bacterial type II topoisomerases are required at the terminal stages of DNA replication.

1.1.3 The roles of gene encoded type II topoisomerase in quinolone resistance

The studies on the molecular basis of quinolone resistance have largely relied on complementation assays of resistant bacteria. For DNA gyrase, this complementation or dominant test is based on the dominance of the susceptibility of

gyrA^{+} over quinolone-resistant *gyrA* in merodiploid strains, which contain both genes - *gyrA*$^{+}$ and *gyrA*. If bacteria revert to a quinolone-susceptible phenotype it is inferred that the resistant phenotype is due to mutations in *gyrA* (Matinez et. al., 1998). With this test, negative results and small decrements in resistance cannot be considered evidence for the absence of chromosomal *gyr A* mutations because plasmid instability, poor plasmid gene expression, and failure of heterologous Gyr subunits to form a funtional enzyme in some host strain may also produce negative results (Power et al., 1992). In *E. coli*, purified DNA gyrase reconstitued with mutant and wild-type GyrA and GyrB subunits was active in the presence of drug concentrations that inhibited the wild-type enzyme (Aoyama et al.,1987 and Sato et al., 1986), similar findings were found in other bacterial species including *Bacillus subtilis* (Sugino et al.,1980), *Enterococcus faecalis* (Nakanishi et al. 1991), *Staphylococcus aureus* (Okuda et al.,1985), *Campylobacter jejuni* (Gootz et al., 1991), *Serratia marcescens* (Masecar et al., 1991), *Pseudomonas aeruginosa* (Robillard et al.,1988), *Haemophilus influenzae* (Setlow et al.,1985) and *Citrobacter freundii* (Aoyama et al., 1988).

There is little information on the role of GyrB in quinolone resistance because the studies of quinolone resistance in DNA gyrase have been mostly focused on GyrA, still, some data have been published. In an analysis of independent *E. coli*

mutants selected with nalidixic acid (at four fold above the MIC), equal number appeared to have gyrA and gyrB mutations. As determined by complementation with *gyrA*$^{+}$ - and *gyrB*$^{+}$ - containing plasmids, respectively (Nakamura et al. 1989).

The discovery of topoisomerase IV in 1990 has demonstrated relevance of *parC* and *parE* in quinolone resistance. The mutation in resistant locus, *nfxD*, isolated from a highly resistant strain serially passaged on increasing concentrations of norfloxacin, has been found in *E.coli* (Ng et al., 1992).This locus is located in the region of *parC* and *parE* genes, which encodes topoisomerase IV and appears to express quinolone resistance conditionally in the presence of *gyrA* resistance mutation. The recessivity of *nfxD* to *gyrA*$^{+}$ suggests that *nfxD* may contribute to resistance only additively in strains that have already acquired at least one mutation (Hooper and Wolfson, 1993). The same finding of mutation in *E. coli parE* has also been identified by Breines and his co-workers (1997). In *Streptococcus pneumoniae*, ciprofloxacin-resistance shows most commonly mutations in *parC* (Pan et al., 1996), while *parE* mutations have been found less frequently (Perichon et al., 1997). Conclusively, mutations in any of these targets might be sufficient for acquisition of quinolone resistance.

1.1.4 Topoisomerase sensitivity to fluoroquinolone

Belland and his colleagues (1994) gave the notion that both *gyrA* and *parC* genes are involved in the establishment of extreme levels of ciprofloxacin resistance. A series of ciprofloxacin-resistance mutants were selected by passage of *Neisseria gonorrhoeae* on increasing concentration of the antibiotic. Sequential passage resulted in the selection of isolates with minimum inhibitory concentrations approximately 10000-fold higher than parental strain. Mutation within *gyrA* resulted in low to moderate levels of resistance, while strains with high–level resistance acquired analogous mutation in both *gyrA* and *parC*. A stepwise-fashion mutation in *E. coli* with high-level resistance to quinolone appears to show similar results (Heisig P., 1996). On the other hand, ParC mutations were found in low-level of clinical resistant isolates of *Enterococcus faecalis* without a mutation in GyrA, with the high-level resistant isolates had mutations in both ParC and GyrA (Kanemutsu et al., 1998). Ciprofloxacin-selected first-step mutants of *Streptococcus pneumoniae* (Pan and Fisher, 1996 and Perichon et al., 1997) also have mutations in the ParC or ParE subunits of topoisomerase IV similar to those reported in *Streptococcus aureus* (Ng et al., 1996 and Fournier and Hooper, 1998). From these observations, a net result in fluoroquinolone resistance target in bacteria has been revealed. In gram-negative bacteria,

DNA gyrase is demonstrated to be a primary target, in particular *gyrA* subunit, while *parC* of topoisomerase IV, which is less sensitive to the drug, is determined to be a secondary target. The inverse situation has been observed in gram-positive bacteria and topoisomerase IV, *parC*, appears to be a primary target as *gyrA* a secondary target (Hooper, 1999).

1.1.5 The mutations in genes encoded type II topoisomerase

The mutation that confers resistance to fluoroquinolone has been studied and most of studies have revealed the hots-pots for mutation with in *gyrA* (Yoshida et al., 1988 and Korten et al., 1994) and *parC* (Vila et.al, 1996 and Wang et al., 1998). This region is called Quinolone-Resistant-Determining-Region (QRDR). In *E. coli*, this cluster is located within the N-terminal domain between amino acid position 67 to 106. It is situated close to the catalytic cleavage residue Tyr122, which is transiently covalent bound to DNA phosphate groups during the enzyme's DNA strand passing reaction (Yoshida et.al, 1990). Two amino acids Ser83 and Asp87, are most commonly mutated in resistant isolates, and a Ser83Trp mutation has been shown to cause reduced binding of norfloxacin to gyrase DNA complexes (Willmott and Maxwell, 1993). Substitutions of amino acids in equivalent position to Ser83 and Asp87 in other bacteria such as *Shigella dysenteriae* (Kim et al., 1994), *Salmonella typhimurium*

(Reyna et al., 1995), *Staphylococcus aureus* (Takenouchi et al., 1995), *Enterococcus faecalis* (Tankovic et al., 1996), *Mycobacterium tuberculosis* (Takiff et al., 1994) and *Chlamydia trachomatis* (Dessus-Babus et al., 1998) have been shown to cause quinolone resistance either by genetic studies or to be associated with resistance in clinical isolates. Thus, models of quinolone resistance from alternations in DNA gyrase developed from studies of *E.coli* will likely to be applicable to a broad range of gram-negative and other bacteria as well.

Likewise the homologous of topoisomerase IV subunits to the DNA gyrase subunits, the QRDRs in the ParC and ParE also highly conserve with QRDR domains of GyrA and GyrB. This statement predicts similarity of drug interaction and resistance mutation for the two enzymes (Hooper, 1999).

The hotspots for fluoroquinolone resistance which were found in several bacteria resistant clinical isolates of *E. coli* (Kumagai et al., 1996 and Lee and Lee, 1998), *Klebsiella pneumoniae* (Deguchi et al., 1997), *Citrobacter freundii* (Nishino et al., 1997), *Pseudomonas aeruginosa* (Nakano et al., 1997), *Haemophilus influenzae* (Georgiou et al., 1996), *Neiserria gonorrhoeae* (Belland et al., 1994 and Tree et al., 1998) and *Staphylococcus aureus* (Fitzgibbon et al., 1998 and Schmitz et al., 1998) have tended to occur in the position equivalent to Ser80 and Glu84 of *E. coli* ParC. In *Streptococcus pneumoniae* similar ParC mutations have been reported,

including Ser79Tyr/Phe and Asp83Asn/Gly/Ala in resistant mutants selected in the laboratory and found in resistant clinical isolates (Pan et,al., 1996; Gootz et al., 1996; Monoz and de la Campa, 1996; Janoir et al., 1996; Tankovic et al.,1996; Pan and Fisher,1997; Pan and Fisher, 1998).

Mutations in GyrB and ParE generally occur less than those in GyrA and ParC. In GyrB, Asp426Asn and Lys447Glu in *E. coli* are most known. Both mutations cause resistance to the nalidixic acid, but they differ in their effects on susceptibility to fluoroquinolone with a piperazinyl substituent at position 7 of the quinolone ring (eg. norfloxacin or ciprofloxacin), causing either low-level resistance (4-8 fold increase in MIC - Asp426Asn) or increase susceptibility (4-fold decrease in MIC– Lys447Glu) to ciprofloxacin (Nakamura et al., 1989). These differences in resistance phenotype have been proposed to be due to difference in direct electrostatic interactions between the affected amino acid in the wildtype and mutant GyrB subunits with or without the positive charged piperazinyl moiety (Yoshida et al., 1991).

A single example of a ParE mutation in gram-negative bacteria contributing to resistance in conjunction with a mutation in GyrA has been reported for *E. coli* -Leu445His (Breines et al., 1997). Other mutations in gram-positive bacteria have been found in *Staphylcococcus aureus* - Asn470Asp (Ng et

al., 1996) and *Streptococcus pneumoniae* – Asp435Asn (Perichon et al., 1997).

1.1.6 Interaction of fluoroquinolone with Type II topoisomerase

Fluoroquinolone plays a role in bacteria inhibition by stabilizing the enzyme-DNA complex during the supercoiling process (Barnard and Maxwell, 2001). To introduce negative supercoiling, DNA gyrase cleaves double-stranded DNA along the 4-bp-staggered cuts resulted in protruding of 5' ends of the DNA chain (Morrison and Cozzarelli, 1979). Then a self assemble of 4-molecules of drug, supermolecule, will bind to the unpaired base via hydrogen bond inside the gyrase-induced DNA pocket (Horowitz and Wang, 1987) (Fig2).

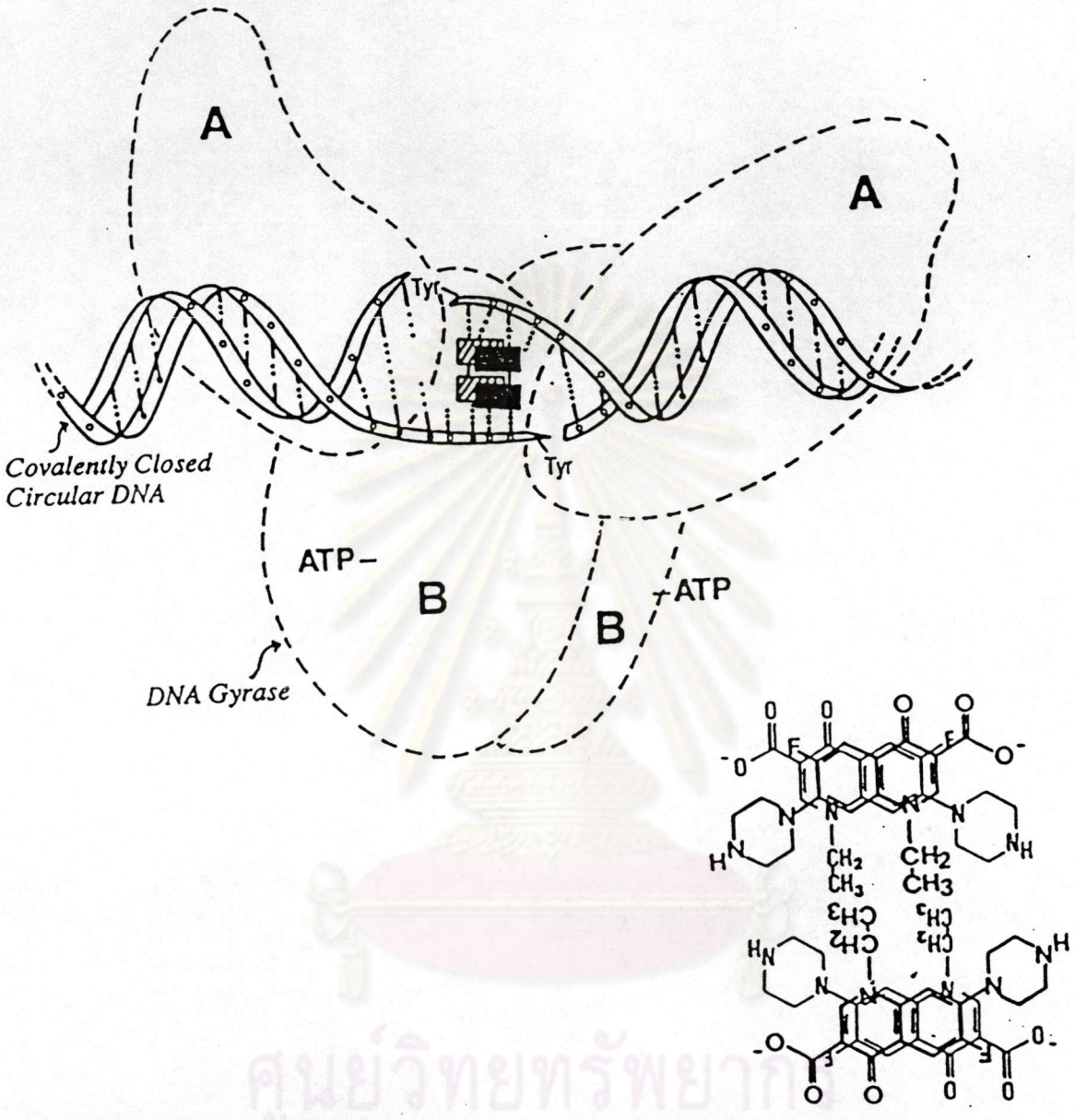

Fig. 2 The proposed quinolone-DNA cooperative binding model for DNA gyrase inhibition (Shen et al., 1989).

Filled and hatched boxes denote the quinolone molecules that self-assemble to form a supermolecule inside the gyrase induced DNA pocket. Dashed curves mimic the shape of the DNA gyrase as reveal by the electron microscopic images of the Micrococcus luteus enzyme.

Besides self-interaction with drug molecules and DNA strands, fluoroquinolone is believed to interact with GyrB subunit. In the study of a quinolone-DNA cooperative binding model for the inhibition of DNA gyrase, Shen and his colleagues (1989) suggests three functional domains on the quinolone molecule as following 1) the DNA-binding domain 2) the drug self-association domain and 3) the drug-enzyme interaction domain (Fig3).

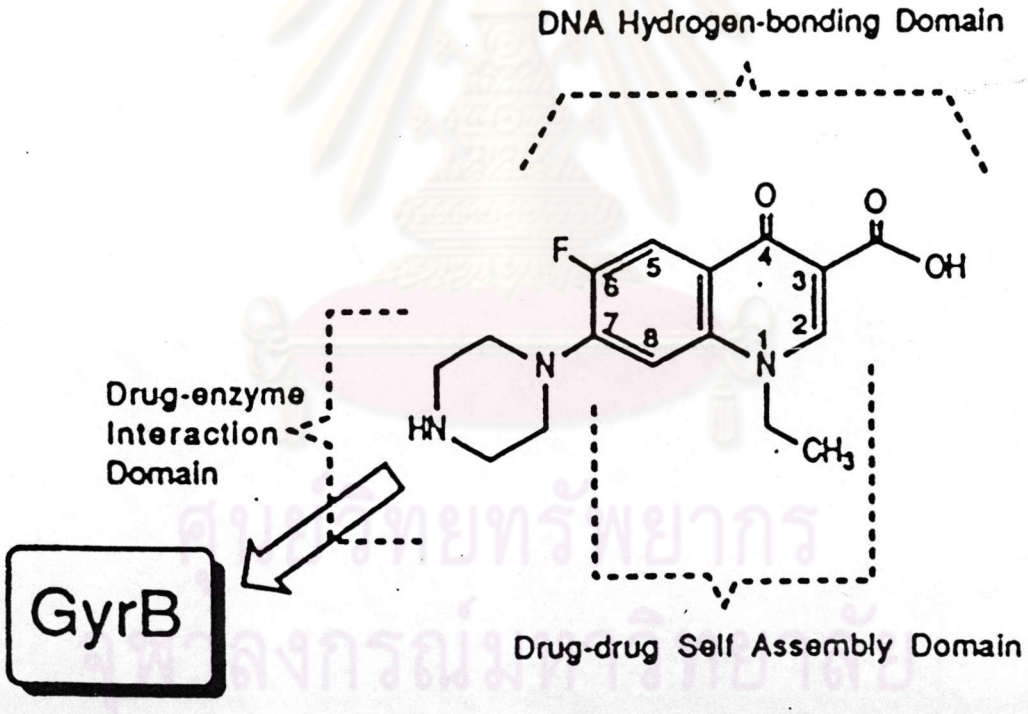

Fig. 3 Functional domains of quinolone antibacterial agent (Shen et al., 1989).

The proposed model also gives the corresponding evidence with the current structure-activity relationships of quinolones (Shen and Pernet, 1985; Chu and Fernandes, 1989; Wentland, 1990) and has provided a general guideline for efforts at synthesizing active quinolones with novel structure (Chu et al., 1988; Hubschwerlen et al., 1992).

Such ternary complexes are also formed with quinolone and topoisomerase IV (Peng and Marians, 1993). A recent study by Hiasa's group (1996) showed that none of the three mutant enzymes of topoisomerase IV, with the wild –type ParE subunits, could block replication fork progression in the presence of norfloxacin while the wild-type enzyme formed a measurable enzyme-DNA-complex with norfloxacin and potently arrested replication fork progression in the presence of drug. This experiment establishes that at least *in vitro*, a quinolone-stabilized-cleavable-complex formed between DNA and topoisomerase IV can cause replication fork arrest. Thus, in the cell, topoisomerase IV might create a lethal effect by a mechanism similar to that described for DNA gyrase.

1.2 Alteration of drug permeation in to target drug

Another mechanism which has been considered to contribute a resistance to fluoroquinolone is the alteration of drug permeation into target enzyme. To reach DNA gyrase and topoisomerase IV, which are

present in the cytoplasm, quinolones must traverse the cell wall and cytoplasmic membrane of gram-positive bacteria and additionally the outer membrane in gram-negative bacteria. The cell wall itself is thought to provide little or no barrier to diffusion of small molecules such as quinolone, which have molecular weight around 300 to 400 daltons (Hooper, 1999). As all active quinolones have a negative charged carboxyl group at position 3, and most current quinolones have an additional positively charged group at position 7 (piperazinyl or perrolidinyl ring derivatives) and thus are zwitterionic (Ross and Riley, 1990). The proportions of positively-charged, negatively-charged, dually-charged and uncharged species of quinolone, which are thought to play a role in penetration through the membrane, vary with pH ranges exceptionally the uncharged species which diffuses freely across the membrane and reaches equilibrium with the cytoplasm (Nikaido et al., 1993). In addition, differences in the pH ranges are thought to affect partitioning of drug by altering the proportions of charged species which are trapped in the cytoplasmic compartment (Hooper, 1999).

1.2.1 Gram negative bacteria

Quinolone-resistant clinical isolates of many species of gram-negative bacteria were found in early reports to have alterations in the amounts of outer membrane proteins, some of which were reduced amounts of general diffusion porins (Hirai

et al., 1987; Hirai et al., 1986; Piddock and Wise, 1986). Subsequent studies have identified the necessary additional role of energy-dependent efflux system that pumps drugs out of the cell and act in concert with reduced diffusion due to reduced porin channel. In *E. coli*, the overexpression of *marA* and *soxS* has been shown to cause quinolone resistance in clinical isolates in addition to mutation in *gyrA* and *parC* (Oethinger et al., 1998).

In the study of Ariza and co-workers (1994) of multiple antibiotic-resistant (Mar) mutants in *E. coli*, which could be selected with tetracycline (George and Levy, 1983), chloramphenicol (George and Levy, 1983), or fluoroquinolone (Cohen et al., 1989), mutations in *marRAB* regulon have been found. *marR* mutants resulting in a defective MarR repressor exhibited increased expression of MarA, a transcriptional activator that led to increase the expression of *micF* and other loci (Cohen et al., 1988). *micF* encodes an anti-sense RNA species that is complementary to the 5' of *ompF* transcripts, which encode OmpF, one of the two major porins of *E. coli* (Andersen et al., 1987). Thus, increased expression of MarA leads to increase *micF*, which in turn leads to reduce translation of *ompF* RNA by formation of a double- stranded RNA that may bind poorly to the ribosome. Reduced amounts of OmpF in the *E. coli* outer membrane appears to slow diffusion of norfloxacin and other fluoroquinolones, leading to slower rates

of accumulation, but reductions in OmpF alone in *ompF* mutants resulted in less resistance and lesser reductions in norfloxacin accumulation than *marR* mutants (Cohen et al., 1989). Moreover, in the presence of proton motive force inhibitors increased norfloxacin accumulation in *marR* mutants, indicating that additional energy-dependent factors contribute to reduce steady-state levels of accumulation (Hooper et al., 1989).

SoxS is a homolog of MarA and appears to affect the expression of an overlapping set of genes, including reductions in OmpF through increase in *micF* RNA (Chou et al., 1998). Mutations in the *E. coli soxS* locus, which is involved in the cellular responses to oxidative stress, shows an overlapping resistance phenotype with *marR* mutants including low-level resistance to quinolone (Miller et al., 1994). Less is known about altered permeation and efflux contributing to quinolone resistance in other gram-negative bacteria, yet MarA has been found in *Salmonella typhimurium* (Sulavik et al., 1997), *Klebsiella pneumoniae* (RamA) (George et al., 1995), *Proteus vulgaris* (PqrA) (Ishida et al., 1995), and several other bacteria species (Alekshun and Levy, 1997), suggesting that similar regulatory mechanisms may underlie some forms of multidrug resistance in these organisms as well.

1.2.2 Gram positive bacteria

In gram-positive bacteria the permeability barrier of the outer membrane does not exist hence an expression of efflux pumps, which confer drugs resistance, is believed to be a major mechanism in this organism. The two best studies that effect quinolone resistance are NorA of *Staphylococcus aureus* (Neyfakh et al., 1993; Kaatz and Seo, 1995; Markham and Nefakh, 1996) and Bmr and Blt of *Bacillus subtilis* (Nefakh, 1992; Ahmed et al., 1994; Ahmed et al., 1995; Markham et al., 1996; Woolridge et.al, 1997).

2. The study of Fluoroquinolone resistance in *Vibrio parahaemolyticus*

In *Vibrio parahaemolyticus*, the primary study of quinolone resistance mechanism has been focused on the alteration in drugs target enzymes. Okuda and his colleagues (1999) have examined the mutations in *gyrA* and *parC* QRDR of ciprofloxacin resistant mutants. They first identified *gyrA* and *parC* QRDR homologues in *Vibrio parahaemolyticus,* in which well-conserved amino acid sequence of the QRDR of *gyrA* and the corresponding ParC regions are utilized. Then induced quinolone ciprofloxacin resistant mutants and examined the mutation in the *gyrA* and *parC* gene. The results showed mutant strains of AQ3815 became resistant to ciprofloxacin in stepwise fashion and had a base substitution at residue position 83 of GyrA and position 85 of ParC with no other mutations than the above were found. Based on these results, the stepwise appearance of a *gyrA* mutation followed by a *parC* mutation in AQ3815 derivatives with increasing ciprofloxacin suggests that DNA gyrase and topoisomerase IV may be the primary and secondary targets of ciprofloxacin respectively in *Vibrio parahaemolyticus*.