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Title of Dissertation:

Contribution of *Neisseria gonorrhoeae* Catalase in Defense against Toxic Oxygen Radicals and Neutrophils, and Its Role during Experimental Genital Tract Infection of Mice

Angel A. Soler García

Candidate, Doctor of Philosophy, 2002

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Neisseria gonorrhoeae (GC) is responsible for a variety of sexually transmitted mucosal infections including urethritis and cervicitis. The hallmark of symptomatic gonorrhea is an intense inflammatory response characterized by an influx of neutrophils (PMNs). The mechanism by which GC resists PMN defenses is not known, however it is hypothesized from in vitro studies that gonococcal catalase protects GC by breaking down H_2O_2 produced by PMNs during the oxidative burst. The primary objective of this research was to assess the role of gonococcal catalase in GC pathogenesis using a genetically defined catalase mutant in *in vitro* assays and in an animal infection model. The *kat* gene of GC strain FA1090 was sequenced and the nucleotide sequence was predicted to encode a 57 kDa (504 amino acid) protein. A GC *kat* mutant was constructed by allelic exchange and demonstrated to be more sensitive to H_2O_2 and paraquat, an

inducer of toxic oxygen radicals, than the wild type (WT) parental strain. The *kat* mutant was also more sensitive to H_2O_2-producing commensal lactobacilli *in vitro*. Complementation of the catalase mutation *in trans* restored wild type levels of catalase activity, and resistance to paraquat and H_2O_2-producing lactobacilli, but not resistance to H_2O_2. The inability to complement the mutant fully is perhaps due to a modification in the recombinant protein as evidenced by an altered mobility of the recombinant catalase on activity gels. The *kat* gene was not essential for infection in that there was no difference in the duration of recovery of the WT or *kat* mutant from the lower genital tract of 17-β estradiol-treated BALB/c mice, even in the presence of an intense PMN response. In mixed infection experiments, however, the recovery of the *kat* mutant was dramatically reduced compared to that of the WT GC. The *kat* mutant was more susceptible to killing by murine PMNs *in vitro*. From these studies we conclude that although GC can infect and persist in the genital tract of estradiol-treated mice without a functional catalase gene, GC catalase confers a competitive advantage *in vivo*, which may be in part due to protecting GC against PMN killing.

Contribution of *Neisseria gonorrhoeae* Catalase in Defense against Toxic Oxygen Radicals and Neutrophils, and Its Role during Experimental Genital Tract Infection of Mice

by

Angel Antonio Soler García

**Dissertation submitted to the Faculty of the
Department of Microbiology and Immunology Graduate Program
of the Uniformed Services University of the Health
Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 2002**

Dedicated to

my mother

Thank you for being the best example of a hard working, loving human being I have ever met. To let me dream and for teaching me to be true to myself. To show me that in life the true success is for the ones that never give up because we hope for a better future. Mami, we are more real when we get closer to what we dream to be. Thanks.

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To my mother-You have always believed I could succeed in any of my endeavors. Thank you for teaching me to be self confident and to stand for my beliefs.

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To the present lab mates-We have laughed a lot together and I have enjoyed.

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Introduction

I- Overview and Purpose

Neisseria gonorrhoeae (GC) catalase is hypothesized to be an important mechanism of defense against H_2O_2 and toxic oxygen radicals produced by polymorphonuclear leukocytes (PMNs) in infected foci. The role of GC catalase as a mechanism of defense against PMNs has not been definitively tested with defined mutants. The present research represents the first description of the role of gonococcal catalase *in vivo* in which a genetically defined mutant was tested in PMN killing assays and in an animal model of genital tract infection. The results of this work further our understanding of the mechanisms of persistence used by *N. gonorrhoeae* during infection. Elucidation of these mechanisms increases the knowledge required for the design of new anti-gonoccocal drugs and vaccines.

In the next sections of the introduction, I will provide information on several topics that are relevant to understanding the present study. First, I will present a summary of the historical impact and epidemiology of *N. gonorrhoeae* and the disease gonorrhea, followed by a description of several aspects of GC pathogenesis such as well characterized virulence factors, and mechanisms of adaptation and immune evasion in the host genital tract. Second, I will present a detailed overview of phagocyte surveillance and well documented paradigms of bacterial mechanisms of defense against phagocytes based on studies with *Escherichia coli* and *Salmonella sp.*, as well as what is known today in regard to GC.

II- Epidemiology and historical impact of *Neisseria gonorrhoeae* and gonorrhea

A. History

Gonorrhea is one of the oldest diseases recorded in human history. References to gonorrhea by the Chinese Emperor Huang-ti date back to 2637 B. C. and to 1500 B.C. in the biblical Old Testament Leviticus 15 (Hook and Handsfield, 1990). The term gonorrhea means “flow of semen or seed” and was coined by the Greek physician Galen in 130 A.D. as the result of confusion between semen and the purulent discharge that is characteristic of gonorrhea in infected males. The etiological agent of gonorrhea was first found in stained smears by Neisser in 1879, and cultivated for the first time in 1881 by Leistikow and Loeffler (Evangelista and Beilstein, 1993; Handsfield, 1990). In 1964, the selective antibiotic-containing medium of Thayer and Martin was introduced (Thayer and Martin, 1964).

B. Epidemiology

Neisseria gonorrhoeae (GC) is a human-specific pathogen that is responsible for a wide variety of common and important infections such as urethritis, cervicitis, pelvic inflammatory disease (Eschenbach et al., 1997; Peipert et al., 1996) and disseminated gonococcal infection (Cohen and Sparling, 1992). It is the second most common cause of sexually transmitted diseases in the U.S.A. after *Chlamydia trachomatis*.

Approximately 750,000 cases of gonorrhea are reported yearly in the U.S.A. and another 750,000 unreported cases are believed to occur mostly in

young adults and teenagers (CDC, 1995; Communicable Diseases, Gonorrhea. Arnot 1998). The U. S. government has estimated the annual cost of gonorrhea and its implications to be 1.1 billion dollars. Given that gonorrhea is a treatable disease and treatment is inexpensive and readily available for anyone who seeks it, why is gonorrhea still so common? In many infected people there are not discernible symptoms. Approximately one third of infected women and as many as 10% of infected men are asymptomatic and consequently the disease is more easily spread. Additionally, GC has evolved highly sophisticated strategies to avoid an immune response via phase and/or antigenic variation of surface molecules.

The rates of gonorrhea in the US declined 72% during 1975-1997. However in 1998 and 1999, an increase of 9.2% and 1.2% (compared to its previous year), was observed, respectively (CDC, 1999; CDC, 2000a). High rates of gonorrhea are still prevalent in the southeastern states among minorities, and among adolescents of all racial and ethnic groups (CDC, 1998b; Fox et al., 1998). The major health impact of gonorrhea is related to its role as a primary cause of pelvic inflammatory disease, which often leads to infertility or ectopic pregnancy (McCormack, 1994; Rhoton-Vlasak, 2000). Recent data have suggested that gonorrhea facilitates HIV transmission (Laga et al., 1993; Cohen et al., 1997).

Through time, the treatment of gonorrhea has become complex due to the emergence of antimicrobial resistant strains. In the 1970s, the use of penicillin and tetracycline was abandoned due to the emergence of penicillinase-producing *Neisseria gonorrhoeae* (PPNG) and chromosomally-mediated penicillin- and

tetracycline-resistant *N. gonorrhoeae* (CMRNG). Today, the recommended therapy for gonorrhea consists of two broad-spectrum cephalosporins, ceftriaxone and cefixime, and two fluoroquinolones, ciprofloxacin and ofloxacin (CDC, 1998b). Unfortunately, fluoroquinolone resistance in GC has been reported in many parts of the world (Knapp et al., 1997; Fox et al., 1997; CDC, 1995; CDC, 1998a; CDC, 2000a).

III- Pathogenesis of *Neisseria gonorrhoeae*

A. General overview

GC is a human specific pathogen that is well-adapted to the microenvironment of the human reproductive tract. In order to survive in this site, GC must overcome fluctuations in local pH (Magnusson et al., 1979; Pettit et al., 1996) and certain inhibitory reproductive hormones (i. e. progesterone and testosterone) (Koch et al., 1950; Morse and Fitzgerald, 1974), specific and non-specific mediators of the immune response (Black et al., 1984; Hamrick et al., 2001; McQuillen et al., 1999, Wetzler et al., 1992) and toxic elements produced by commensal flora (McBride et al., 1978; Saigh et al., 1978) (**Fig. 1**). Gonococci utilize multiple sophisticated mechanisms to adapt to selective pressures in the host, including those that allow GC to withstand periods of intense inflammatory response (Cohen and Sparling, 1992). Adaptation mechanisms utilized by GC include phase and/or antigenic variation of surface components (Meyer et al., 1990) and transcriptional regulation of genes in response to external stimuli.

Figure 1. Factors encountered by GC in the genital tract. *Neisseria gonorrhoeae* is a well-adapted human pathogen that has evolved several mechanisms that allow the bacterium to survive during fluctuations of pH, specific and non-specific mediators of the immune response and competition with commensal flora.

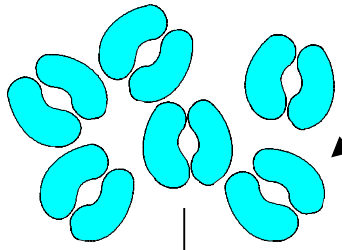
Environmental Changes

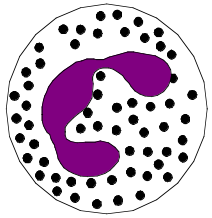
-Inhibitory hormones
-Local pH
-Oxygen tension

Neisseria gonorrhoeae

Immune Response

-Specific mediators
-Non-specific mediators



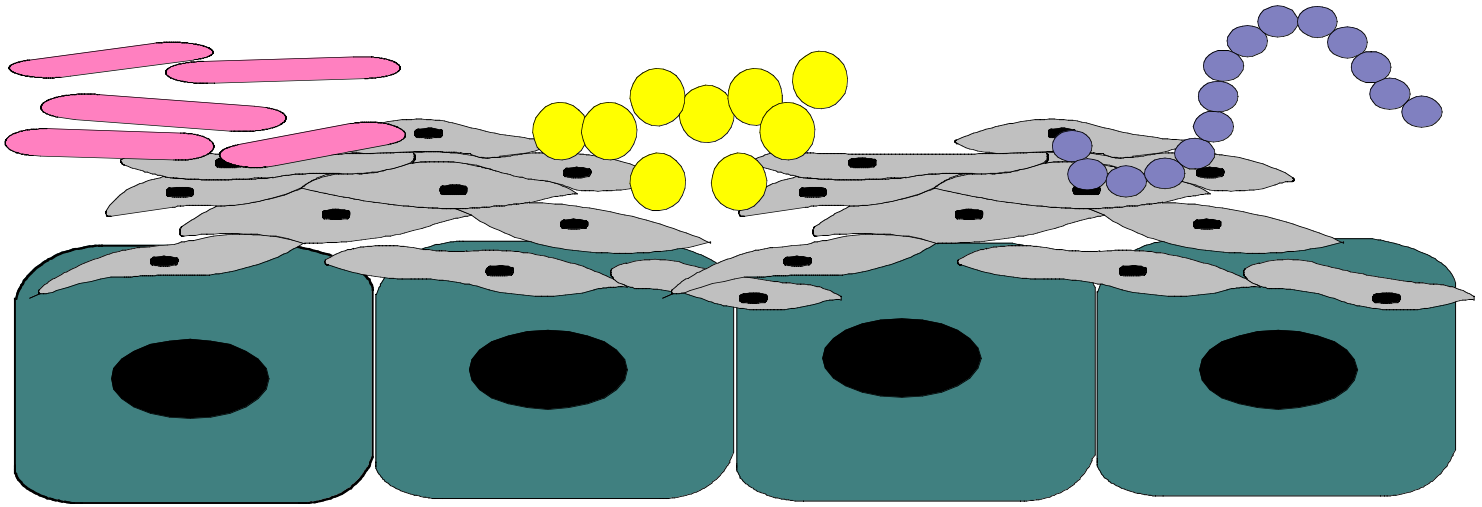


Commensal Flora

Bacteriocins

Acidification

Hydrogen peroxide

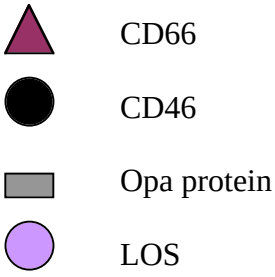


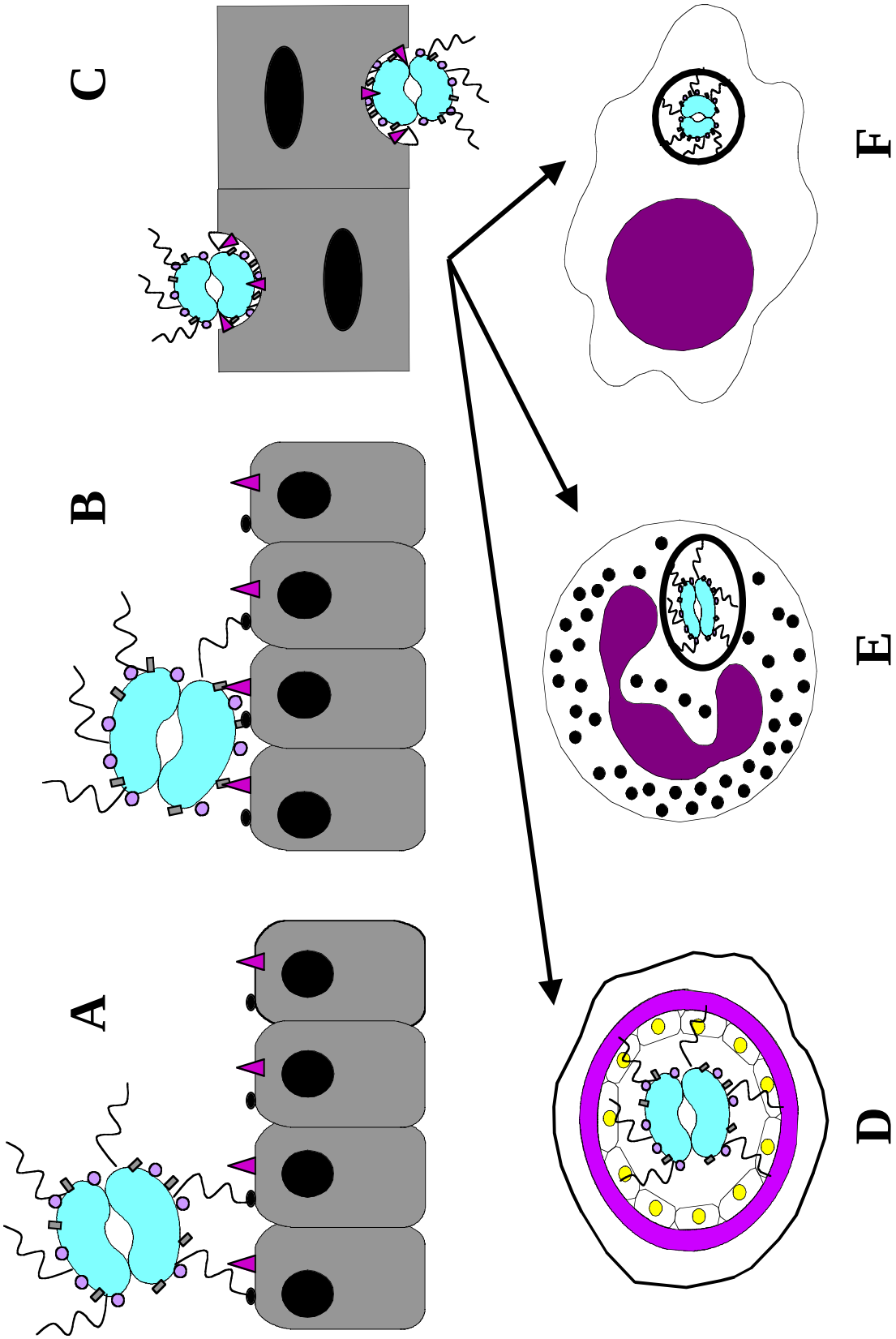
N. gonorrhoeae utilizes several virulence factors during the different steps of infection (Koomy, 2001; Naumann et al., 1999). Pili, porin, opacity proteins (Opa), lipooligosaccharide (LOS) and iron-binding proteins, among others, are utilized by gonococci to infect, invade and survive in the human genital tract (**Fig. 2**).

B. Major virulence factors

1) **Pili**- Pili are filamentous appendages composed primarily of two distinct proteins, PilE and PilC. PilE is the major pilin subunit that forms the fiber. PilC is located at the tip of the pilus and is also believed to be involved in pilus maturation (Tonjum and Koomey, 1997). Pili mediate initial binding of gonococci to epithelial cells (Scheuerpflug et al., 1999; Swanson, 1972), and are required for natural competence for DNA (Fussenegger et al., 1997; Seifert et al., 1990). PilE and PilC also exhibit phase and/or antigenic variation (Hamrick et al., 2001; Seifert et al., 1988; Seifert, 1996). Membrane cofactor protein (MCP) or CD46 may serve as the GC pilus receptor (Källström et al., 1997; Källström et al., 2001).
2) **Porin**- Also known as P. I protein, porin is used in epidemiological studies for serotyping based on its antigenic variability between strains (Knapp et al., 1984). Porin is a very immunogenic protein (Elkins et al., 1994a) that has been shown to promote invasion of eukaryotic

Figure 2. Steps in GC infection. Colonization of the urogenital mucosa by *Neisseria gonorrhoeae* involves pili as the first factor to promote adherence (**A**). The interaction between pili and the epithelial membrane of the host is mediated by the CD46 receptor. After initial contact by the pili, a more intimate interaction is developed by the Opacity proteins of GC and the vaginal epithelium through the CD66 family of receptors and/or the heparin sulfate proteoglycan receptors (**B**). GC can invade and transcytose epithelial cells. Invasion of endothelial cells (**C**) can lead to a systemic infection. GC also encounters phagocytes such as granulocytes (**D**) and monocytes (**E**). There is evidence suggesting that GC can survive inside phagocytes and it also has been postulated that GC may utilize phagocytes for dissemination.




A
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cells (van Putten et al., 1998; Bauer et al., 1999), translocate and insert in the membranes of host cells to form channels (Lynch et al., 1984), inhibit exocytosis from human neutrophils (Haines et al., 1988), induce signaling in murine B cells (Snaper et al., 1997) and induce apoptosis in epithelial cells by induction of a transient increase in calcium and activation of calpain and caspase (Müller et al., 1999, Müller et al., 2000).

3) Opacity (Opa) proteins -The Opa proteins are also known as P. II proteins. GC strains carry 11-12 *opa* genes (Barritt et al., 1987; Bhat et al., 1991; Black et al., 1984; Connell et al., 1990), each of which exhibits phase variation (Black et al., 1984; Kupsch, et al., 1993; Malorney et al., 1998). Phase variation of individual *opa* genes leads to antigenically variable Opa phenotypes. Phase variation of *opa* genes occurs via a frame shift mechanism and may be driven by differences in the promoter strength in the individual *opa* genes (Belland et al., 1997). The opacity proteins mediate intimate attachment and/or invasion of gonococci to epithelial cells (Grassmé et al., 1996; Weel et al., 1991) and uptake by neutrophils in a nonopsonic fashion (Naids et al., 1991). The capacity of Opa proteins to mediate epithelial cell invasion has been corroborated by the demonstration that antibodies directed to Opas inhibit attachment of GC to tissue culture cells (Sugasawara et al., 1983), and *E. coli* expressing these proteins invades epithelial cells (Simon and Rest, 1992).

Two Opa receptors, heparin sulfate proteoglycan (HSPG) and the CD66 family (members of the carcinoembryonic antigens) have been identified; the presence of multiple Opa-receptor specificities defines the cellular tropism of gonococci in the host (Billker et al., 2000; Bos et al., 1997; Bos et al., 1998; Chen et al., 1997; Dehio et al., 1998; Grant et al., 1999; Gray-Owen et al., 1997; Hauck et al., 2000; van Putten et al., 1997). Variable expression of Opa proteins also influences the production of proinflammatory cytokines by human macrophages (Makepeace et al., 2001). Recently, a correlation between Opa expression and elevated rates of transformation was reported (Hill, 2000).

4) **Lipooligossaccharide (LOS)**-Gonococcal LOS is believed to be responsible for most of the symptoms of gonorrhoea by triggering an intense inflammatory response (Ellis et al., 2001). *Neisseria* express a variety of lipooligosaccharides that lack the O-specific side chain of enteric LPS (Mcleod Griffiss et al., 1988; Nassif, 2000). Monoclonal antibodies have been useful tools for identifying LOS immunotypes, and one strain can express more than one LOS type simultaneously via phase variation of glycosyl-transferase genes (Erwin et al., 1996; Preston et al., 1996; Schneider et al., 1984). The most common sugar motif in clinical isolates is lacto-*N*-neotetraose, a structure shared by the precursor of ABH antigens found in human erythrocytes (Campagnari et al., 1990). This motif has been implicated in the Opa-

independent invasion of GC into epithelial cells. Recently, phosphorylation of the lipid A portion of LOS has been implicated in gonococci intracellular survival in male urethral epithelial cells (Song et al., 2000; Post et al., 2002). John et al. (1999) showed that gonococci infecting males express a specific LOS terminal structure. This result suggests that this particular LOS structure is either preferentially expressed or selected for *in vivo*. Induction of a local production of TNF-α by GC LOS is thought to be the main cause of tissue damage in infected fallopian tubes. LOS also contributes to serum resistance, an important trait for strains that cause systemic disease (Schneider et al., 1985; van Putten, 1993). Some strains can catalyze the addition of the activated form of *N*-acetylneuraminic (sialic) acid (Apicella et al., 1990) to one particular LOS variant. As a consequence, sialylated LOS renders the bacteria more resistant to serum (de la Paz et al., 1995; Kim et al., 1992; Robertson et al., 1993). One of the reasons for increased resistance via LOS sialylation is because sialic acid, a ubiquitous molecule in the host, does not activate complement (Mandrell and Apicella, 1993). LOS sialylation also prevents access of bactericidal antibodies to P.I. and other surface proteins (Wetzler et al., 1992). Although LOS sialylation can be thought of as simply a mechanism of host mimicry (Harvey et al., 2001), it has been demonstrated in the male volunteer model that modification of LOS with sialic acid decreased infectivity, as

evidenced by a longer asymptomatic stage in individuals infected with sialylated GC compared to non-sialylated infected individuals (Schneider et al., 1996). LOS binds to the asialoglycoprotein receptor (ASGP-R) on HEp2G cells (Porat et al., 1995). Harvey et al. (2000) demonstrated that GC binds to human sperm via interaction between LOS and ASGP-R, an interaction that may be utilized by GC to disseminate from male to female. Sialylation of GC LOS allows the bacteria to interact with Siglecs (Sia-binding Ig like lectins), which are receptors in hematopoetic cells that can mediate inhibitory signals in the innate immune response (Crocker and Varki, 2001). Finally, another possible function of LOS is to confer resistance against hydrophobic antimicrobial agents (Lucas et al., 1995).

5) **Transferrin- and Lactoferrin-binding proteins**- Pathogenic *Neisseria* have developed several mechanisms of iron acquisition from the host (Schryers and Stojiljkovic, 1999). This topic will be discussed below.

C. Adaptation to the genital tract microenvironment

1) **Reproductive Hormones**-Besides controlling the balance of commensal flora in the vaginal ecosystem, hormones may exert a direct effect on *N. gonorrhoeae*, in that GC is inhibited by progesterone and testosterone *in vitro* (Koch et al., 1950; Miller and Morse, 1977; Morse and Fitzgerald, 1974). Consistent with the hypothesis that progesterone challenges the survival of GC during infection is the report that

gonococci were more frequently isolated from infected women during the proliferative phase of the menstrual cycle than during the secretory phase (Koch, 1947). Cyclical differences in complement function and the antibacterial activity of serum may explain this observation. Cyclical differences in the recovery of gonococci have also been observed in experimentally infected female mice. When GC were inoculated intravaginally or intrauterinally during the proestrus (high-estrogen) stage, short-term recovery was observed. In contrast, inoculation during the post-ovulatory (high-progesterone) stage of the estrous cycle, no colonization occurred (Braude et al., 1978; Johnson et al., 1989; Kita et al., 1981; Streeter and Corbeil, 1981).

The relatively high incidence of disseminated gonococcal infection (DGI) during menstruation and pregnancy suggests that hormonal changes may also lower systemic resistance to gonococcal infection (Braude et al., 1978). Nowicki et al. (2000) hypothesized that these fluctuations may occur due to changes in complement levels during the menstrual cycle. In this study, GC sensitivity to serum from female volunteers drawn at different stages of the menstrual cycle was tested. Changes in the expression of surface molecules such as Opa proteins have been correlated with hormonal changes in women. James and Swanson (1978), showed that cervical isolates from women in the pre-ovulatory phase of the menstrual cycle expressed one or more Opa proteins, in contrast to gonococci obtained post-ovulation or during

menses, which were Opa-negative. Changes in Opa phenotype have also been observed in estradiol-treated mice inoculated with a predominantly Opa-negative population. The majority of vaginal isolates recovered post-inoculation expressed different Opa proteins, suggesting that some sort of selection for Opa protein expression is present in the female mouse, which may be similar to that in women (Jerse, 1999). An apparent selection for Opa expression was also observed during urethral infection of male volunteers (Jerse et al., 1994).

2) **Local pH**- Fluctuation in pH is a particularly relevant environmental stress in gonococcal pathogenesis, given that infected host tissues exhibit a wide rage of pHs. For example, the average vaginal pH in infected and uninfected women is 5.5 (range, 4.1 to 7.4), and the average pH of male urethral exudates is 7.3 [(range, 6.2 to 8.4), Hebeler et al., 1979). The pH of vaginal mucus is lower than that of endocervical mucus (Singer, 1975; Wolf and Litt, 1986), a characteristic that may restrict the gonococcus from colonizing the vaginas of females of reproductive age. McCutchan et al., (1977) reported that pH is the major anti-bacterial factor in urine samples that exhibit killing activity against GC. This phenomenon might explain the reason why the bladder and kidney are never infected in patients with gonococcal urethritis (Holmes et al., 1970). The effect of low pH on GC includes decreased bacterium hydrophobicity, and increased

negative charge on the surface of the bacterium (Magnusson et al., 1979). Expression of outer membrane molecules such as LOS and Hsp63 (heat shock protein 63), among others, is affected by acidic pH, suggesting a possible role for pH as a stimulus of gene regulation in the urogenital tract (Pettit et al., 1996; Pettit et al., 1999; Pettit et al., 1995; Pettit et al., 2001).

3) Iron sequestration-The requirement for iron is recognized as one of the critical steps in the growth survival of any pathogen inside a mammalian host. Indeed, the ability to obtain iron from host transferrin, ferritin, hemoglobin, and other iron-containing proteins is a central step in whether or not a pathogen lives or dies (Ratledge and Dover, 2000). Pathogenic *Neisseria* have developed several mechanisms of iron acquisition (Schryers and Stojiljkovic, 1999). Gonococci express receptors for transferrin (Anderson et al., 1994; Cornelissen and Sparling, 1994; Cornelissen et al., 1992), lactoferrin (Biswas and Sparling, 1995; Biswas et al., 1999) and hemoglobin (Chen et al., 1996; Chen et al., 1998), thereby increasing the availability of host iron resources for successful colonization and persistence in the human genital tract. Originally, transferrin was not thought to be an important source of iron during genital tract infection, due to the low levels of transferrin on mucosal surfaces (Bridges and Seligman, 1995). However, the importance of the gonococcal transferrin receptor as a mechanism of iron acquisition during infection, and therefore, the

importance of transferrin as a iron source during urethritis, was evidenced by Cornelissen et al. (1998). In this study, a *N. gonorrhoeae* transferrin receptor mutant was attenuated in a male volunteer urethritis model, compared to the wild-type strain. This result indicated that the concentration of transferrin in the urethra was sufficient to support *in vivo* growth of wild-type gonococci. Lactoferrin is generally thought to be the preferred iron source for bacterial pathogens of the mucosal surfaces. Lactoferrin, however, appears to have no critical role during mucosal GC infection based on the fact that only 50% of gonococcal strains can utilize iron from lactoferrin (Cornelissen et al., 1998; Mickelsen et al., 1982). Gonococci can also utilize iron from hemoglobin (Hb), haptoglobin-hemoglobin (Hp-Hb) and heme, but not heme-hemopexin or heme-albumin for growth (Chen et al., 1996; Chen et al., 1998; Dyer et al., 1987; Turner el at., 1998). The *hpuAB* operon encodes for the HpuA and HpuB proteins that mediate iron acquisition from Hb and Hb-Hp (Lewis et al., 1997). Although the levels of heme on mucosal surfaces are low, gonococci can be exposed to an abundant supply of heme iron during menstrual bleeding probably in the forms of free Hb, Hb-Hp, complexed and free heme.

Siderophore-mediated iron acquisition is utilized by other pathogenic bacteria as a means to acquire iron (Ratledge and Dover, 2000). Siderophores are not produced by pathogenic *Neisseria*; however, siderophores released into the extracellular milieu of mucosal

surfaces by commensal flora may provide a potential source of iron. In 1985, West and Sparling demonstrated that the addition of siderophores to culture medium supported the growth of *Neisseria* sp. This result supports the presence of pathways for the uptake of iron-siderophore complexes. The gonococcal FetA protein, formerly known as FrpB, has been shown to bind ferric enterobactin and may be part of a system responsible for transporting the siderophore into the cell (Biegel Carson et al., 1999).

4) **Commensal Flora-** The lower genital tract of women is colonized by commensal microbes in contrast to the male urethra, in which no commensal flora are normally present except for in a very short region of the tip (Fontaine et al., 1982). The ecosystem of the female genital tract changes under the pressure of external stimuli, such as variations in hormone levels and their effects, and returns to the normal state when the stimulus is removed. Vaginal flora composition varies with age and hormonal levels (Gorbach et al., 1973; Hillier et al., 1993; Milson et al., 1993; Redondo-Lopez et al., 1990). In healthy women, the most predominant members of the vaginal flora are lactobacilli (Redondo-Lopez et al., 1990; Baron et al., 1993). The lack of lactobacilli and the predominance of anaerobic species results in the disturbed vaginal ecosystem that is characteristic of bacterial vaginosis (Eschenbach, 1993; Fredricsson et al., 1993). The consequence of bacterial vaginosis is an increased risk in acquiring some genital tract infections and their

adverse effects given that the antagonism normally exerted by the commensal flora is not present. Martin et al. (1999) and Sewankambo et al. (1997) demonstrated a correlation between HIV-1 acquisition and bacterial vaginosis. On the other hand, Faro et al. (1993) could not establish the same relationship between bacterial vaginosis and *Neisseria gonorrhoeae* or *Chlamydia trachomatis*, the two major causes of pelvic inflammatory disease (PID).

Lactobacilli utilize several mechanisms to antagonize potential pathogens of the genital tract, and species such as *L. acidophilus*, *L. crispatus* and *L. jensenii* have been the most commonly studied in antagonism studies (Antonio et al., 1999; Eschenbach et al., 1989; Hughes and Hillier, 1990; Klebanoff et al., 1991; Ocaña et al., 1999; Osset et al., 2001; Redondo-Lopez et al., 1990). Ways by which lactobacilli can antagonize other microorganisms include the following:

a. **Adherence and competitive exclusion**-Long-term colonization of the vagina requires adherence to epithelial cells. *In vitro*, bacterial adherence to the vaginal epithelium is dependent on factors such as temperature and pH (Zawaneh et al., 1979). Several studies have demonstrated the ability of lactobacillus to adhere to vaginal epithelial cells (Andre et al., 1995; Boris et al., 1998; Mardh and Westrom, 1976; Osset et al., 2001; Sobel et al., 1981). It has also been shown that lactobacilli interfere with uropathogen colonization of

uroepithelial and vaginal cells *in vitro*. Steric hindrance is most likely the mechanism by which this interference occurs, rather than specific receptor blockage (Chan et al.,1985; Reid et al., 1987), although specific receptor blockage by lactobacilli has been reported against *Gardenella vaginalis* and *Candida albicans* (Boris et al., 1998).

b. **Low vaginal pH and production of lactic acid**-The acidic pH of the vagina appears to be the primary mechanism by which vaginal flora is controlled. The average vaginal pH of reproductive-aged women typically ranges from 4 to 4.5. Based on this low pH, it is commonly believed that only acidophilic or aciduric species such as *Lactobacillus sp*. can colonize the vaginal microenvironment. Fatty acids and lactic acid secreted by vaginal epithelial cells are one source of vaginal acidification (Preti and Higgins, 1975). Lactic acid and fatty acids produced by lactobacillus metabolism also contribute to vaginal acidity. Boskey et al., 1999, determined the culture acidification rate of eight vaginal lactobacilli isolates to be 10^6 protons/bacterium/s. This rate, together with the estimated number of lactobacilli in the vagina, suggests that the presence of lactobacilli alone accounts for the degree of acidification in the vagina. *In vitro* studies have shown that lactobacillus acidification inhibits urogenital

pathogens (Young et al., 1956; Reid et al., 1985; Skarin and Sylwan, 1986).

c. **H_2O_2 production**- Vaginal commensal lactobacilli and other bacteria possess the ability to produce and release appreciable amounts of H_2O_2 (Eschenbach et al., 1989). The amount of H_2O_2 produced is dependent on the strain and certain environmental conditions, such as pH, which might increase or decrease its production (Barnard and Stintson, 1999; Pericone et al., 2000). H_2O_2 produced by bacteria can be autoinhibitory or toxic to surrounding microorganisms. Eschebanch et al., (1989) and Hawes et al., (1996) observed that approximately 6% of women with bacterial vaginosis were colonized by H_2O_2-producing lactobacilli, in contrast to 96% of normal women. These studies suggest that H_2O_2-producing lactobacilli play a role in preventing bacterial vaginosis (BV); however, BV still can occur in the presence of H_2O_2-producing lactobacilli (Rosenstein et al., 1997).

d. **Bacteriocins, bacteriocin-like substances and biosurfactants**- Bacteriocins and bacteriocin-like substances are small molecules of proteic origin produced by bacteria that have a narrow or a broad spectrum of activity, respectively. These substances can inhibit strains of the same or of closely related species (Jack et al., 1995), a property

that is advantageous to microorganisms in polymicrobial ecosystems such as the female genital tract (Boris and Barbes, 2000). Although *in vitro* studies have shown that some species of vaginal lactobacilli produce bacteriocins that are inhibitory against other bacteria, no study has been conducted to directly elucidate the role of these substances in the female genital tract (Coconnier et al., 1997; Kanatani et al., 1995; McGroarty and Reid, 1988; Ocaña et al., 1999). Biosurfactants comprise a wide range of compounds such as glycolipids, lipopeptides, phospholipids, substituted fatty acids and lipopolysaccharide. These compounds facilitate the entry of water-immiscible substrates, and can also exhibit antimicrobial activity. *L acidophilus* produce a biosurfactant called surlactin that interferes with adherence of *E. faecalis*, *E. coli* and *S. aureus* to silicone rubber (Millsap et al., 1994; Velraeds et al., 1998).

e. **Stimulation of the immune system**-The capacity of *Lactobacillus sp*. to stimulate the immune system has not been studied in depth (Perdigon et al., 1986 and 1987). Perdigon et al. (1988) observed when mice were fed fermented milk containing *L. casei* and *L. acidophilus* for 8 days, an increase in both phagocytic and lymphocytic activity was achieved. This result suggests that *Lactobacillus sp.*

might enhance the immune response in the gut mucosa. No study has yet been conducted to measure the immune response in the genital tract of mice colonized with *Lactobacillus sp.*

5) **Antagonism of GC by commensal flora**-Studies describing antagonism by genital flora species against gonococci are very limited (McBride et al., 1978; Saigh et al., 1978; Zheng et al., 1994). Saigh et al. (1978) carried out a survey of endocervical flora from 229 women. The flora were tested for their ability to inhibit gonococci in an overlay assay. Streptococci, staphylococci and lactobacilli were the most active in terms of gonococcal inhibition. It was also observed in this study that women with gonorrhea were less often colonized by lactobacilli. This result suggests that lactobacilli may prevent gonorrhea. Zheng et al. (1994) found that when GC was co-cultivated with H_2O_2-producing and non H_2O_2-producing lactobacilli at pH 7.2, the viability of GC was not affected. An increase in gonococcal catalase activity was observed, however in the presence of H_2O_2-producing lactobacilli. The effect was abrogated with the addition of exogenous catalase. At low pH (4.8-5.0), co-cultivation of GC with H_2O_2-producing lactobacilli resulted in decreased GC cell viability, and the addition of exogenous catalase partially abrogated the effect. The inhibitory activity of lactobacillus lysate was heat stable but was eliminated by proteinase

K treatment. This study concluded that lactobacillus use several mechanisms to antagonize GC, including acidification, H_2O_2-production and the release of inhibitory proteins.

D. Phagocytic surveillance and bacterial mechanisms against phagocytic defenses

1) Oxygen-independent killing

a. Definition and overview

Neutrophils utilize both O_2-independent and -dependent mechanisms to kill bacteria. O_2-independent killing is mediated by acid production (decrease of pH in the vacuole after phagocytosis), the release of lactoferrin which sequesters iron, and an array of proteins with diverse functions such as hydrolytic enzymes, lysozyme, cationic proteins and neutral proteases. Vacuolar acidification is a process driven by the vacuolar proton-ATPase and the alkalinization effect of the NADPH oxidase activity. In neutrophils, alkalinization of the vacuole occurs early after ingestion of microorganisms, but by 60 min the phagosome acidifies to a pH of 5-6 (Cech and Lehrer, 1984). Some microbes are inhibited by this mechanism, but many others are either resistant to a mildly acid pH or block the acidification process (Horwitz and Maxfield, 1984; Rathman et al., 1996).

One of the effects of vacuolar acidification is to control the intracellular growth of pathogens. A correlation between vacuolar acidification and the role of the Nramp 1 protein (natural resistance-associated macrophage protein) has been demonstrated in macrophages. The Nramp 1 protein is recruited to the phagosome membrane in infected macrophages. The pH of the phagosomes from macrophages infected with live *Mycobacterium* expressing Nramp1 protein are more acidic as compared to macrophages obtained from Nramp1-deficient mice. This data suggests the possible role of Nramp 1 protein in phagosomal pH modulation and as a consequence in control of microbial intracellular growth (Hackman et al., 1998).

Another mechanism employed by macrophages and non-phagocytic cells to limit microbial intracellular replication is the induction of indoleamine 2,3-dioxygenase to deplete intracellular tryptophan (Murray et al., 1989; Taylor and Feng, 1991). A similar strategy can be delineated for iron, the transport of which is diminished by macrophage activation (Byrd and Horwitz, 1989; Gebran et al., 1994).

Neutrophils deliver high concentrations of preformed antimicrobial substances to the phagosome that directly damage the microbial target (Ganz, 1999). These antimicrobial components are contained in granules, and are classified based on density,

composition and response to various stimuli (Borregard and Cowland, 1997; Egesten et al., 1994; Rice et al., 1987). The granule formation occurs during the promyelocyte and myelocyte stages of granulocyte development in the bone marrow (Borregaard and Cowland, 1997). The densest primary granules (or azurophilic granules) consist mostly of small cationic microbicidal peptides called defensins. The classic azurophils or primary granules contain the larger cationic proteins such as MPO, elastase, cathepsin G, azurocidin and lysozyme. Lighter granules, also known as secondary specific granules, contain lysozyme, lactoferrin and a group of microbicidal peptides called cathelicidins. The contents of dense and intermediate primary granules are released primarily to the phagososme, while the secondary granules are largely secreted into the extracellular milieu (Leffel and Spitznagel, 1974; 1975).

b. Gonococcal defense mechanisms against phagocytes

Oxygen-independent killing of GC by PMNs has not been studied in depth. The role of phagosome formation in PMN killing of GC was suggested by Densen and Mandell (1978). In this study, treatment of PMNs with cytochalasin B abrogated killing of GC. This result suggests that internalization and probably phagosome formation were necessary. Exposure of the bacteria to neutrophil

granular extracts resulted in decreased viability of GC (Casey et al., 1986; Rest, 1979; Rock and Rest, 1988). Some granular components such as the cationic proteins, cathepsin G (Cohen and Sparling, 1992; Rest, 1979) and protegrins (Qu et al., 1996; Qu et la., 1997) have antimicrobial activity against GC.

Neisseria gonorrhoea IgA1 protease has been proposed to be used by GC to prevent phagolysosome formation as a consequence of acidification. GC produces two phenotypically distinct types of IgA1 proteases, each of which cleaves a specific peptide bond in the hinge region of the human IgA1 heavy chain (Lin et al., 1997; Mulks and Knapp, 1987; Mulks et al., 1987; Shoberg and Mulks, 1991; Simpson et al., 1988). Human IgA1 is not the only molecule that can serve as a substrate. Lysosome-associated membrane protein (LAMP) has been identified as another substrate for GC IgA1 protease (Lin et al., 1997). Lamps are present in vertebrate species. Only two distinct molecules, h-lamp-1 and 2, have been characterized in humans (Carlsson et al., 1988; Fukuda et al., 1988). Although conserved during evolution, the function of Lamp proteins is unclear. It is believed that they may protect the lysosomal membrane from the action of degradative enzymes within the lysosome by forming a carbohydrate coat on the luminal face of the membrane (Hunziker and Geuze, 1996; Peters and von Figura, 1994). Hauck and Meyer

(1997) demonstrated the presence of GC in Lamp-1 positive vacuoles after uptake by professional phagocytes and epithelial cells. Furthermore, purified Lamp-1 from epithelial cells, but not from phagocytes, was cleaved by GC IgA1 protease. The role of IgA1 protease in dissemination of GC from infected epithelial cells was tested by comparing the capacity of an IgA1 protease mutant and wild-type strain to transit through polarized T84 cells (Ayala et al., 1998; Hopper et al., 2000a,b). The role for IgA1 protease in gonococcal dissemination is supported by the finding that fewer numbers of the IgA1 protease mutant exited from the epithelial cells compared to the wild-type strain

It has also been hypothesized that gonococcal IgA1 protease may allow GC to escape the acidic environment of the phagolysosome by preventing its formation or by promoting the release of GC to the cytoplasm (Hedges et al., 1998). Recently the ability of gonococcal IgA1 protease to inhibit TNFα-mediated apoptosis was demonstrated (Beck and Meyer, 2000). This result suggests that gonococci might use phagocytes as a mechanism to evade host defenses and to disseminate to other tissues. The role for IgA1 protease in dissemination of GC from the mother to the fetus is implicated by the demonstration that gonococcal IgA1 protease can also cleave human chorionic gonadotropin hormone (HCGH) (Senior et al., 2001). Although, it is important to mention

that during a typical GC infection the bacteria do not encounter human HCGH, and as consequence the proposed mechanisms is not generally relevant to gonococcal pathogenesis.

2) Oxygen-dependent killing

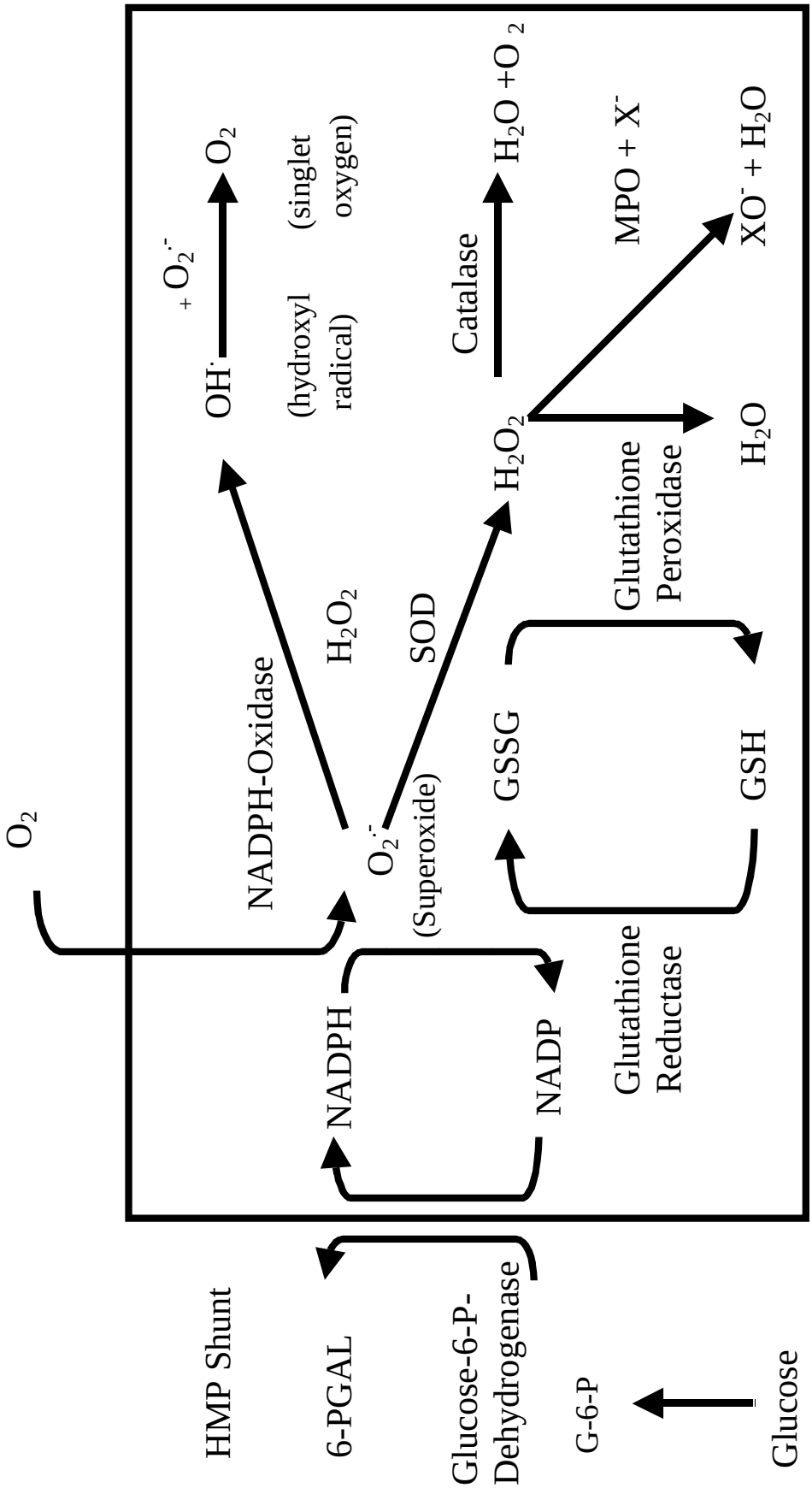
a. Definition

O_2-dependent killing by PMNs is a consequence of the pathogen-phagocyte interaction triggering the oxidative burst (**Fig. 3**). The oxidative burst is characterized by high consumption of oxygen and glucose, and the formation of toxic oxygen radicals such as superoxide, hydrogen peroxide, hydroxyl radical and halogenated products that are deleterious to the pathogen (Clark, 1990).

b. Toxic Oxygen radicals

Neutrophils, monocytes, monocyte-derived macrophages and eosinophils generate superoxide ($O_2^{\cdot}$) through a single electron transfer from NADPH to molecular oxygen. The electron transport chain in phagocytes is different from that in other cells since all the components needed must first assemble, which requires several steps of activation (Leusen et al., 1996). Subsequent to the formation of superoxide, a variety of important oxygen intermediates with greater antimicrobial effects are produced downstream. Superoxide is unstable and will react with itself to

Figure 3. Oxidative burst. The process of oxidative burst is characterized by the high consumption of glucose and molecular oxygen (O_2). The generation of superoxide, catalyzed by the enzyme NADPH oxidase, is a committed step towards the production of toxic oxygen radicals. From this point on, derivatives of superoxide ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2) are formed in subsequent reactions. Abbreviations: G-6-P (glucose-6-phosphate), 6-PGAL (6-phosphoglyceraldehyde), GSSG (oxidized glutathione), GSH (reduced glutathione), SOD (superoxide dismutase), MPO (myeloperoxidase), X^- (halide ion such as Cl^-) and XO^- (hypochloride). Diagram modified from Bellanti and Kladec, 1985.


Glucose
G-6-P
Glucose-6-P-Dehydrogenase
6-PGAL
HMP Shunt
NADPH
NADP
Glutathione Reductase
GSH
GSSG
Glutathione Peroxidase
H_2O
H_2O_2
Catalase
$H_2O + O_2$
MPO + X^-
$XO^- + H_2O$
SOD
$O_2^{\cdot -}$ (Superoxide)
H_2O_2
NADPH-Oxidase
$OH^{\cdot}$ (hydroxyl radical)
$+ O_2^{\cdot -}$
O_2 (singlet oxygen)
O_2

form H_2O_2 in a pH-dependent fashion (Fridovich, 1978). H_2O_2 formation is promoted by the acidification of the phagocytic vacuole.

Hydroxyl radical can be generated by chemical combination of $O_2^{\cdot}$ and H_2O_2 in the presence of a catalyst such as iron (Henle and Linn, 1997). This chemical reaction is know as the Haber-Weiss reaction. In PMNs, the formation of hydroxyl radical can be limited by the secretion of lactoferrin and MPO, resulting in a reduction in the iron and H_2O_2, respectively, that are available to react with each other (Cohen et al., 1988). As a consequence, the magnitude of hydroxyl radical formation is decreased (Britigan et al., 1989; Winterbound, 1986). Macrophages are more likely to generate hydroxyl radicals by the Haber-Weiss reaction in the presence of the correct catalyst, than are neutrophils, given that they do not produce MPO or lactoferrin (Britigan et al., 1986). Singlet oxygen, another oxygen intermediate, is a high energy form of O_2 and its formation has been demonstrated indirectly (Krinsky, 1988).

In contrast to macrophages, neutrophils contain myeloperoxidase (MPO). The interaction between MPO, H_2O_2 and a halide ion such as chloride can lead to the formation of an OCl^- potent antimicrobial derivative (Dunkan and Touati, 1996; Klebanoff, 1988). Eosinophils possess their own peroxidase

(EPO), with a preference for bromide over chloride (Hurst and Barrette, 1989). There is evidence suggesting that EPO confers more efficient killing of some microorganisms (Locksley et al., 1982).

c. Bacterial mechanisms of defense

Pathogens have developed a variety of mechanisms to prevent and/or overcome the diverse defenses utilized by phagocytic cells (Hassett and Cohen, 1989; Storz et al., 1990). Three major types of strategies have evolved in microorganisms to promote survival in response to phagocytes.

The first mechanism consists of detoxifying enzymes and free-radical scavenging substrates. This mechanism includes enzymes such as superoxide dismutase (SOD), catalase and peroxidase. SOD is involved in the conversion of superoxide to hydrogen peroxide (Fridovich, 1997). Basically all bacteria that use oxygen as a terminal electron acceptor generate one or more SOD. In *E coli*, two types of SODs have been identified, based on the co-factor used. The SODs encoded by the gene *sodA*, utilize Mn^{++} as a co-factor. The SOD encoded by *sodB* uses Fe^{+}. Recently, a periplasmic Cu-Zn SOD encoded by *sodC* was identified in *E. coli* (Battistoni et al., 2000) and *Salmonella* (Canvin et al., 1996). Previously, Cu-Zn SOD was believed to be

only present in eukaryotes (Bannister et al., 1987). The activity of these enzymes depends on the cellular microenvironment. Regulation of *sodB* in *E. coli* is believed to be controlled by an iron sensitive transcriptional repressor (Moody and Hassan, 1984; Pugh and Fridovich, 1985). The importance of SOD as a defense mechanism against toxic oxygen radicals has been demonstrated by the increased sensitivity of SOD-deficient mutants to superoxide ion (Cantoni et al., 1989; DiGuiseppi and Fridovich, 1982).

Catalase, a second detoxifying enzyme, is the enzyme responsible for the conversion of H_2O_2 to water and molecular oxygen (Heimberger and Eisentark, 1988; Winquist et al., 1984). Catalases are classified into three different groups based on enzymatic properties and amino acid sequence. The first group that contains the monofunctional catalases is divided into two subcategories, typical and atypical catalases. The typical catalases are enzymes that closely resemble eukaryotic catalases in both size and heme content (Loewen, 1997). Catalases belonging to this group share significant similarity in a core region of 370 amino acids, based on sequence alignment. Typical monofunctional catalases consist of homotetramers of 55 to 65 kDa subunits containing heme b. They differ in the pH optimum for activity and thermal sensitivity. Examples of microorganisms possessing

typical catalases are *Micrococcus luteus* (Herbert and Pinsent, 1948), *Bordetella pertussis* (DeShazer et al., 1994), and *Campylobacter jejuni* (Grant and Park, 1995). In contrast to the typical monofunctional catalases, the atypical enzymes consist of larger protein subunits ranging form 80 to 84 kDa. They contain heme d instead of heme b, retain stability at 70^{o}C, and in 7M urea or 1% SDS, and have a broader pH range of activity (3.0 to 11.0). Catalases from *Bacillus firmus* (Hicks, 1995), *Escherichia coli* (Claiborne et al., 1979) and *Klebsiella pnemoniae* (Goldberg and Hochman, 1979) belong to this group.

The second group of catalases consists of the catalase-peroxidases. These enzymes are characterized by the ability to use o-dianisidine as a substrate, and by similarity to plant peroxidases. Catalase peroxidases consist of homotetramers of 80 kDa with a sharp pH dependency for activity. Examples of microorganisms possessing this enzyme are *Escherichia coli* (Claiborne and Fridovich, 1979), *Salmonella typhimurium* (Loewen and Stauffer, 1990) and *Mycobacterium tuberculosis* (Zhang et al., 1992). The peroxidase activity of these enzymes catalyzes the conversion of H_2O_2 to non-toxic species by the reduction of H_2O_2 in the presence of a second substrate.

The last group is composed of non-heme catalases. Originally this group of enzymes was referred to as the pseudo-

catalases due to insensitivity to common catalytic inhibitors such as azide and cyanide. The protein subunits of the non-heme-catalases are smaller in size compared to heme-containing catalases, but their thermal stability can be as high as 80^{o} C. The co-factor required for their activity is Mn^{++}. Microorganisms such as *Lactobacillus plantarum* (Kono and Fridovich, 1983), *Thermoleophilum album* (Allgood and Perry, 1986) and *Salmonella enterica* (Robbe-Saule et al., 2001) are examples of non-heme containing catalases.

The number of different catalase isoenzymes that are produced by a bacterium depends on the species. *E. coli* has two distinct catalase-hydroxyperoxidases, designated HPI and HPII (Claiborne and Fridovich, 1979; Claiborne et al., 1979; Triggs-Raine and Loewen, 1987). The HPI enzyme is encoded by the *katG* gene (Loewen et al., 1985). HPII synthesis is encoded by the *katE* gene, and is regulated by the *katF* loci (Loewen, 1984; Loewen and Triggs, 1984; Loewen et al., 1984). It is known that catalases from *E. coli* and *Salmonella* are regulated by environmental stimuli such as growth phase, pH, H_2O_2 and anaerobiosis (Finn and Condon, 1975; Meir and Yagil, 1990; Moustafa Hassan and Fridovich, 1978; Mulvey et al., 1990; Schellhorn and Stones, 1992). These two hydroxyperoxidases are controlled by a positive regulon of H_2O_2-inducible genes termed

oxyR (Christman et al., 1985; Mukhopadhyay and Schellhorn, 1997; Storz et al., 1990). Mutants in *oxyR* are more sensitive to H_2O_2 than the parental strain.

During aerobic growth of any microorganism, intracellular H_2O_2 is generated from different sources (Farr and Kogoma, 1991). Therefore, microorganisms that can grow in the presence of oxygen should possess catalase. Sources of H_2O_2 include the reduction of oxygen as a terminal electron acceptor, reactions catalyzed by oxidases and dehydrogenases, as well as oxidation of thiols, flavins, and ascorbate (González-Flecha and Demple, 1995; González-Flecha and Demple, 1997). H_2O_2, unlike superoxide ion, can diffuse through biological membranes and interact with a wide variety of cellular substrates. In a catalase deficient background, the rate of spontaneous mutation is increased due to DNA damage (DeRose and Greeg Claycamp, 1991). The killing of *E. coli* by H_2O_2 is bimodal; meaning that low (1-3 mM for mode-one killing) and high (>20 mM for mode two-killing) concentrations of H_2O_2 are more lethal than are intermediate concentrations (Imlay and Linn, 1986). Mode-one killing has been attributed to DNA damage by H_2O_2. The mechanisms behind mode-two killing is unclear, although it is known that it does not require iron or an electron source, and it is not due to DNA damage.

The second mechanism used by microorganisms against the effects of toxic oxygen radicals consists of DNA repair systems. Oxygen radicals can damage DNA and also membranes by targeting proteins and lipids. Molecules such RecA, Exonuclease III, and polymerase I and II play a role in DNA repair (Demple and Harrison, 1994). The effect of superoxide ion on DNA has not been studied in depth. It is known that exposure of *E. coli* to paraquat, an inducer of superoxide ion, activates the SOS response (Brawn and Fridovich, 1985). Chan and Weiss (1987) showed that superoxide generation increased the activity of endonuclease IV by ten-fold, an effect not observed in the presence of H_2O_2 (Demple et al., 1986). DNA repair components are clearly involved in defense against H_2O_2 and have been proposed to have a role in mode-one killing (Hagensee and Moses, 1989; Imlay and Linn, 1987; Yonei et al., 1987). RecA is a protein that mediates recombinational repair (Sancar and Sancar, 1988) and the role of the *recA* gene as a defense mechanism against toxic oxygen radicals has been explored extensively in *E. coli* and *Salmonella. E. coli* and *Salmonella* strains deficient in *recA* were attenuated in a mouse model of infection compared to the parental strain. In contrast, strains deficient in catalase and SOD did not show attenuation *in vivo*, although they were more sensitive to toxic oxygen derivatives *in vitro*. This result suggests that the *recA* gene is more relevant

than the detoxifying enzymes as a defense mechanism against toxic oxygen radicals (Buchmeier et al., 1995; Carlsson and Carpenter, 1980). *E. coli* strains deficient in other components of the repair system, such as exonuclease III, were 20 times more sensitive to H_2O_2 than the parental strain (Demple et al., 1983). Although the role of DNA repair components in the presence of toxic oxygen derivatives has been demonstrated, it is important to mention that most DNA repair enzymes are not inducible by H_2O_2 (Demple and Halbrook, 1983; Greenberg and Demple, 1988) with the exception of endonuclease IV (Chan and Weiss, 1987).

Finally, competition between bacteria and phagocytic cells for substrates is a very interesting mechanism of bacterial adaptation to oxidative stress. Regardless of the capacity of phagocytic cells to generate antimicrobial elements, several different microorganisms survive and multiply in inflammatory, infected foci (Veale et al., 1979). The effect of microbial competition for substrates varies from decreased production of toxic oxygen radicals due to a decrease in aerobic conditions (Britigan et al., 1988) to increased antioxidant proteins (Hassan and Fridovich, 1978) and production of microbial heat shock proteins (Young et al., 1987).

d. Gonococcal mechanisms of defense

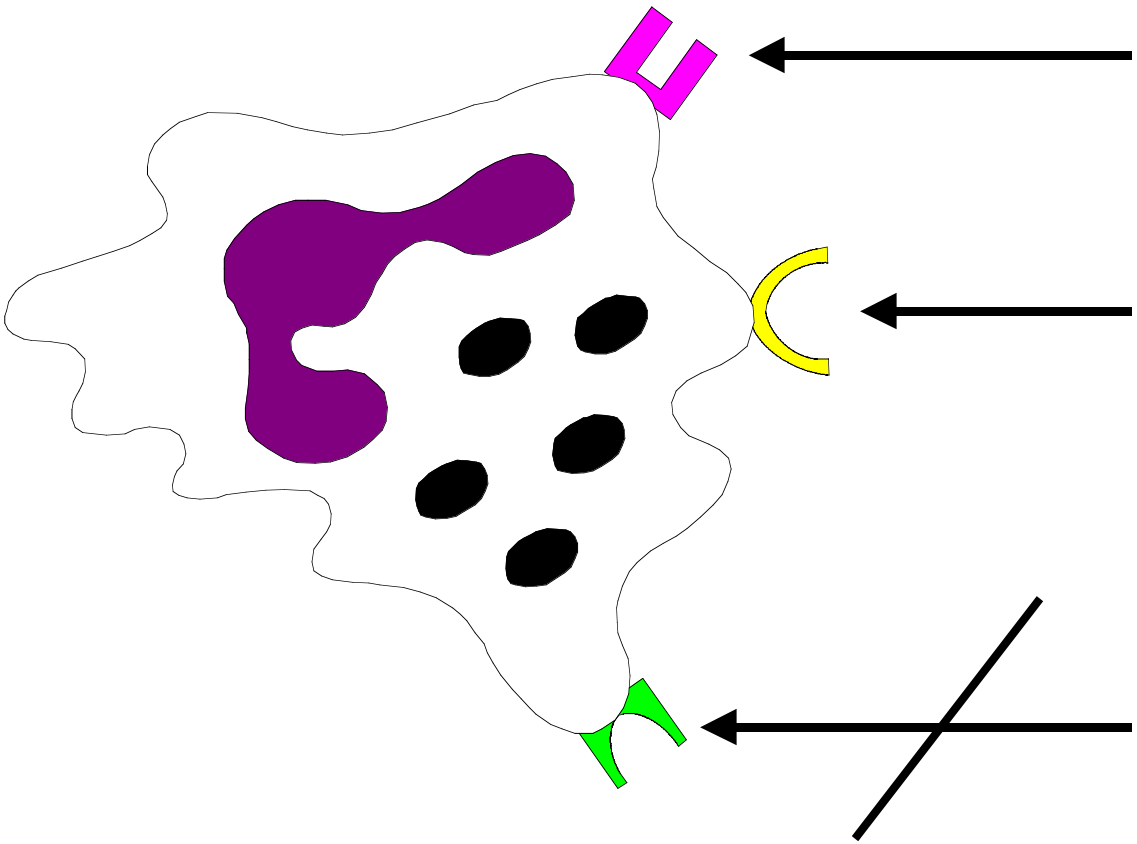
The hallmark of a symptomatic gonococcal infection is an intense inflammatory response characterized by an influx of neutrophils at the site of infection (Shafer and Rest, 1989). Neutrophils have been shown to play an important role in diseases produced by HSV-2 (Milligan, 1999) and *Chlamydia psittaci* (Buendía et al., 1999). Although neutrophils can phagocytize and kill gonococci, there is evidence suggesting that at least 3% of intracellular GC survive for 6 to 14 hours following ingestion (Casey et al., 1979; Casey et al., 1986; Daly et al., 1982; Parsons et al., 1981; Rest, 1979; Swanson and Zeligs, 1974a; Veale et al., 1976; Veale et al., 1979). Early studies suggested an association between gonococcal colony morphology and GC resistance to phagocytes (Dilworth et al., 1975; Ofek et al., 1974; Punsalang and Sawyer, 1973; Swanson and Zeligs, 1974b; Swanson et al., 1974; Thongthai and Sawyer, 1973). An increased resistance to phagocytes may occur in vivo as suggested by the observation that GC grown *in vivo* in chick embryos or in guinea pigs in subcutaneous chambers exhibited a higher resistance to phagocytosis compared to bacteria grown *in vitro* (Gibbs and Roberts, 1975; Witt et al., 1976).

How do PMNs take up GC? The initial association of GC with PMNs is proposed to be energy dependent. This hypothesis is supported by the decreased association of GC to human

neutrophils following inhibition of the respiratory chain with inhibitors such as KCN and amobarbital (Kenimer and Lapp, 1978; Weber et al., 1989). The gonococcus can be taken up by PMNs via three different portals of entry such as CGM1, and the complement and Fc receptors (**Fig. 4**). Entry into PMNs can be classified into two categories, nonopsonic and opsonic (Ross and Densen, 1985). In the opsonic pathway phagocyte receptors such as Fc and complement are used.

Nonopsonic uptake of GC by phagocytes is mediated by Opa proteins (Fisher and Rest, 1988; Naids et al., 1991). Rest et al. (1985), first showed that stimulation of human leukocytes by Opa-positive GC resulted in a vigorous oxidative burst (Naids and Rest, 1991). The role of Opa proteins in nonopsonic uptake by PMNs was confirmed by Elkins and Rest (1990) using monoclonal antibodies against Opa proteins to block the interaction of GC with human neutrophils. The PMN receptors utilized by Opa proteins are the CGM1 receptors of the CD66 family (Chen et al., 2001; Endes-Pener et al., 1994). Neutrophils can express 3 types of CD66, to which different Opa proteins exhibit different degrees of adherence (Naumann et al., 1999) (**Fig. 5**). Certain Opa protein-CD66 molecule interactions lead to activation of the cell death pathway or PMN apoptosis. This cell death probably correlates

Figure 4. Receptors expressed in PMNs. Fc, complement (C3a), and CGM1 (CD66) are some of the different receptors expressed in the surface of granulocytes that can be used to interact with microorganism during infection. The CGM1 receptor mediates nonopsonic uptake of GC in human PMNs. This receptor is not present in murine PMNs suggesting that nonopsonic uptake of GC by mouse PMNs will not occur.

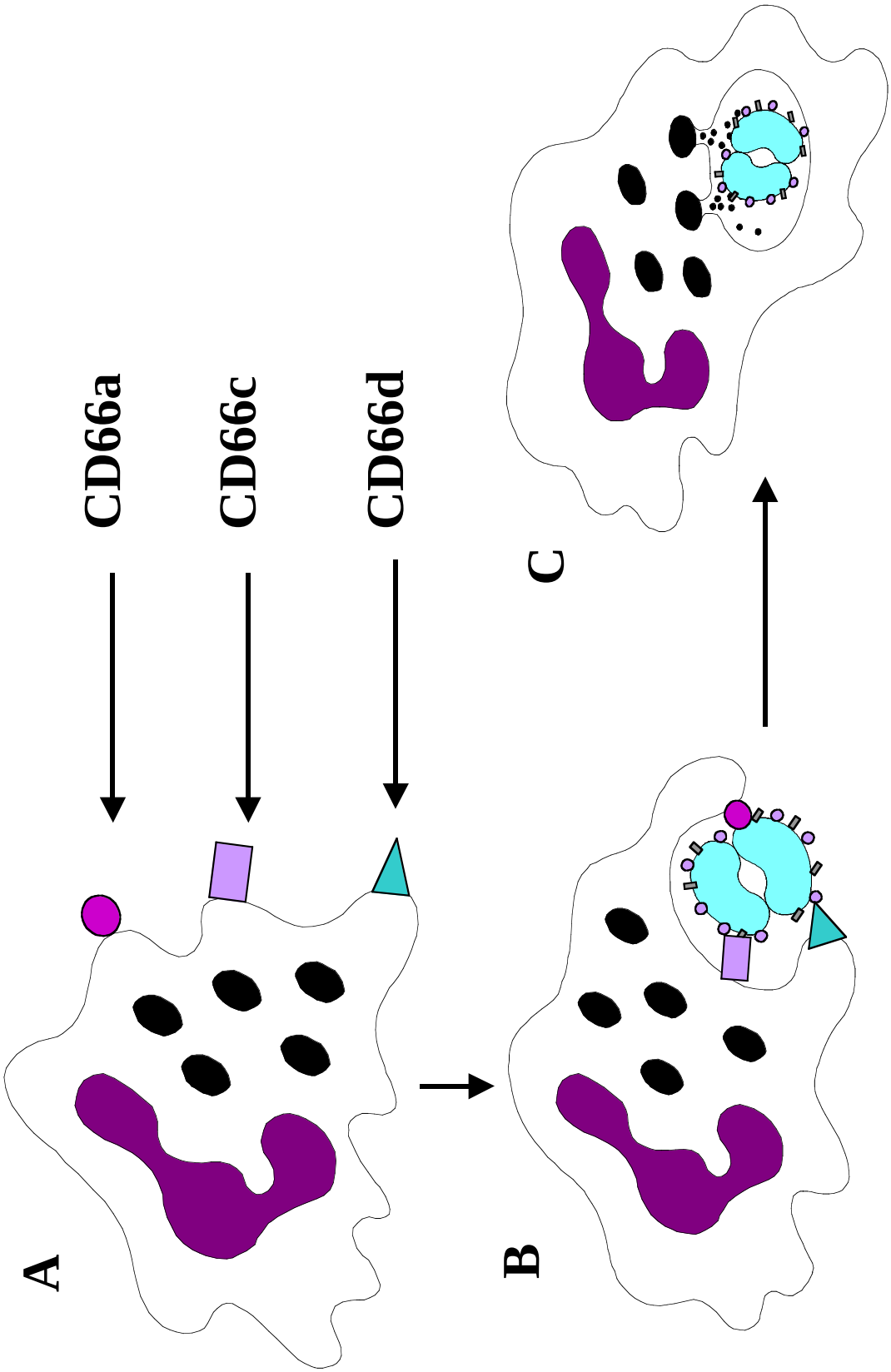


Fc Rceptor
(Immune serum)

Complement receptor
(Normal serum)

CGM1 Receptor
(Opa Mediated)

Figure 5. Interaction of Opa proteins with CD66 family of receptors (**A**). The interaction of GC with the members of the CD66 family of carcinoembriogenic receptors is mediated by the Opa proteins expressed on the surface of the GC. The CD66 receptors are not only expressed in granulocytes but also in epithelial cells. The type of Opa protein expressed determines cell tropism, given that specific Opas have been demonstrated to interact with specific members of the CD66 family of receptors. Interaction between the Opa proteins and their corresponding receptor leads to signaling in the host cell (not depicted), cytoskeleton rearrangement (**B**) and, finally, phagocytosis of the bacteria (**C**).


CD66a
CD66c
CD66d
A
B
C

with the previously described cytotoxic effects that GC has on human PMNs (Casey et al., 1983).

Porin, unlike the Opa proteins, interacts with PMNs apparently but does not mediate uptake. Instead, porin may play a role in evasion of phagocyte killing based on the demonstration that mutations in the gonococcal porin gene actually increased uptake of GC by human PMNs (Bauer et al., 1999). Porin also modifies the oxidative burst of human professional phagocytes, inhibiting degranulation and phagocytosis (Bjerknes et al., 1995; Haines et al., 1988; Lorenzen et al., 2000). Like Opa proteins, porin can activate the apoptotic pathway in neutrophils (Müller et al., 1999; Müller et al., 2000). Finally, sialylated LOS, in contrast to porin and Opa proteins, inhibits both opsonic and nonopsonic uptake of GC (Kim et al., 1992; Rest and Frangipane, 1992; Wetzler et al., 1992).

Once taken up by PMNs, GC encounters superoxide and other toxic oxygen radicals. However, despite the fact that GC are isolated from purulent exudates containing PMNs which are vigorously evolving superoxide radical ($O_2^{\cdot}$) and H_2O_2, many GC strains do not produce superoxide dismutase (Archibald and Duong, 1986; Ismail et al., 1977; Norrod and Morse, 1979). Norrod and Morse (1979) reported that only 20% of GC strains expressed detectable SOD activity and that this activity was

restricted to *sodB*. Tseng et al. (2001) detected the presence of *sodB* gene in different strains of *Neisseria gonorrhoeae* by Southern blot. Mutation of this gene in gonococcus strain 1291 did not alter sensitivity to superoxide anion. Although SOD does not exhibit a relevant role as a gonococcal mechanism against superoxide ion, GC may utilize Mn^{++} accumulation as an alternative defense. The hypothesis that Mn^{++} accumulation is a critical mechanism of defense against superoxide ion toxic effects is supported by the report that mutation in a gene responsible for the accumulation of Mn^{++} in GC rendered the bacteria more sensitive to superoxide ion compared to the wild-type strain, suggesting that The Mn^{++} can act as an $O_2^{\cdot}$ quenching agent by its oxidation to Mn^{+++}. Although Mn+++ normally is a highly reactive oxidant it can be stabilized by co-ordinating ligands such as pyrophosphate or carboxylic acids that can lower the redox potential of the Mn^{++}-Mn^{+++} (Coassin et al., 1992). This type of mechanism has been proposed for *L. plantarum* (Archibald and Fridovich, 1981).

It is hypothesized that gonococci use catalase as a primary defense against H_2O_2 released by PMNs during phagocytosis. GC generates high concentrations of catalase in the presence of sublethal concentrations of H_2O_2 and upon brief exposure to neutrophils (Zheng et al., 1992). A spontaneous gonococcal

catalase mutant from *N. gonorrhoeae* strain 2821 was shown to be extremely susceptible to H_2O_2 (Johnson et al., 1993). The nature of the catalase defect in this mutant was not characterized, however. Interestingly, Alcorn et al. (1994), reported that the H_2O_2 sensitivity of *N. gonorrhoeae* depends on factors in addition to catalase, since all the strains tested produced the same amount of catalase, but H_2O_2 sensitivity varied among strains.

In contrast to catalase activity, which is produced by all GC strains, peroxidase activity is a strain-dependent characteristic. Archibald and Duong (1986), as well as Jonhson et al., (1993) reported peroxidase activity in pathogenic strains of GC but not in nonpathogenic *Neisseria sp*., and a homolog of glutathione peroxidase was identified in *N. meningitidis* by Southern blot (Moore and Sparling, 1995). No peroxidase gene homolog was identified in GC.

An additional system that GC might utilize as a mechanism of defense against oxidative burst is DNA repair via the product of the *recA* gene. RecA is involved in DNA repair after damage from UV light and H_2O_2 or its derivatives. The *recA* gene was demonstrated to be more important than catalase and superoxide dismutase in H_2O_2 resistance in *E. coli* (Cantoni et al., 1989, Carlsson and Carpenter, 1980), and more important than catalase in *Salmonella typhimurium* (Buchmeier et al., 1995). Based on the

killing kinetics following exposure to increasing concentrations of H_2O_2, the *recA* gene of *E. coli* and *Salmonella* is thought to be an important mechanism of defense when concentrations of H_2O_2 are low, while catalase protects the organisms from high concentrations of H_2O_2 (Buchmeier et al., 1995; Imlay and Linn, 1986; Yonei et al., 1987). The gonococcal *recA* gene has been cloned; however, the role of RecA as a defense mechanism against H_2O_2 has not been studied in depth (Fyfe and Davies, 1990; Hassett et al., 1990; Koomey and Falkow, 1987). One study established that a GC *rec*A mutant was no more sensitive to H_2O_2 than the wild-type strain (Hassett et al., 1990). This result suggests that RecA may not confer protection against oxidative burst by PMNs; however, more detailed studies are needed to address the role of RecA in GC infection.

GC as a defense mechanism also utilizes competition with PMNs for substrates. For example, GC obtain iron from the lactoferrin secreted by neutrophils via expression of a lactoferrin-binding receptor (Biswas and Sparling, 1995; Biswas et al., 1999; Cohen et al., 1988). This mechanism is an important defense given that hydroxyl radical can be generated by the chemical combination of $O_2^{\cdot}$ and H_2O_2 in the presence of a catalyst such as iron (Henle and Linn, 1997). The potent anti-gonococcal activity

of hydroxyl radical has been demonstrated *in vitro* (Cohen et al., 1987; Hassett et al., 1989; Hassett et al., 1987).

A second substrate that may be utilized by GC to its advantage is lactate. Lactate is secreted by phagocytes during oxidative burst. Lactate consumption by GC is mediated by the presence of two lactate dehydrogenases (Fischer et al., 1994). Among the two isoenzymes, the membrane-bound species exhibits a broad substrate specificity (Bhatnagar et al., 1989; Hendry et al., 1990). When GC was exposed to human serum, an increase in gonococcal metabolism, as reflected by increased O_2 consumption and glucose, was observed (Cohen and Cooney, 1984; Britigan et al., 1988). The serum factor stimulating gonococcal metabolism was lactate (Britigan et al., 1988). Competition for the lactate produced by phagocytes can lead to the generation of an anaerobic environment, and as a consequence, may decrease the O_2 available for the PMN oxidative burst (Britigan and Cohen, 1986; Britigan et al., 1988; Fu et al., 1989). Another reason why lactate consumption is critical for pathogens is that lactate, which is an α-hydroxy acid, enhances hydroxyl radical generation in the Fenton reaction (Aktar Ali et al., 2000).

PMNs have been shown to generate nitrite, albeit in low concentrations (McCall et al., 1989). Interestingly, gonococci can utilize nitrite as a terminal electron acceptor under anaerobic

conditions via the function of the lipoprotein AniA (formerly known as Pan1), a nitrite reductase (Boulanger and Murphy, 2001; Clark et al., 1987; Hohen and Clark, 1992a,b; Householder et al., 1999; Mellies et al., 1997; Knapp and Clark, 1984). Expression of *aniA* enhances resistance against normal human serum (NHS), and therefore the generation of an anaerobic environment via lactate consumption by GC, may promote survival from O_2-dependent killing (Cardinale and Clark, 2000; Frangipane and Rest, 1992).

Another substrate used by gonococci is pyruvate. Once internalized by epithelial cells, GC binds to pyruvate kinase via its Opa proteins, and uses host pyruvate for growth (Williams et al., 1998). Pyruvate, as well as lactate, can increase sialic acid transferase activity (McGee and Rest, 1996). Also, lactate has been shown to increase gonococcal catalase activity (Hassett et al., 1990; McGee and Rest, 1996). It has been hypothesized that depletion of these substrates leads to the generation of an anaerobic environment, and as a consequence, activation of O_2-independent mechanisms such as defensins. It has been hypothesized, however, that GC cannot replicate in PMNs, due to the lack of nutrients. Therefore, defensins may not be effective against GC because their antimicrobial activity is exerted in metabolically active bacteria (Casey et al., 1985; Lehrer et al., 1989).

E. Serum resistance and evasion of the specific immune response

1) **Serum resistance**- The role of complement in killing GC was first elucidated with sera from patients exhibiting deficiencies in some of these components (Ellison et al., 1987; Harriman et al., 1981; Petersen et al., 1979). *Neisseria gonorrhoeae* is exposed to bactericidal factors derived from NHS during various stages of infection (Vogel and Frosh, 1999). For example, local inflammation at the site of infection exposes the bacteria to moderate concentrations of complement components. Complement activation products are deposited on the surface of gonococci in genital secretions of infected individuals (McQuillen et al., 1999).

GC strains differ in their ability to resist complement-mediated killing in the presence of NHS. Strains isolated from disseminated infection in the absence of symptoms are typically serum resistant. In contrast, serum sensitive strains tend to produce an acute localized disease (Rice and Kasper, 1982). Originally, the gonococcal serum resistance phenotype was explained in genetic terms. Two different loci, *sac-1* and *sac-3*, confer different levels of NHS susceptibility (Cannon et al., 1981; Shafer et al., 1982). More recently, the serum resistant phenotype has been classified as unstable and stable (Ram et al., 1999). Unstable serum resistance is mediated by sialylated LOS. Sialylation of LOS blocks the binding of bactericidal anti-LOS antibodies present in the NHS. Blocking

of anti-gonococcal antibodies also is attributed to the gonococcal P.III protein. The P. III protein is an analog of OmpA (porin). Antibodies against P. III block bactericidal antibodies against P. I and LOS (Joiner et al., 1985; Rice et al., 1986).

Stable serum resistance was recognized by the demonstration that some strains of GC remained serum-resistant even when they were grown in the absence of CMP-NANA (cytidine 5'-monophospho-N-acetylneuraminic acid, sialic acid). The stable serum resistance phenotype is attributed to porin type (Carbonetti et al., 1990). Two porin alleles have been identified, porin PIB is more frequently associated with disseminated strains while PIA is mostly associated with acute, localized strains (Brunham et al., 1985). PIB was demonstrated to bind factor H and the C4B factor of the complement components, and as a consequence, interferes with complement activation (Ram et al., 1998; Ram et al., 2001). The inhibition of complement activation via the mechanism described above, along with blocking of anti-gonococcal antibodies might explain the innate levels of resistance of some GC strains to killing by serum. It has been shown that specific anti-gonococcal antibodies can induce chemotaxis of neutrophils *in vitro* (Densen et al., 1987; Densen et al., 1982). Given that fact, blocking of this process would facilitate gonococcal dissemination by evasion of phagocyte surveillance.

2) **Evasion of the specific immune response**-Stimulation of the immune system during GC infection has been demonstrated by the production of pro-inflammatory cytokines in male volunteers (Ramsey et al., 1995). Evidence of lymphocytic reactivity (Rosenthal and Sandström, 1978), and the activation of NF-κB (Naumann et al., 1997) and the transcription factor AP-1 (Naumann et al., 1998) by GC further support the activation of the immune system. The production of local and systemic opsonic antibodies in infected individuals has also been demonstrated (Bisno et al., 1975). Repeated infections with *N. gonorrhoeae* are common among patients seen at sexually transmitted disease clinics. Interestingly, Hedges et al. (1999) showed that repeated gonococcal infections in males and females did not alter the local (IgA) and systemic (IgG) immune responses. The responses were modest and slightly higher compared to uninfected individuals. This result suggests that immunological memory is not induced during uncomplicated gonorrhea and/or gonococci have developed mechanisms to evade the humoral immune response.

Phase and antigenic variation of gonococcal molecules such as LOS (Wetzler et al., 1992), Opa proteins (Black et al., 1984; Kupsch, et al., 1993; Malorney et al., 1998) and pili (Hamrick et al., 2001; Seifert et al., 1988; Seifert, 1996) may contribute to evasion of the specific immune response. This

mechanism is best exemplified by pilin antigenic variation. Antibodies against pili block adherence to cells (Punsalang and Sawyer, 1973) and immunization with purified pilin protects against infection with a homologous pilin variant, presumably by the same mechanism demonstrated *in vitro* (Brinton et al., 1982).

The failure of a pilus vaccine trial, however, in which a specific pilin variant was used as the vaccine, clearly demonstrates how antigenic variation of GC surface molecules contributes to evasion of the immune response (Boslego et al., 1991; Tramont et al., 1981).

Finally, cleavage of the hinge region of IgA1 produced in the mucosa by gonococcal IgA1 protease may be another mechanism of immune evasion. An IgA1-protease deficient mutant was equally infective as a wild-type strain in the male volunteer model (Cohen and Cannon, 1999); however, it is important to mention, that the role of IgA1 is probably more critical in previously infected individuals.

IV- Tools utilized to study gonococcal pathogenesis

A. Genetic systems

The study of the genetic basis of gonococcal pathogenesis has been impaired by the lack of reliable genetic systems. In contrast to *E. coli* and other pathogens, gonococci lack suitable well-characterized vectors that can

be adapted for laboratory manipulation. Bacteriophages have not been identified in pathogenic *Neisseria*, although they have been isolated and studied from commensal species (Phelps, 1967; Steinberg et al., 1976; Stone et al., 1956). To address this need for genetic systems in *Neisseria* sp., cryptic (Korch et al., 1985; Sarandopoulus and Davies, 1993; Seifert and Wilson, 1992), conjugative (Roberts and Falkow, 1978; Sox et al, 1978) and penicillinase-encoding (Attardo Genco and Clark, 1988; Pagotto et al., 2000) plasmids have been isolated from GC and have been used as the backbone for the construction of shuttle vectors.

1) **Introduction of DNA into GC**- Genetic transfer in GC is mediated by two different mechanisms, transformation and conjugation (Cannon and Sparling, 1984). A variety of gonococcal genetic systems for generating transcriptional or translational fusions take advantage of these two mechanisms of DNA transfer in neisseriae.

    a. **Transformation**-Transformation consists of the uptake of naked linear or plasmid DNA, and in *N. gonorrhoeae*, is mediated by the surface pili present on the surface of the bacteria. Uptake of DNA by GC is restricted to molecules containing a 10 bp uptake sequence (Elkins et al., 1991; Goodman and Scocca, 1988), although uptake sequence-independent transformation has been reported (Boyle-Vavra and Seifert, 1996). One problem encountered with natural transformation is that once the DNA is taken up, the fragment is exposed to restriction

modification systems within the gonococcus. During transformation the DNA is transferred as a double-strand molecule, that is the substrate for restriction enzymes. Restriction modification systems can therefore reduce the efficiency of transformation as a way of introducing DNA to strains (Stein et al., 1988; Stein et al., 1995).

b. Conjugation-Conjugation is the direct transfer of DNA from a donor bacterium to a recipient. GC possess conjugative plasmids, which can be transferred from GC strain to another, and basically all gonococcal strains, and both piliated and nonpiliated variants, can serve as recipients during conjugation. In the laboratory the conjugal transfer of DNA from *E. coli* into GC can be mediated by an *E. coli* strain specifically engineered for this purpose. The transfer of DNA by conjugation is advantageous because the DNA is transferred as a single-stranded molecule, and therefore is not a substrate for innate restriction enzymes within the recipient bacterium.

2) Transposons-The attempt to utilize transposition as a way to mutate GC has not been extremely fruitful. *Tn1545*, a derivative of a transposon found in gram-negative bacteria, has been used in GC with limited success (Nassif et al., 1991; Piffaretti and Soldati, 1990). Transposition into cloned GC genes carried in *E. coli*, followed by

transformation of the mutated genes into GC to recombine the mutated piece of DNA into the gonococcal chromosome has been more successful. This technique called shuttle mutagenesis has been used to introduce mutations into pilin genes (Seifert et al., 1990) and genes involved in invasion (Kahrs et al., 1994), and LOS (Stephens et al., 1994).

3) Shuttle vectors-Shuttle vectors have been constructed for use in GC with fragments of the cryptic and conjugative plasmids from GC as the backbone. pLES2 and derivatives (Stein et al., 1983a; Stein et al., 1983b), Hermes vectors (Kupsch et al., 1996), and pJD5 derivatives (Pagotto et al., 2000) are good examples of shuttle vectors used in GC research. Problems with these vectors include large size of the plasmid, incomplete characterization of the backbone making genetic manipulation difficult, a limited number of restriction sites, and availability to the scientific community. As a consequence, complementation of mutations in gonococci via expression of cloned genes *in trans* is not customary in this field (Chaussee et al., 1999; Ramírez-Arcos et al., 2001).

4) Chromosomal integration systems-A few chromosomal integration systems have been designed. They consist of plasmid-based vectors that can replicate in *E. coli* for the performance of genetic manipulation but once transformed into the gonococcus, these vectors cannot replicate as an independent episome (Johnston and Cannon,

1999; Salvatore et al., 2000; Silver and Clark, 1995). The plasmid therefore must integrate into the gonococcal chromosome, an event that results in the desired mutation or chromosomal fusion. The sites of integration for some of these plasmids are silent loci in the GC genome such as the *proBA* (Silver and Clark, 1995) and *leuS* (Salvatore et al., 2000) loci.

5) Reporter gene fusions-Transcriptional regulation of gonococcal genes has been studied with limited success when compared with *E. coli* or *Salmonella* due to a lack of reliable genetic systems that are based on reporter genes to measure transcriptional activity. Plasmids such as pLES94 (Silver and Clark, 1995; Stein et al., 1984), pAErmC'G (Zhou and Apicella, 1996) and transposons to generate β-galactosidase (Boyle-Vavra and Seifert, 1993) and *phoA* (Boyle-Vavra and Seifert, 1995) fusions have been designed, and their functionality has been tested with known gonococcal promoters. It is important to mention that most of these systems generate a translational fusion and not a transcriptional fusion. Some of the gonococcal genes that have been studied using gene fusions are *pilE* (Laskos et al., 1998; Long et al., 2001), *tbpB* (Ronpirin et al., 2001), *aniA*, and other genes involved in gonococcal anaerobic adaptation (Lissenden et al., 2000). Techniques such as Northern blot and RT-PCR are more frequently used to generate data on how gonococcal genes are regulated (Tauschek et al., 1997).

B. Animal models for studying GC pathogenesis

Neisseria gonorrhoeae is a human specific pathogen, a characteristic that challenges the use of laboratory animals as an experimental host. A male volunteer model of urethritis has been used to study gonococcal pathogenesis, however information about pathogenesis in the female genital tract has been limited to *in vitro* systems ranging from morphological examination of clinical isolates on solid agar plates to fallopian tube organ cultures. Tissue culture systems have been used to characterize host cell-GC interactions (Christodoulides et al., 2000; Edwards et al., 2000; Giardina et al., 1998; Harvey et al., 1997; Hauck et al., 1997; Mosleh et al., 1997; Nassif and So, 1995). Tissue culture systems have led to the characterization and discovery of host receptors (Källstrom et al., 1997; Spence et al., 1997) and the corresponding gonococcal ligand (Hopper et al., 2000; Hopper et al., 2000; Ilver et al., 1998; Merz and So, 1997; Scheuerpflug et al., 1999; Spence and Clark, 2000), and the identification of cytokines induced by GC (Jarvis et al., 1999; Nakova et al., 2001). These models are limited in that they may not sufficiently mimic all the factors present in the complex microenvironment of the host, including the influence of an intact immune system on infection.

Despite the host restriction of GC, several animal models of gonococcal infection have been used in the elucidation of gonococcal pathogenesis in the genital tract.

1) **Disseminated gonococcal infection**- Injection of GC following pre-incubation with C1q into rat pups results in bacteremia and dissemination to different tissues. C1q is one of the three subcomponents (C1q, C1r, and C1s) of the first component of the complement system. The C1q model has been used to address questions related to serum sensitivity in GC strains isolated from DGI (Nowicki et al., 1995; Nowicki et al., 1997; Nowicki et al., 1999).

2) **Gonococcal arthritis**-Gonococcal arthritis is the clinical hallmark of disseminated gonococcal infection (DGI). Viable bacteria are not frequently isolated from the affected joints, and the damage observed in the tissues seems to be due to immunological injury. A model using rats or rabbits injected intra-articularly with GC or some of its components (LOS, peptidoglycan, etc.) has been used to study gonococcal arthritis. The resultant pathology consists of acute polymorphonuclear synovitis similar to that which is observed in cases of gonococcal arthritis (Flemming et al., 1986; Goldenberg et al., 1983; Goldenberg et al., 1984)

3) **Subcutaneous chambers**-Gonococcal infection of subcutaneous chambers implanted in rabbits, guinea pigs, hamsters, mice, and rats has also been described. This system consists of the implantation of a

plastic chamber into the subcutaneous tissue of the dorso-lumbar region of the animal. Among many animals tested, guinea pigs and mice are the two most frequently used. The original chamber consisted of a hollow polyethylene practice golf ball with holes in the wall that was sterilized before surgical implantation (Arko, 1972). Modifications such as using estradiol-treated mice as the host for the chamber have been made to enhance susceptibility to GC (Arko et al., 1997). The subcutaneous chamber model has been useful in studying the infectivity of clinical isolates and mutant GC, the immune response to infection, and the testing of vaccine candidates (Arko et al., 1997; Arko et al., 1979; Genco et al., 1991; Lambden et al., 1982; Novotny et al., 1978; Penn et al., 1976; Wannemuehler et al., 1982).

4) **Genital tract infection**-As previously mentioned, experimental gonococcal urethritis in male volunteers has been used to study different aspects of GC pathogenesis (Cohen and Cannon, 1999; Cohen et al., 1994; Schmidt et al., 2001). The male volunteer model has provided information about the role and expression dynamics of surface molecules such as Opa proteins (Jerse et al., 1994; Scheneider et al., 1995), LOS (Schneider et al., 1991; Schneider et al., 1995), and pili (Seifert et al., 1994; Schneider et al., 1995). A number of mutant GC strains have also been tested in this model including mutants in RecA, pilin, IgA1 protease, and the transferrin receptor. As an example, a mutant unable to express pilin due to a deletion in the

promoter region of the *pilE* gene was expected to be non-infectious given the fact that pili are critical for the initial contact of GC to host cells. Surprisingly the pilin-deficient mutant was able to colonize, and caused a mild urethritis, suggesting that pili are not essential for colonization of the male urethra. A *rec*A mutant, which was deficient in DNA recombination and antigenic variation of pilin, colonized the majority of subjects inoculated and caused asymptomatic infection (Cohen and Cannon, 1999). A gonococcal transferrin receptor mutant has thus far been the only mutant tested in the male volunteer model that was completely attenuated. This result suggests that transferrin is a critical iron source for GC in the male urethra (Cornelissen et al., 1998).

In spite of the high relevance of the male volunteer model to GC infection, some practical limitations must be considered. Restriction in the number of subjects tested, and the administration and cost of such a program limits the use of the model to a few laboratory settings. Additionally, only early stages of infection can be studied due to the ethical need to treat volunteers upon the onset of symptoms, and the data collected cannot be used to reliably explain gonococcal pathogenesis in women given the obvious differences between the male urethra and female cervix.

Laboratory animal models of GC genital tract infection may circumvent some of the limitations of the male volunteer model.

Productive genital tract infection has been reported in chimpanzees (Lucas et al., 1971) and mice (Kita et al., 1981; Taylor-Robinson et al., 1990; Kita and Kashiba, 1984; Kita et al., 1991). Other laboratory animals such as rats (Flynn, 1972) and macaques (Bowie et al., 1978) have been tested without a successful outcome. Urethral infection of male chimpanzees closely mimics that of men as evidenced by the presence of purulent exudates following urethra inoculation. Furthermore, infection was transferred from a male chimpanzee to two female chimpanzees (Lucas et al., 1971). Although the chimpanzee may be an excellent model for gonococcal pathogenesis, the size, maintenance and cost make this model unsuitable for most laboratory settings.

Gonococcal infection of the genital tract of female mice has also been successful. Kita et al., (1981) inoculated ddY mice intravaginally at different stages of the estrous cycle. These workers and others (Dalal et al., 2001) found that recovery of GC from the mouse genital tract was dependent on the stage of the estrous cycle. Further studies by Kita et al., compared the ability of Opa-positive and Opa-negative gonococci to infect mice. The results of these studies suggest that gonococcal Opa phenotype might affect the survival capacity of GC in the mouse genital tract, similar to that observed in clinical surveys (James and Swanson, 1978; Kita et al., 1991). A long-term recovery of gonococci was observed in ddY mice as compared to

other strains of mice tested when inoculated in the proestrus stage (Johnson et al., 1989). Manipulation of mice via estradiol treatment increases susceptibility to gonococcal infection. Specifically, administration of estradiol to two different strains of female mice, germ-free BALB/c and Caw:CF1, increased susceptibility to intravaginal infection with GC (Kita et al., 1985; Taylor-Robinson et al., 1990). Estradiol-treated female mice have also been used to study *Ureaplasma urealyticum* (Furr and Taylor-Robinson, 1989). Although long-term recovery of the bacteria was obtained, no influx of PMNs was observed at the site of infection. Jerse et al., (1999) also developed a murine model of gonococcal genital tract infection using estradiol-treated BALB/c mice that are also treated with antibiotics to reduce the commensal flora. In this model, GC was recovered from mice for 12-13 days following intravaginal inoculation with 10^6 CFU of strain FA1090. An influx of vaginal PMNs higher than that which occurs in placebo control occurred in 80% of infected mice.

IV- Specific aims

The main goal of my research project was to determine the contribution of *Neisseria gonorrhoeae* catalase in gonococcal pathogenesis. The following steps were taken to address this objective. First, the catalase gene of GC strain FA1090 was cloned and characterized. Second, a well-defined catalase mutant was constructed and characterized. Third, the role of the catalase gene was tested *in vivo,* using the newly

developed experimental murine model of genital tract infection, and fourth, the role of catalase GC catalase in defense against neutrophils was tested using isolated murine PMNs in *in vitro* assay.

Materials and Methods

I- Bacterial strains and culture conditions

All the bacterial strains and plasmids used and constructed in this study are listed in **Tables 1** and **2**. *N. gonorrhoeae* strain FA1090 and MS11 B2 were from the authors collection, and gonococcal strain F62 was obtained from D. S. Stein, University of Maryland at College Park; *Lactobacillus acidophilus, Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus jensenni* strains were from American Type Culture Collection, Manassas, VA; these strains produced H_2O_2 in the qualitative assay described by McGroarty et al. (1992).

Neisseria sp. were cultured on GC medium base with 5g/L additional Bacto agar with Kellogg's supplement I and II (Kellogg et al. 1968; Kellogg et al., 1963). Supplement I (400 g Glucose, 10 g L-glutamine and 20 mg co-carboxylase per L) and supplement II (1.25 g $Fe(NO_3)_3$ in 250 ml of H_2O) were added to media at a 1/100 and 1/1,000 dilution, respectively. For growth curves and H_2O_2 sensitivity assay, GC was grown in GC broth (15 g proteose peptone #3, 4 g potassium phosphate, dibasic; 1 g potassium phosphate, monobasic; and 5 g of NaCl per L) with Kellogg's supplements I and II. Broth was also supplemented with $NaHCO_3$ to a final concentration of 5 mM when cultures were grown in room air. For bacterial transformation experiments, broth was supplemented with 10 mM $MgCl_2$. Lactobacilli were cultured on "Lactobacillus MRS agar". GC strains were maintained in freezer storage medium (3 g trypticase soy broth and 25 ml of glycerol per 100 ml) at -84^{o}C. When antibiotic selection was necessary the following concentrations were used: kanamycin 50 μg/ml; erythromycin, 0.5 μg/ml; and streptomycin, 100 μg/ml.

Table 1. Bacterial strains utilized and constructed in this study

Strains	Relevant marker or genotype	Comments
1) ***Escherichia coli***		
a. DHαmcr	*lacZM15 hsdR hsdM mcrA mcrB mcrC mrrA*	Gibco- BRL
b. HB101	F^- *hsdS*20 (r_B^-, m_B^-) *thi*-1 *sup* E44 *ara* 14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20 (StrR) *xyl*-5 *mtl rec*A13 *mcr*B *leu*B6	Boyer and Roulland-Dussoix, 1969.
c. HS-4	*lacZ* $^-$, NaI ^{R}	Levine et al., 1983
d. S17-1	RP4 2-Tc*::*Mu-*Km::Tn*7, *pro*, *res*$^-$, mod$^+$	Simon et al., 1983
e. Top 10	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end* A1 *nup*G	*Invitrogen*, CA
f. UM255	*kat*G, *kat*F; double catalase mutant	Schwartz et al., 1983
2) ***Neisseria gonorrhoeae***		
a. FA1090	Sm ^{R}	Cohen et al., 1994
b. FA1090 P^-	Wild-type (nonpiliated variant)	This laboratory
c. NGAAS-500	FA1090 *kat* Δ1.2 kb*::aph*A-3	This study
d. NGAAS-501	FA1090 *kat* Δ 1.2 kb	This study
e. NGAAS-502	NGAAS-500 carrying plasmid pAAS20-*kat* 30	This study

f. NGAAS-503	NGAAS-501 carrying plasmid pAAS20-*kat* 30	This study
g. NGAAS-505	NGAAS-501 (nonpiliated variant)	This study
h. MS11 B2	Sm^R, nonpiliated	Boyle-Vavra and Seifert, 1996
i. F62		Clark et al., 1987
3) ***Staphylococcus aureus***		
a. RN6390B	agr +, derivative of RN450	Novick, 1967
4) ***Lactobacillus***		
a. *L. acidophilus* 4356	H_2O_2 producing; human isolate	ATCC
b. *L. jensenii* 25258	H_2O_2 producing; human isolate	ATCC
c. *L. crispatus* 33197	H_2O_2 producing; human isolate	ATCC
d. *L. gasseri* 33123	H_2O_2 producing; human isolate	ATCC

Table 2. Plasmid and cloning vectors utilized and constructed in this study (**Fig. 6**, **7** and **8**)

Plasmid	Relevant marker	Source
1) **pACYC184**	Cm and Tc resistance	Chang and Chen, 1978 Rose, 1988.
a. pAAS184-*kat* 30	2.4 kb *Cla* I-*Dra* I fragment carrying the GC *kat* gene	This study
b. pAAS184-*kat* Δ30.1d *km*	pAAS184-*kat* 30 Δ 1.2 kb *::* *aphA*-3	This study

c. pAAS184-*kat* Δ30.2d	pAAS184-*kat* 30 Δ 1.2 kb	This study
d. pAAS184-*kat*Δ (-)400	pAAS184-*kat* 30 $_{\Delta (-) 400*}$	This study
e. pAAS184-*kat*Δ(–)427	pAAS184-*kat* 30 $_{\Delta (-) 427*}$	This study
f. pAAS184-*kat* Δ(–)492	pAAS184-*kat* 30 $_{\Delta (-) 492*}$	This study
g. pAAS184-*kat* Δ(–)605	pAAS184-*kat* 30 $_{\Delta (-) 605*}$	This study
h. pAAS184-*kat* Δ(–)669	pAAS184-*kat* 30 $_{\Delta (-) 669*}$	This study
i. pAAS184-*kat* Δ(+) 47	pAAS184-*kat* 30 $_{\Delta (+) 47 *}$	This study
2) **pCR-Blunt**	Km and Zeocin resistance	*In Vitrogen*, CA
a. pCRAAS-*kat* 700I	PCR amplified internal 700 bp of the GC *kat* gene cloned into pCR-blunt	This study
3) **pLEE20**	Shuttle vector, *erm* resistance	Nassif et al., 1991 Stein et al., 1983 Stein et al., 1983
a. pAAS20-*kat* 30	2.4 kb *Cla* I-*Dra* I fragment carrying the GC *kat* gene cloned into pLEE20	This Study
4) **pUC18Km**	*Aph*A-3 non polar cassette	Menard et al., 1993

* Numbers refer to the number of nucleotides deleted upstream (-) and downstream (+) of the ATG of the cloned GC *kat* gene.

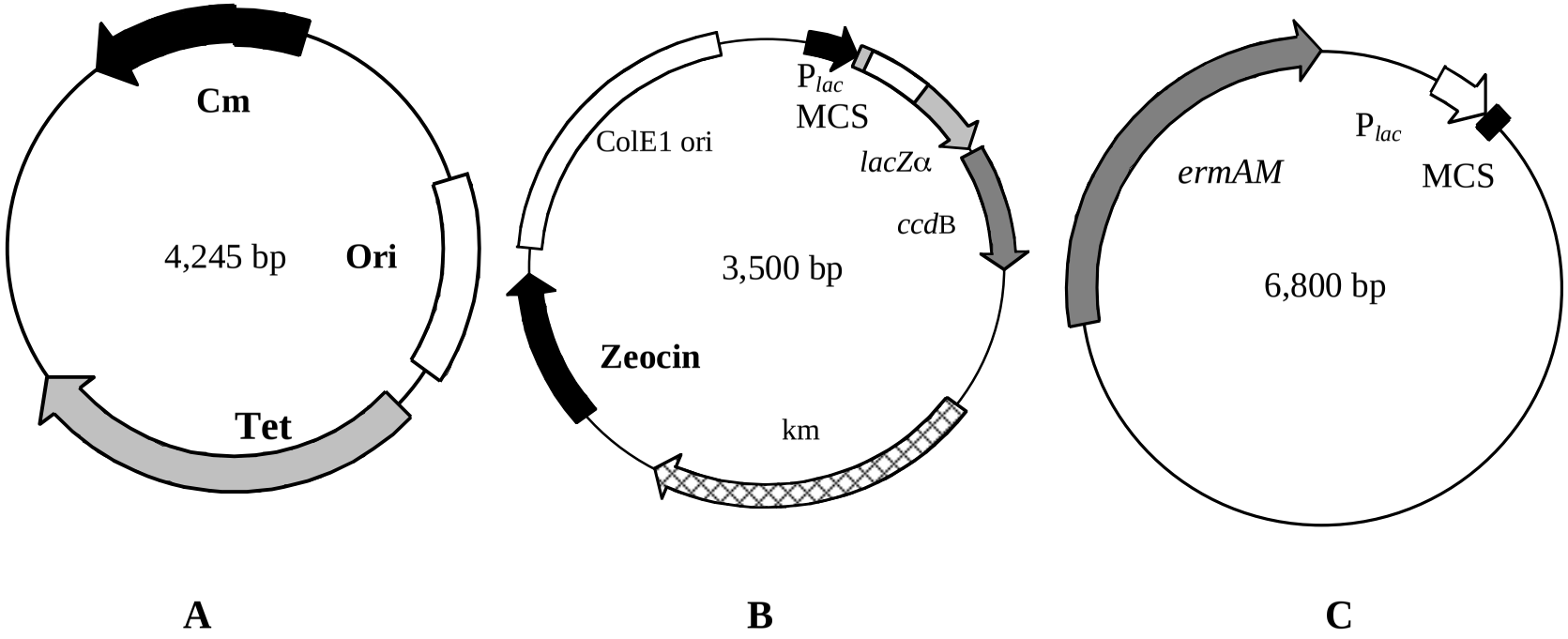


Figure 6. Cloning vectors used in the present research. (**A**) pACYC184, plasmid vector utilized in the cloning of the catalase gene. (**B**) pCR-blunt 3.5, vector used to clone PCR-amplified fragments. The presence of inserts is determined by blue/white selection on Xgal and also by successful interruption of the lethal gene *ccd*B. Only transformants with inserts in the plasmid are recovered. (**C**) pLEE20, shuttle vector that can replicate in *E. coli* and GC. This vector can be transferred to *N. gonorrhoeae* by conjugation and the screening of inserts can be done by blue/white selection on agar containing Xgal.

Figure 7. Schematic representation of deletions made in pAAS184-*kat* 30 to study the promoter region of the catalase gene. (**A**) pAAS184-*kat* 30, (**B**) pAAS184-*kat* 30 $_{\Delta (-) 400}$, (**C**) pAAS184-*kat* 30 $_{\Delta (-) 427}$, (**D**) pAAS184-*kat* 30 $_{\Delta (-) 492}$, (**E**) pAAS184-*kat* 30 $_{\Delta (-) 605}$, (**F**) pAAS184-*kat* 30 $_{\Delta (-) 669}$, and (**G**) pAAS184-*kat* 30 $_{\Delta (+) 47}$. RLS, *rpoN*-like sequence; RBS, ribosomal binding site; UPS, uptake sequence.

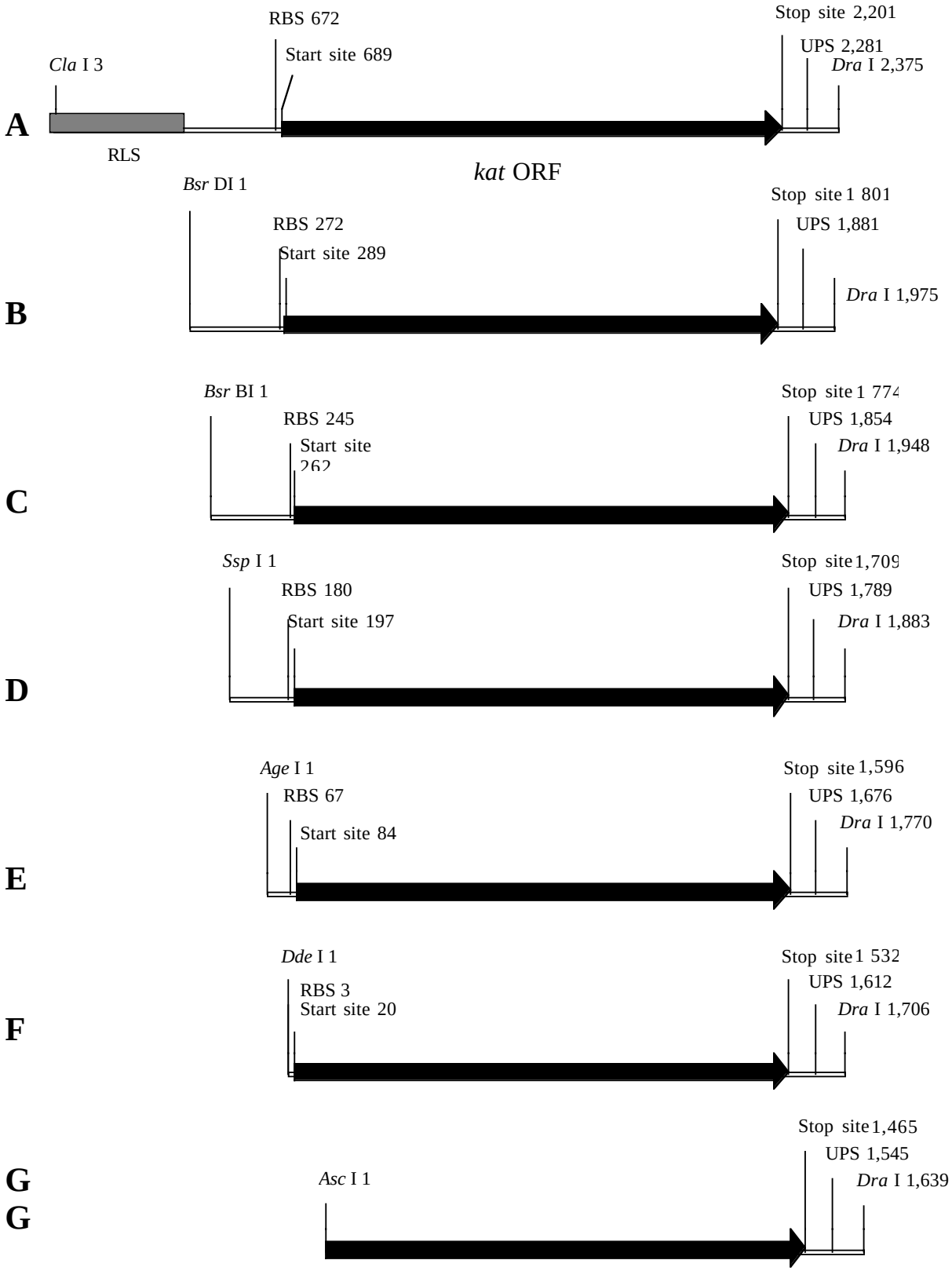
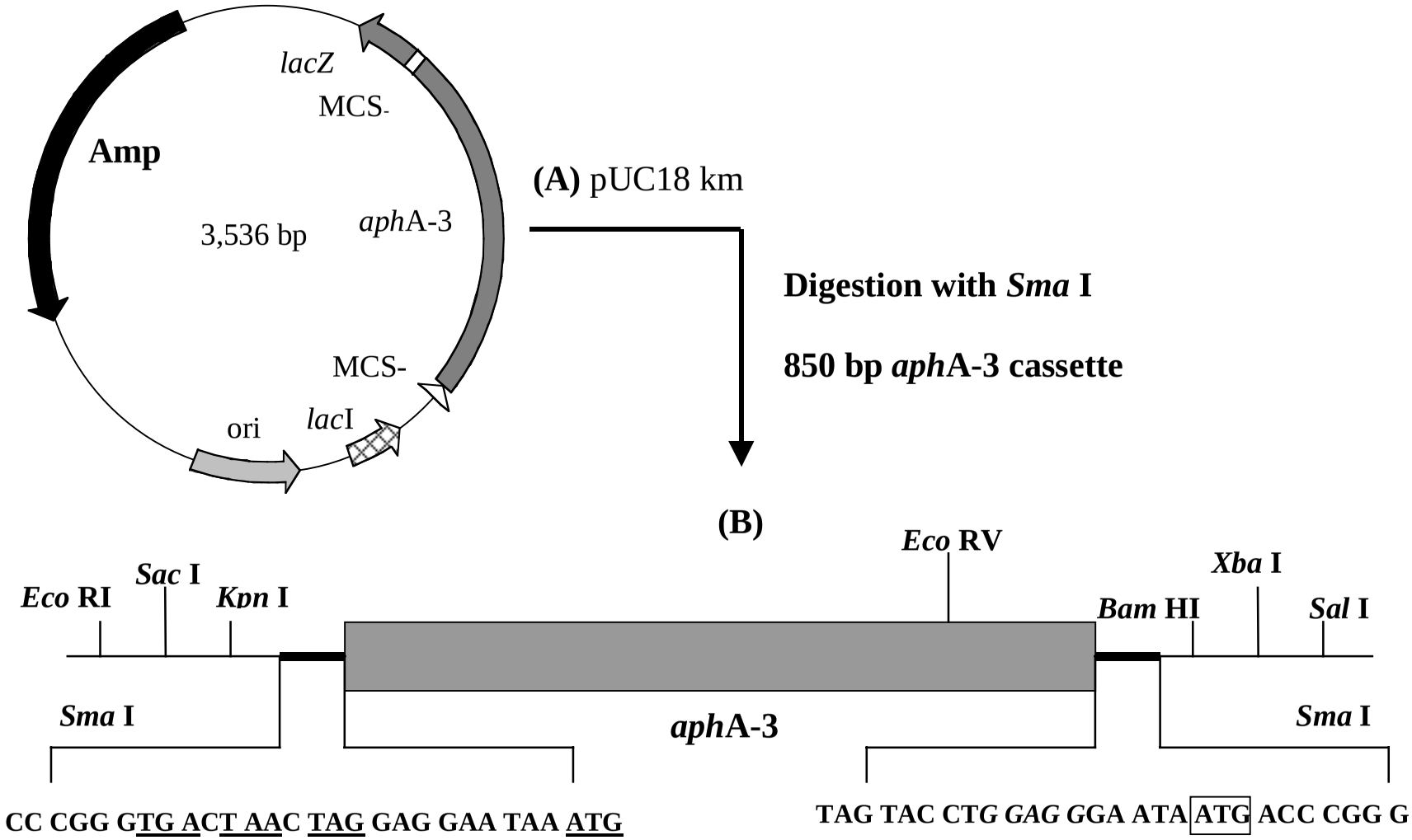

A
Cla I 3
RBS 672
Start site 689
Stop site 2,201
UPS 2,281
Dra I 2,375
RLS
kat ORF
B
Bsr DI 1
RBS 272
Start site 289
Stop site 1 801
UPS 1,881
Dra I 1,975
C
Bsr BI 1
RBS 245
Start site 262
Stop site 1 774
UPS 1,854
Dra I 1,948
D
Ssp I 1
RBS 180
Start site 197
Stop site 1,709
UPS 1,789
Dra I 1,883
E
Age I 1
RBS 67
Start site 84
Stop site 1,596
UPS 1,676
Dra I 1,770
F
Dde I 1
RBS 3
Start site 20
Stop site 1 532
UPS 1,612
Dra I 1,706
G
G
Asc I 1
Stop site 1,465
UPS 1,545
Dra I 1,639

Figure 8. Schematic representation of pUC18 km (**A**) and *aph*A-3 (**B**). The plasmid pUC18km contains a 850 bp kanamycin resistance cassette encoded by the gene *aph*A-3 inserted into the *Sma* I site (Menard et al., 1993). The kanamycin resistance cassette contains a start site preceded by 3 translational stop codons, which correspond to all three reading frames. A consensus ribosomal binding site, GGAGG (italics) and a start codon (box) are shown in the 3' stippled box. The downstream ATG provides the non-polar effect by reinitiating translation. Restriction enzymes sites found in the multiple cloning site of the vector are shown.


lacZ
MCS-
Amp
3,536 bp
aphA-3
MCS-
ori
lacI
(A) pUC18 km
Digestion with Sma I
850 bp aphA-3 cassette
(B)
Eco RV
Xba I
Sac I
Eco RI
Kpn I
Bam HI
Sal I
Sma I
aphA-3
Sma I
CC CGG GTG ACT AAC TAG GAG GAA TAA ATG
TAG TAC CTG GAG GGA ATA ATG ACC CGG G

In the case of mouse experiments, GC agar plates were supplemented with vancomycin, colistin, nystatin and trimethoprim (VCNT) GC and lactobacillus strains were incubated under 7% CO_2 at 37^{o}C.

E. coli strains were grown in LB (10 g tryptone, 5 g yeast extract, 10 g NaCl per L) and plates were solidified with 15 g of Bacto agar per L. All the strains were stored at -84^{o}C in LB containing 15% glycerol and the selective antibiotic. When antibiotic was necessary, the following antibiotic concentrations were used: erythromycin, 300 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 50 μg/ml; nalidixic acid, 100 μg/ml; tetracycline, 50 μg/ml; and ampicillin 100 μg/ml (broth) and 200 μg/ml (plates).

All media were purchased from Difco Laboratories (Detroit, MI) and prepared per manufacturer instructions.

II- DNA manipulation and recombinant techniques

Total genomic DNA from all bacterial strains, preparation of chemical and electrocompetent cells; electroporation and spin column purification were performed as described by Ausubel et al. (1994). Plasmid preps and DNA agarose gel extraction were done using Qiagen kits (Qiagen, CA). DNA restriction enzymes, DNA polymerase Deep Vent and 100 bp and 1 kb DNA molecular weight markers were obtained from New England Biolabs (NEB, MA). Cloning of PCR products and ligations were done by using Zero Blunt PCR cloning kit (*Invitrogen*, CA) and Ligation kit (Stratagene; La Jolla, CA) respectively. Primer and peptide synthesis and DNA and protein sequencing was performed by the USUHS Bio Instrumentation Center (BIC; USUHS, MD). PCR kits for Digoxigenin labeling of PCR products and chemiluminescence detection digoxigenin were

obtained from Roche Molecular Biochemicals, IN. Pre-stained protein molecular weight markers and the ECL Western blot detection system were obtained from Life-Technologies (MD) and Amersham-Pharmacia (UK), respectively. Nytran plus membranes and the Turboblotter system for agarose gel downward transfer were obtained from Schleicher & Schuell, NH. PVDF membranes for Western blot were obtained from BioRad (CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless stated.

Computer analysis such as Blast search and alignment was done using the NCBI website (ncbi.nlm.nih.gov). Primer design was performed using Program GeneRunner for Windows (Hasting Software, Inc.; Hasting, NY). Restriction mapping and identification of motifs were performed by Clone Map from CGC Scientific, Inc. (Balwin, MO). Protein hydrophilicity, hydrophobicity analyses, and analysis for surface exposed regions were performed using Clone Manager software (Durham, NC).

Statistical analyses were performed using the standard version of computer software SPSS for Windows. Susceptibility of wild-type and *kat* mutant GC to H_2O_2 and to inducers of toxic oxygen radicals was analyzed by unpair t-test. PMN killing assays were first analyzed by the nonparametric test Mann-Whitney U followed by t-test for comparison of the means. Recovery of bacteria from infected mice with either wild-type or *kat* mutant GC was analyzed by the unpair t-test.

Table 3. Sequences of oligonucleotides primers utilized in this study

Primer designation	Sequence	Use
18-97 F (M13)	5'-GTAAAACGACGGCCAG-3'	General sequencing
19-97 B (M13)	5'-GGAAACAGCTATGACCATG-3'	General sequencing
20-98 F	5'-AAGTTCGATACCCGGCATCTG-3'	GC *kat* gene sequencing
21-98 B	5'-GCCACAAATCTTGCGTCAGC-3'	GC *kat* gene sequencing
22-98 F	5'-TATGCGTTCCGCCACAAACAAC-3'	GC *kat* DNA probe
23-98 B	5'-GAAGCTGTTGGGTTCGTAGTGC-3'	GC *kat* DNA probe
25-99 F	5'-GCATATCTTCGCCTTCGTCTC-3'	GC *kat* gene sequencing
26-99 B	5'-CGTCTGAAACGGTCAAACGAC-3'	GC *kat* gene sequencing
32-99 B	5'-GAGACGAAGGCGAAGATATGC-3'	GC *kat* gene sequencing
33-99 F	5'-GCTGACGCAAGATTTGTGGC-3'	GC *kat* gene sequencing
34-99 B	5'-GTTGTTTGTGGCGGAACGCATA-3'	GC *kat* gene sequencing
35-99 F	5'-GCACTACGAACCCAACAGCTTC-3'	GC *kat* gene sequencing
36-99 F	5'-TTGCGCGTGCAGGTATTGCTTG-3'	GC *kat* gene sequencing
37-99 F	5'-AGCTGCTGGGTCTGTTTGAG-3'	GC *kat* gene sequencing
38-99 B	5'-AGTACGATTTGGCGGAAAGC-3'	GC *kat* gene sequencing
39-99 F	5'-GTATGCACGCCAAAGGTTCG-3'	GC *kat* gene sequencing
40-99 B	5'-TTTGTGGCGGAACGCATATTG-3'	GC *kat* gene sequencing

43-99 F	5’-AATGTAGCACCTGAAGTCAGCC-3’	Upstream of *Cla* I site in pACYC184
44-99 B	5’-GTGCGCATAGAAATTGCATC-3’	Downstream of *Eco*R V site in pACYC184
45-99 F	5’-ACAGCCGGTATAAAGGGACCAC-3’	Km^R cassette probe
46-99 B	5’-ACGCAGAAGGCAATGTCATACC-3’	Km^R cassette probe
47-99 F	5’-GTGGTCCCTTTATACCGGCTGT-3’	Km^R gene sequencing
48-99 B	5’-GGTATGACATTGCCTTCTGCGT-3’	Km^R gene sequencing
50-01 F	5’-CGTATAATCGCATCCATAG-3’	GC *kat* gene sequencing
51-01 B	5’-GGGCATTTGGAGGTAGTCAT-3’	GC *kat* gene sequencing
57-01 B	5’-GTGATGTCGGCGATATAGGC-3’	Downstream of *Bam* HI site in pACYC184

III- Cloning and characterization of gonococcal catalase gene

A. Cloning of gonococcal catalase gene

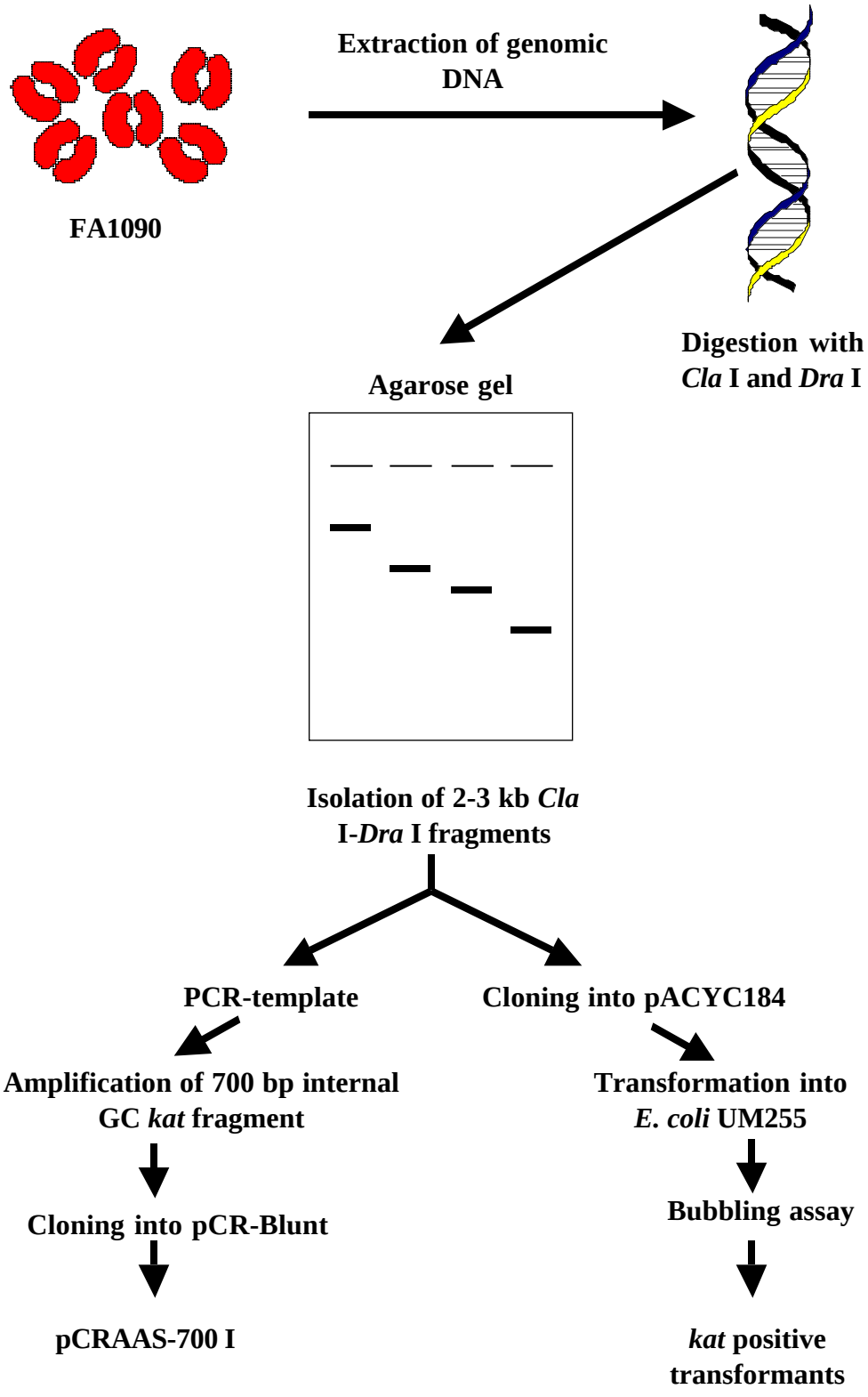
The gonococcal catalase gene was cloned by conventional methods. **Figure 9** explains the procedure.

B. PCR-amplification and labeling of DNA probes

Three different probes were used in the Southern blots. All three probes were labeled using the Genius PCR-Dig labeling kit following the instructions of the manufacturer. The catalase probes consisted of a 700 bp internal fragment and the whole 2.4 kb *Cla* I-*Dra* I DNA fragment containing the gonocococcal *kat* gene. The third probe consisted of 560 bp of the km^R gene. The 700 bp fragment was amplified by PCR using primers 22-98 F and 23-98 B and size fractionated (2 to 3 kb) fragments from *Cla* I, *Dra* I digested FA1090 genomic DNA as the template. The resultant PCR fragment was cloned into PCR-blunt plasmid following the manufacturer's instructions, and transformed into *E. coli* Top10. Putative clones were selected on LB plates containing 50 μg/ml kanamycin. The presence of the 700 bp insert was confirmed by PCR-screening using the primers 22-98 F and 23-98 B following a protocol from Perkin Elmer.

A quick colony screening protocol using PCR was performed as follows. A small portion of overnight colony growth was transferred to 50 μl of colony lysis buffer (20 ml of TE pH 8.0, and 20 μl of Tween 20). The colony was resuspended and the tube was heated at 95˚C for 10 min. The tube was centrifuged for 3 min at

Figure 9. Cloning of GC *kat* gene and generation of the 700 bp gonococcal catalase probe.


Extraction of genomic DNA
FA1090
Digestion with *Cla* I and *Dra* I
Agarose gel
Isolation of 2-3 kb *Cla* I-*Dra* I fragments
PCR-template
Cloning into pACYC184
Amplification of 700 bp internal GC *kat* fragment
Transformation into *E. coli* UM255
Cloning into pCR-Blunt
Bubbling assay
pCRAAS-700 I
kat positive transformants

high speed in a microcentrifuge. The resultant supernatant lysate was transferred to a clean tube. Three µl of the lysate was used for PCR. The clone pCRAAS-700I was selected for sequencing using 18-97 F (M13 forward) and 19-97 B (M13 reverse) primers.

The 2.4 kb whole catalase gene probe and the km^R cassette probe were obtained in a similar manner, and labeled using the Genius PCR-Dig labeling using specific primers 43-99 F and 44-99 B; 45-99 F and 46-99 B, respectively. The PCR amplified whole catalase probe was cloned into PCR-blunt and transformed into *E. coli* UM255. Bubbling assay was performed to identify *kat* $^+$ clones. This assay consists in the addition of few drops of 3% H_2O_2 directly over the colony. The formation of bubbles can be observed in catalase-positive expressing clones due to the degradation of H_2O_2 to H_2O and O_2. Sequencing and purification reactions were performed according to BIC.

C. Characterization of the catalase gene

After cloning the gonococcal catalase gene, the entire 2.4 kb insert was sequenced. The obtained nucleotide sequence was analyzed for the presence of motifs using the computer program CloneMap. The minimum portion of the catalase promoter required for catalase activity in *E. coli* was determined as follows. Clone pAAS184-*kat* 30 was digested with restriction enzymes that cut upstream of the 5' end region of the gene and ligated into the cloning vector pACYC184. Ligated DNA was transformed into E. coli UM255 and transformants were selected on LB plates containing 50 µg/ml of chloramphenicol. The presence

of the desired insert was confirmed by PCR screening. Digested 5' ends were sequenced as above to verify the length of the upstream region. Clones with insert were tested for catalase activity by the addition of 3% H_2O_2 to the colony, and by brown colony color phenotype.

D. *N*-terminal sequencing

A 7.5% SDS-PAGE was run according to Laemmli (1970). After electrophoresis, the gel was transferred to a PVDF membrane overnight at 200 mAmps according to Matsudaira (1987). The gel was soaked in transfer buffer (10 mM CAPS in 10% methanol) to reduce the amount of Tris and glycine prior to transfer. The PVDF membrane was activated by soaking in 100% methanol, and then transferred to transfer buffer. After transfer, the membrane was washed in deionized water for 5 min and then stained with 0.1% Coomassie blue R-250 in 50% methanol for 5 min. The membrane was then destained in 50% methanol:10% acetic acid for 10 min at RT, rinsed in deionized water for 5-10 min, air dried and stored at -20^{o}C until processing. *N*-terminal sequencing was performed by BIC facilities.

E. Southern blot

Gonococcal genomic DNA was isolated, and the DNA concentration and purity were determined spectrophotometrically by reading the absorbance at 260 nm and 260/280 nm, respectively. Seventy-five micrograms of genomic DNA were digested with *Cla* I, *Dra* I, *Hinc* II and *Cla* I-*Dra* I following the specific

conditions described by the manufacturer. The digestion reaction was incubated overnight in a water bath at 37°C and stopped with 5 µl per 20 µl reaction with 6X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll in water). Twenty µl of digested DNA (10 µg) were loaded in a 0.8% 0.5X TAE agarose gel containing 0.5 µg/ml of ethidium bromide. Gels were run at 100 volts using an 11 x 14 gel box. After electrophoresis, DNA was transferred to a nylon membrane using the Nytran plus downward transfer system, and the membrane was processed according to the manufacturer. Pre-hybridization, hybridization and detection in the Southern blot was performed according to Genius system protocol.

F. Generation of anti-catalase antibody for Western blot.

Antibody specific for GC catalase was produced by subcutaneous immunization of female BALB/c mice with the peptide NH_2-RDPRKFPDLNKAVKRDPRTNM-COOH. This peptide corresponds to amino acids 140 to 160 of the deduced gonococcal catalase amino acid sequence. Selection of the peptide was based on analysis of the predicted protein for hydrophobic, hydrophilic and surface exposed regions. The peptide was synthesized by BIC. Mice were immunized 3 times with 20 µg (two doses) and 40 µg (one dose) of KCL-conjugated peptide using Quil-A as an adjuvant every two weeks. Eye bleeds were collected at each time of immunization to measure anti-catalase antibody titers by ELISA (Enzyme Linked Immunoabsorbent Assay).

ELISA plates were coated with non-conjugated peptide. Pre-immune mouse sera were tested in parallel to provide a baseline.

IV-Measurement of SOD, catalase and peroxidase activity

A. Lysate preparation- *E. coli* and GC strains were cultured in 500 ml of LB or supplemented GC broth, respectively. Antibiotic selection was used to maintain recombinant plasmids. After 24 h, bacteria were harvested by centrifugation at 6,000 rpm for 10 min. Pellets were then resuspended in 10 ml of PBS and disrupted by passaging twice through a French pressure cylinder at 20,000 psi. After bacterial cell breakage, lysates were ultracentrifuged for 1 h at 40,000 rpm to eliminate cell debris. Protein concentration of the supernatant was determined by the method of Lowry (1951).

B. Activity gels and detection of enzymatic activity

Native electrophoresis gels composed of 7.5% acrylamide were pre-run for 2 to 3 hours in running buffer containing 0.1 mM sodium thioglycollate.

1) SOD-Superoxide dismutase activity was detected by the method of Mirsa and Fridovich (1977). The method consisted of soaking the gel in 10 mM potassium phosphate at pH 7.2 containing 2 mM o-dianisidine and 0.1 mM riboflavin for 1 h. After incubation, the gel was rinsed with deionized water and illuminated in a transluminator for 5 to 15 min. SOD positive bands were observed as brown bands against a clear background.

2) Peroxidase-The native gel was soaked for 1 hour in 10 mM potassium phosphate buffer at pH 7.2 containing 2 mM dianisidine. The protein gel was then transferred to potassium buffer containing 0.1 mM H_2O_2 for 15 min. Peroxidase positive bands were observed as dark bands against a clear background (Mirsa and Fridovich, 1977).

3) Catalase-The native gel was soaked in 50 mM potassium phosphate buffer at pH 7.0 containing 50 mg/ml of HRP for 45 min. Then H_2O_2 was added to a final concentration of 5 mM and the gel was soaked for an additional 10 min. The gel was rinsed with water and transferred to potassium buffer containing 0.5 mg/ml of diaminobenzidine (Clare et al., 1984). The catalase positive bands were observed as colorless bands against a dark brown background.

C. **Quantitative enzymatic assays**

1) Catalase- The method of Worthington (1993) was utilized to quantitate catalase activity. This method measures the disappearance of peroxide at 240 nm and is based on the method described by Beers and Sizer (1952). The reaction mixture consisted of 950 µl of reagent grade water and 500 µl of 0.059 M H_2O_2. The mix was incubated for 4-5 minutes to achieve temperature equilibration. Fifty µl of the test sample were added, and the decrease in $Abs_{240\ nm}$ over 5 minutes was measured in aspectrophotometer. The slope of the change in the absorbance was calculated in the linear portion of the curve. Units were defined as one unit decomposes one µM

of H_2O_2 per minute at 25°C and pH 7.0. Units/mg of protein were calculated using the following formula: Units/mg = ΔA_{240}/min X 1000/43.6 X mg enzyme/ml reaction mixture.

2) Peroxidase-four different enzymatic reaction mixtures were used to detect peroxidase activity in lysates.

a. 4-aminoantipyrine as hydrogen donor (Trinder 1969; Worthington, 1993)- This assay measures the increase in absorbance at 510 nm. The reaction consisted of 675 µl of phenol/aminoantypirine solution (0.17 M phenol/0.0025 M 4-aminoantypirine), 725 µl of 0.0017 M H2O2, and 100 µl of lysate or 50 µl of 1/12,000 dilution of HRP in a final volume of 1.5 ml. The solutions were made in 0.2 M potassium phosphate at pH 7.0. A cuvette lacking the enzyme was used as a reference. One unit results in the decomposition of one µM of hydrogen peroxide per minute at 25°C and pH 7.0. Units/mg protein were calculated as follows:

Units/mg=ΔA510/6.58 X mg enzyme/ml reaction mixture

b. Diaminobenzidine as the hydrogen donor at pH 4.5- This assay, described by Herzog and Fahimi (1973), measures the increase in absorbance at 465 nm. The reaction consisted of 1,400 µl of DAB-gelatin solution in 0.2N citric acid/Na2HPO4, 50 ml of 0.6% H2O2

in reagent grade water and 50 µl of bacterial lysate or HRP (1/12,000 dilution). A cuvette containing the DAB-gelatin solution and H_2O_2 was used as a reference.

c. **Diaminobenzidine as the hydrogen donor at pH 7.0**-In this method, the increase in absorbance was measured at 482 nm. A mixture of DAB-H_2O_2 solution consisting of 0.5 mM diaminobenzidine, 2.0 mM H_2O_2 in 50 mM potassium phosphate buffer at pH 7.0 was used. Nine-hundred µl of DAB- H_2O_2 solution and 100 µl of lysate or 50 µl of HRP (1/12,000 dilution) were combined to measure the activity. One ml of DAB- H_2O_2solution was used as a reference (Archibald and Duong, 1986).

d. o-**dianisidine as hydrogen donor**-This enzymatic assay is an adaptation of the peroxidase activity gel described by Mirsa and Fridovich (1977) and Lundquist and Josefsson (1971). The assay buffer consisted of a mixture of 2.0 mM o-dianisidine and 2.0 mM H_2O_2 in 50 mM potassium phosphate buffer at pH 7.0. The reaction consisted of 900 µl of o-dianisidine- H2O2 and 100 µl of lysate of 50 µl of HRP as before. The peroxidase activity was measured as an increase in absorbance at 440 nm.

V- Sensitivity to toxic oxygen radicals

A. Disc diffusion

A disc diffusion assay was used to measure the sensitivity of the wild-type GC, the *kat* mutant, and *E. coli* UM255 expressing recombinant gonococcal catalase to H_2O_2. Sensitivity to several inducers of toxic oxygen radicals such as paraquat (Hassett et al., 1987), streptonigrin (Cohen et al., 1987; Gutteridge, 1984; White, 1977; White and Yeowell, 1982), tetracycline (Quinland and Gutteridge, 1988), gentamicin (Sha and Schacht, 1999) and *N*-morpholino sydnonimine (SIN-1) (Groves, 1999; Motohashi and Saito, 2002) was tested similarly. GC were grown overnight on GC agar plates and resuspended in GC broth to a OD_{600} of 0.1 (10^8 cfu/ml), and 100 μl of the cell suspension was spread onto GC agar plates. Filter paper discs (1/4 inch in diameter) saturated with various concentrations of H_2O_2 (0.05,0.1,0.5, 1, 5, 10 and 20 mM), paraquat (1, 5, 10, 20, 40, and 60 mM), streptonigrin (0.25, 0.5, 1, and 2.5 μg/ml), tetracycline (1, 5, 10, 15, 25, and 50 μg/ml), gentamicin (1, 5, 10, 15, 25, and 50 μg/ml) and SIN-1 (1, 5, 10, 15 and 20 mM) were placed on the surface of the plate. After 24 h incubation at 37˚C in 7% CO2, plates were examined for zones of growth inhibition. The zone of growth inhibition was determined by measuring the diameter (mm) of the clear zone surrounding the disc, minus the diameter of the disc. The mean zone of inhibition and standard deviation was calculated from the results of three separate experiments (Chen and Morse, 1999).

B. H_2O_2 susceptibility in broth culture

Wild-type and *kat*- mutant GC were grown on GC agar plates for 18 to 20 hours, and then harvested and resuspended in GC broth containing supplement I

and II, and adjusted to OD_{600} of 0.1. One-hundred µl samples of bacteria were mixed with 100 µl of H_2O_2 of twice the desired final concentration. The mixture was incubated either statically or in a rotary shaker for 30 min at 37°C. Exogenous catalase (1,400 U) was added to neutralize H_2O_2 before inoculating serial dilutions onto GC agar. The number of colonies was counted after 36-48 hours of incubation. The susceptibility of the mutant to H_2O_2 was determined by comparing the viability of the mutant strain in the presence or absence of H_2O_2 with that of the wild-type GC strain.

C. Determination of H_2O_2-induced damage to bacterial DNA

Breakage of gonococcal DNA due to exposure to H_2O_2 was analyzed by alkaline agarose gel electrophoresis (Johnson et al., 1993). Bacteria were exposed to H_2O_2 as described for the H_2O_2 susceptibility experiments above, with the difference that catalase was added directly to each reaction after 30 min. and no dilutions were made. The bacteria were harvested by centrifugation and suspended in 25 µl of water. Alkaline loading dye (300 mN NaOH, 6 mM EDTA, 18% Ficoll in water, 0.15% bromocresol green and 0.25% xylene cyanol FF) was added prior to agarose gel analysis. Breakage of DNA was evaluated by comparing the degree of DNA degradation in samples exposed to H2O2 with unexposed samples.

VI- Construction of GC catalase mutant

Two approaches for constructing the catalase mutant were used. First, an in-frame deletion in the plasmid pAAS184-*kat*30 was made by cutting with *BssH* II and religating, resulting in loss of an internal 1.2 Kb *BssH* II fragment. Transformants were selected on LB agar with chloramphenicol. Second, the plasmid pAAS184-*kat*30 was digested with *BssH* II and blunt-ended. A kanamycin resistance cassette designed to produce non-polar mutations was obtained from pUC18Km on a 850 bp *Sma* I fragment and was ligated to the plasmid (Menard et al., 1993). Transformants carrying the mutated plasmid with the kanamycin resistance cassette were selected on LB containing kanamycin and chloramphenicol. Both modified plasmids were transformed into *E. coli* UM255, and transformants were screened for the absence of catalase activity by the bubbling assay. The orientation of the kanamycin resistance cassette in selected transformants was determined by PCR. The modification to the catalase gene was verified by sequencing.

The mutated catalase gene in each of the above plasmids was released by cutting with Cla I-Nhe I. Construction of mutants NGAAS-500 and NGAAS-501 by introduction of gel purified linear DNA containing the mutated gene was performed by spot dilution transformation as follows. Suspensions of piliated strain FA1090 in GC broth containing 10 mM $MgCl_2$ were mixed with 100 ng of the mutated linear catalase gene. DNA uptake was mediated by the recognition of a 10 bp uptake sequence at the 3' end of the cloned catalase gene (Elkins et al., 1991). The DNA/cell mixture was spotted onto GC agar plates and incubated overnight. Individual colonies that grew within the spots were streaked for isolation and passaged severaltimes for

purity (Gunn and Stein, 1996). The isolated colonies were screened for the loss of catalase activity by the bubbling assay.

Loss of catalase expression in the mutant was confirmed by catalase activity gels (Ausubel et al., 1994; Clare et al., 1984) and by quantitative enzymatic assay (Beers and Sizer, 1952; Worthington, 1993). Correct insertion of the non-polar kanamycin resistance cassette into the native *kat* gene was confirmed by Southern blot.

VII- Characterization of GC catalase mutant

A. Growth characteristics of GC strains

Growth curves of wild-type and catalase mutant GC were analyzed by culturing each in 100 ml of GC broth in a 250 ml flask. The OD_{600} of the cultures were adjusted between 0.05 to 0.07. OD_{600} readings was taken at 1 h intervals for 12 h. One-hundred µl samples of each strain were also serially diluted in GC broth containing 0.05% saponin and inoculated onto GC agar plates. Colony forming units were determined after 24 to 36 h post incubation at 37oC in a CO2 incubator. To determine if co-cultivation of the two gonococcal strains might affect catalase mutant viability, growth curves were performed using broth cultures containing a 1:1 ratio of wild-type and catalase mutant. Wild-type and catalase mutant GC strains were distinguished on culture plates by bubbling assay.

B. Monitoring of phase variable phenotypes

1) **Piliation**-Wild-type and mutant gonococci were assessed for piliation by looking for the colony morphology that is characteristic of piliated GC and by demonstrating natural competence for genetic transformation (Biswas et al., 1989). For transformation, recipient strains were cultured on GC agar plates for 18 hrs at 37^{o}C under 5% CO_2. GC was suspended in GC broth containing 10 mM $MgCl_2$ to an OD of 0.05 (ca. 5.0 x 10^7 cfu/ml). One-hundred μl of DNA from a rifampicin resistant GC strain (100, 500 or 1,000 ng) in sterile 1X SSC were added to 0.9 ml of the suspended bacteria. The mixture was then incubated at 37^{o}C in a water bath for 30 min. A tube containing bacteria alone was used as a control. After 30 min of incubation, the DNA-bacteria mixture was transferred to 5 ml of pre-warmed GC broth containing 10 mM $MgCl_2$ with supplements. The cultures were incubated aerobically for 5 hrs before plating diluted and undiluted samples onto GC agar containing 50 μg/ml rifampicin. Transformants were counted after 24-48 hrs.

2) **Opacity proteins**-The predominant Opa phenotype of wild-type and *kat* mutant stocks was determined by colony immunoblotting according to Jerse et al. (1994). Suspensions of individual colonies were spotted onto multiple nitrocellulose membranes and probed with Opa specific monoclonal or polyclonal antibodies. Bound antibody was detected with goat anti-mouse IgG conjugated to HRP, followed by enhanced chemiluminescence substrate (ECL).

3) **Lipooligosaccharide (LOS)**-LOS phenotype was determined by SDS-PAGE gradient gel electrophoresis (Noda et al., 2000) of proteinase K-treated cell lysates and bands visualized by silver staining (Tsai and Frasch, 1982). GC strains were grown on solid medium, harvested with sterile swab and resuspended in 10 ml of cold PBS, pH 7.2. The suspension was adjusted to OD_{650} of 0.4 (Hitchcock and Brown, 1983). One and a half ml of the adjusted bacterial suspension were centrifuged for 2 min at high speed. The pellet was solubilized in 50 µl of lysing buffer containing 2% SDS, 4% 2-mercaptoethanol, 10% glycerol in 1M Tris, pH 6.8 containing bromophenol blue. The lysed bacteria were heated at 100˚C for 10 min. Twenty-five µg of proteinase K were added to the boiled lysate and incubated for 1 h at 60°C. Nucleic acid and protein contamination was determined by agarose gel electrophoresis and the method of Lowry et al. (1951), respectively.

C. Complementation of catalase mutant

Complementation of the mutant *in trans* was performed by supplying a wild-type copy of the catalase gene subcloned into the C*la* I and S*ph* I sites of pLEE20 (Nassif et al., 1991; Stein et al., 1983). The resultant *kat*-containing plasmid, pAAS20-*kat* 30, was transformed into *E. coli* UM255, and catalase positive transformants were identified by the bubbling assay. Transfer of the complementing plasmid to GC was performed by conjugation between the GC catalase mutant and *E. coli* S17-1 carrying pAAS20-*kat* 30 as donor. Conjugation was performed by *in vitro* filter mating (Schwan et al., 1999).

Briefly, 3 ml of LB broth containing 300 μg/ml of erythromycin were inoculated with S17-1 (donor) carrying the pAAS20-*kat* 30, and incubated overnight at 37^{o}C statically. Fifteen ml of filtered GC broth were then inoculated with GC from a fresh overnight plate culture. GC was incubated aerobically with shaking until bacteria reached log phase (A_{600} = 0.1 to 0.2). The OD_{600} of S17-1 was determined and adjusted to 0.5 (1 x 10^{8} CFU/ml). Bacteria were harvested and washed once with an equal volume of GC broth to eliminate residual antibiotic. The optical density of recipients was adjusted to OD_{600} of 0.1 (1 x 10^{7} CFU). The number of donor and recipients was determined by serial dilution of broth cultures and plates on LB or GC agar plates. The mating reaction consisted of a mixture of 1 ml of donor and 10 ml of recipient, resulting in a ratio of 1:1. The mixture was transferred to a filter apparatus, and bacteria were collected on a 0.45 μm filter. The filter was then placed onto the surface of a GC agar plate without selection. The mating was carried for 1 to 5 hours, the exact time of which was optimized for the recipient strain being used. After mating, the filter was resuspended in 1 ml of GC broth, serially diluted, and cultured on GC agar plates containing VCNTS and erythromycin to select for the desired progeny. Plates were incubated for 24-48 hrs. In order to verify recipient viability after mating with *E. coli*, serial dilutions of the mating mixture were plated on GC VCNTS. As a control, donors and recipients were plated in GC agar containing VCNTS and erythromycin to verify sensitivity. Conjugation frequency was determined by the following formula:

Conjugation frequency = 100 X $\frac{\text{Num. of transconjugants/ml of resuspended filter}}{\text{Num. of GC used in mating}}$

VIII- Effect of H_2O_2-producing lactobacilli on *N. gonorrhoeae*.

The inhibitory properties of H_2O_2-producing lactobacilli were determined using the agar overlay technique of Saigh et al. (1978). *Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Lactobacillus crispatus* and *Lactobacillus jenseni* were grown overnight on MRS plates. Saline suspensions of the four different species of lactobacilli were prepared and the OD_{600} was adjusted to 0.3. Serial dilutions of each strain were cultured on MRS plates to determine number of CFU/ml. Fifty µl of dilutions 10^{-1} to 10^{-3} and undiluted suspension were inoculated onto Heart Infusion Agar (HIA) plates. Plates were kept at room temperature for 30 to 60 min until the inocula dried. The plates were then incubated overnight at 37˚C under CO_2 atmosphere for 24 hrs.

Plates inoculated with *Lactobacillus* sp. were overlaid with 7.5 ml of enriched GC agar. The overlay was allowed to solidify for at least 30 min at room temperature. Suspensions of 18 to 21 hr old cultures of wild-type, catalase mutant and complemented strains were prepared by suspending the bacteria in PBS and filtering through a 1.2 µm Acrodisc syringe filter unit to remove bacterial aggregates. The OD_{600} of the filtered bacteria was adjusted to 0.1. Serial dilutions of the adjusted cultures were plated on GC agar plates to determine the CFU/ ml, and 100 µl were spread over the GC agar ovelay. Plates were incubated for 24 hrs at 37˚C in a CO_2 incubator. After 24 hours, the diameter of the growth formed by

the lactobacilli and the clear zone of inhibition around the lactobacilli were measured. The ratio of inhibition was calculated by the following formula:

$$\textbf{Ratio of Inhibition} = \frac{\text{diameter of GC growth inhibition zone}}{\text{diameter of LB growth}}$$

The effect of pH on the inhibitory properties of H_2O_2-producing lactobacilii was assessed by performing this assay using HIA plates at pH 7.0 and 5.8.

IX- Role of gonococcal catalase *in vivo*.

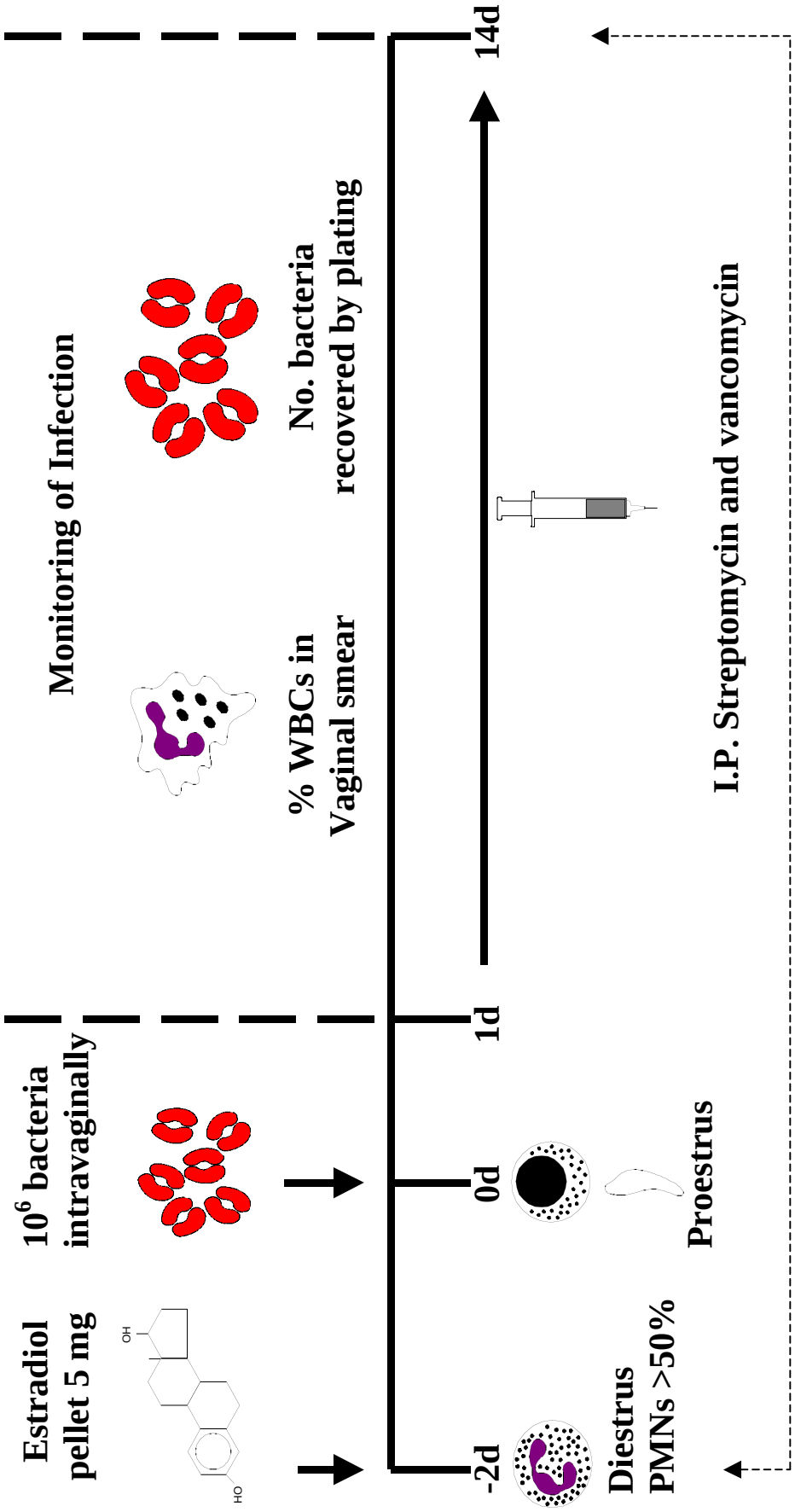
A. Preparation of inoculum

Growth from solid agar cultures (19 hours incubation) was harvested with a sterile swab, and resuspended in PBS containing 1 mM $MgCl_2$ and 2 mM $CaCl_2$. Bacteria were filtered through a 1.2 µm Acrodisc syringe filter to eliminate clumped bacteria. Suspensions of wild-type GC and catalase mutant OD_{600} were adjusted to an OD_{600} = 0.08 and 0.1 units respectively, which was previously determined to give equal numbers of both strains.

B. Mouse Infections

Experimental infection of female BALB/c mice was performed as described by Jerse (1999) and as shown in **Fig. 10**. Estradiol-treated female BALB/c mice (4-6 weeks old) were inoculated intravaginally with 20 µl of the wild-type and *kat* mutant strains. The duration and degree of colonization over 12 days was monitored by enumeration of viable bacteria on vaginal swabs collected at different time points following inoculation using a standard quantitative culture

Figure 10. Estradiol-treated mouse model of GC genital tract infection. Estradiol-treated mice were infected intravaginally with GC wild type, *kat* mutant or a 1:1 mixture of the strains. The degree of colonization was monitored by enumeration of viable bacteria from vaginal swabs at different time points during murine infection using a standard quantitative culture method. The percentage of PMNs among vaginal cells in stained vaginal smears was used as an indicator of inflammatory response. The degree of inflammation was determined by comparison of the percentage of PMNs present in mice infected with bacteria vs. the inoculum diluent (placebo).


Estradiol pellet 5 mg
10^6 bacteria intravaginally
Monitoring of Infection
% WBCs in Vaginal smear
No. bacteria recovered by plating
I.P. Streptomycin and vancomycin
-2d
0d
1d
14d
Diestrus PMNs >50%
Proestrus
OH
HO

method. The recovery of mutant bacteria was compared with that of the wild-type strain to evaluate the capacity of the mutant strain to survive the murine genital tract. Infection experiments using mixed inocula containing 1:1 ratios of mutant and wild-type strains were also performed. Daily vaginal swab suspensions were cultured on GC agar. The degree of attenuation of the mutant was measured as the ratio of catalase negative: catalase positive isolates as determined by bubbling assay. An average of 42 colonies or more were tested from each time point. A ratio equal to that of the inocula was interpreted as lack of attenuation. A ratio $\geq$ 10 fold lower than that of the inocula was interpreted as attenuation.

C. Determination of inflammation

The percentage of PMNs among 100 vaginal cells in stained vaginal smears was used as a indicator of inflammatory response, and to establish if a correlation between clearance of the infection and degree of inflammation existed. Slides were stained with a modified Wright's stain (HEMA 3) and viewed by light microscopy.

X- GC Interactions with PMNs

A. Isolation of murine PMNs

Murine PMNs were elicited by intraperitoneal injection of 2.5 ml of 3% thioglycollate broth. Thioglycollate broth was prepared as follows. Glassware was baked overnight at 225°C and autoclaved for 1 h. Thioglycollate broth media

(29.5 g/L) in one liter of endotoxin free water was stirred and heated to boil. The heat was then reduced to a gentle boil for 20 min. Media was transferred to 1 L flask and autoclaved for 30-45 min. After cooling, the broth was poured in 50 ml conical tubes. The thioglycollate broth (2.5 ml) (Baron and Proctor, 1982) was injected into female BALB/c mice (estradiol-treated and untreated). After 5 hours, mice were sacrificed by CO_2 asphyxiation and the peritoneal cavity was opened without disruption of the membrane. PMNs were obtained by peritoneal lavage with 10 ml of cold Hank's Balanced Salt Solution (HBSS) buffer using a 10 ml syringe and 18 gauge needle. The needle was removed, and the murine PMNs were transferred to 50 ml conical tubes to make the pools. Normally two mice were used to make the pools. PMNs were then transferred to 15 ml conical tubes, centrifuged for 5 min at velocity 3 in clinical centrifuge and then washed with an equal volume of HBSS. After washing, the PMNs were centrifuged again as above and resuspended in 4 to 5 ml of HBSS complete buffer II (HBSS, 10 mM glucose, 0.1% gelatin, 1mM $CaCl_2$ and $MgCl_2$). The number of PMNs per ml was calculated using a hemacytometer. Trypan blue exclusion staining was used to confirm that most of the PMNs were viable.

B. Serum sensitivity

The bactericidal activity of estradiol-treated and untreated murine serum against GC was determined as described by McQuillen et al. (1997) with some modifications. Overnight grown GC was harvested from GC agar plates with a sterile swab and resuspended in HBSS complete buffer. Bacterial suspensions were

filtered through a 1.2 μm syringe filter to eliminate clumps. The OD_{600} of the different suspensions was adjusted to 0.08. Ten μl of the bacterial suspension were exposed to different concentrations of estradiol-treated and untreated serum ranging from 5 to 25% in a final volume of 100 μl in a microtiter plate. A portion of murine serum was heat-inactivated (56^{o}C, 10 min) and tested in parallel. As a positive and negative control of killing, normal and heat-inactivated human serum from a healthy female donor was used. Bacteria were incubated for 30 min at 37^{o}C aerobically. Serial dilutions of each reaction were cultured onto GC plates. CFU were determined after 24 to 36 hrs.

C. Acridine orange-trypan blue assay

This assay is a modification of the protocols described by Belland et al. (1992) and Miliotis (1991). Conditions such as presence or absence of serum, percentage of serum, PMA, multiplicity of infection (MOI) and time of incubation were tested to determine the optimum conditions of the assay. Twelve mm round glass coverslips were sterilized by flaming after immersion in 95% ethanol. Each coverslip was placed in a separate well of 24-well tissue culture plate. Coverslips were overlaid with 0.25 ml of HBSS complete buffer containing 5.0 x 10^5 PMNs. The PMNs were allowed to attach to the glass during 30 min incubation at 37˚C in a CO_2 incubator. Wild-type or catalase mutant GC were added at a ratio of 10:1 bacteria per PMN in 0.25 ml of HBSS complete buffer to the previously attached PMNs. PMA was added 10 min before the bacteria were added to the wells. Infected PMNs were incubated for 30, 60, 90 and 135 minutes. One set of

duplicate coverslips was washed with 1 ml of HBSS, stained for 45 s with acridine orange (Difco), rinsed with another 1.0 ml of HBSS, and mounted onto a slide. A second set was stained as above, then counter-stained with 0.5 ml of 0.4% trypan blue for 45s before a final wash with 1 ml of HBSS. Trypan blue was used to distinguish intracellular from extracellular bacteria since it quenches the fluorescence of acridine orange as seen under a fluorescence microscope. Intracellular bacteria are protected from the quenching due to the exclusion of trypan blue by the PMNs. Stained coverslips were inverted, mounted onto microscope slides and sealed with nail polish. Each time point included three pairs of coverslips stained with acridine orange alone or acridine orange with trypan blue. The number of GC associated with PMNs was recorded using the following categories 0, 1-2, 3-10 or more than 10 GC per PMN to better interpret the degree of association between GC and PMNs.

D. PMN Killing assay

This assay is a modification of the protocol described by Fischer and Rest (1988). Assay conditions such as bacteria:PMN ratio, opsonization of bacteria, the percent of serum in the assay and incubation conditions (incubation time and CO_2 versus air incubation) were first optimized. The final assay was performed in 2 ml polypropylene microcentrifuge tubes. Bacteria were opsonized with 10% mouse serum for 15 min at 37^{o}C in an air incubator with a rotary shaker. PMNs were placed on a rotary shaker for 10 min prior to the addition of the bacteria. Wild-type, catalase mutant or 1:1 mixtures of both strains were mixed with murine

PMNs at a ratio of 2:1or 1:1 PMN (5.0×10^5) per bacteria ($2.5\text{-}5.0 \times 10^5$) in HBSS complete buffer containing 10 % murine serum. At 45, 90 and 135 min of incubation, 10 μl samples were taken and serially diluted in GC broth containing 0.05% saponin and cultured on GC agar. The percentage of killing was determined by comparing the number of viable bacteria recovered in the presence of PMNs plus 10% heat-inactivated serum. *Staphylococcus aureus* was used as a killing control (Kalmar, 1997).

E. Measurement of chemiluminescence

This assay is an adaptation of various protocols (Belland et al., 1992; Brihem et al., 1984; De Pablo et al., 1998; Fisher and Rest, 1988; Lock and Dahlgren, 1988; Magrisso et al., 1995; Oldenborg et al., 2000; Westman, 1986). Murine PMNs, 5.0×10^5/ml in complete HBSS buffer HBSS + 1mM $CaCl_2$, 0.1 mM $MgCl_2$, 10 mM glucose and 1% gelatin) were pre-incubated with 10 ml of luminol (0.1mg/ml in HBSS) in a 5 ml scintillation vial for 2 minutes at 37^{o}C in a final volume of 1 ml. Gonococci were opsonized for 15 min at 37^{o}C in 10% murine serum, centrifuged and washed once with HBSS complete buffer. Bacteria were added at a 10:1 ratio of bacteria to PMN. Chemiluminescence signal was measured in a scintillation counter (model LS6100) at 0.2 min intervals using the pre-set full tritium window with the counter in normal in-coincidence mode for a defined period of time Intracellular chemiluminescence was measured by the addition of 2,000 units of catalase to remove extracellular H_2O_2. The extracellular chemiluminescence was measured by addition of sodium azide (an inhibitor of

MPO) to a final concentration of 1 mM and 4U of horseradish peroxidase [(insensitive to sodium azide), Briheim et al., 1984; Lock and Dahlgren, 1988].

F. Measurement of glucose consumption

Glucose consumption was measured by adaptation of the colorimetric method described by Ngo and Lenhoff (1980); and Lundquist and Josefsson (1971). Murine PMNs (1.0×10^5) were exposed to a 10:1 ratio of opsonized wild-type or catalase mutant GC in microcentrifuge tubes. For comparison of estradiol-treated and untreated PMNs, neutrophils were exposed to wild-type GC at an MOI of 10. Unlike the PMN killing assay, the reaction was performed in HBSS buffer containing only 25 μM glucose. Tubes were incubated for 90 minutes at 37°C in a rocking shaker as for the PMN killing assay. After incubation the tubes were centrifuged for two minutes and the supernatant transferred to a clean tube. The assay reaction consisted of 130.5 ml of glucose reagent (1000 U glucose oxidase, 300 U peroxidase and 1 ml of 1% o-dianisidine in ethanol) diluted to 100 ml in 0.5M Tris-HCl buffer pH 7.0 Nineteen and a half μl of supernatant were placed in wells of a microtiter plate in triplicate. The reaction mix was incubated for 90 min at 37°C and the absorbance was read at 450 nm. A glucose blank solution was used as a control. To determine the concentration of glucose in experimental samples, a glucose standard curve ranging from 5 to 25 μM was made and the extinction coefficient was estimated.

G. Measurement of superoxide production

Generation of superoxide ion was measured by two different assays. Extracellular production was followed by cytochrome C reduction and intracellular generation of superoxide by nitroblue tetrazolium reduction (NBT). Cytochrome C reduction was measured as follows. Fifty μl of murine PMNs (2.0×10^6 cells/ml) were pipetted into wells of a 96-well microtiter plate. Fifty μl of 320 μM cytochrome C plus the stimuli (such as PMA (100 ng), wild-type or catalase mutant strains at ratio of 10:1) were added. Bacteria were pre-opsonized as above. PMNs exposed to bacteria or other stimuli were incubated for 90 min. The reaction was stopped by the addition of 30 U of SOD. Absorbance was measured at 550 or 600 nm in a microtiter plate reader. Wells with PMNs alone, PMA or with SOD were used as a control. One mole of superoxide ion reduces 1 μM of cytochrome C. The concentration of superoxide in nM per well was calculated by multiplying the absorbance at 550 by 15.87. Also the concentration can also be determined by the extinction coefficient $\Delta E_{550} = 21 \times 10^3\ M^{-1}cm^{-1}$ (Pick, 1986).

The NBT reduction assay that was used was based on a change in color due to the formation of oxygen radicals (superoxide). NBT originally is colorless or yellow. Interaction of NBT with superoxide produces an insoluble precipitate in aqueous solution and a dark blue derivative called formazan. NBT reduction was measured as follows: 5.0×10^5 murine PMNs resuspended in HBSS complete buffer in a final volume of 100 μl were added to wells of a microtiter plate. One-hundred μl of a 1:1 mix of NBT (4 mg/ml in HBSS) with opsonized WT GC and catalase mutant were added to wells containing PMNs and the plates were incubated at 37°C for 30 to 90 minutes. Absorbance was measured at 550 nm and

600 nm. Wells containing PMNs, SOD or PMA were used as controls (Rest, 1997).

H. Determination of H_2O_2 production

The assay was performed *in situ* in a 96-well microtiter plate. One-hundred μl of PMNs (2.0×10^6 cells/ml) were transferred to the wells. An equal volume of assay solution (1.12 mM phenol red and 40 U/ml HRP in complete buffer) containing stimuli (GC strains or PMA) was added. The microtiter plate was incubated at 37°C for 90 min. The reaction was stopped by adding 20 μl of 1N NaOH. Wells only containing PMNs were used as a negative control. Wells containing assay solution and 1N NaOH were used as a blank. The absorbance was measured at 600 nm. To determine the concentration of experimental samples, a standard curve consisting of μM concentrations of H_2O_2 was used. An extinction coefficient was estimated.

I. Determination of myeloperoxidase activity (MPO)

Myeloperoxidase activity was measured by an adaptation of Bradley et al. (1982) and Parkos et al. (1985). Murine PMNs were exposed to GC strains as before. MPO was extracted by adding an equal volume of 0.5% HTAB (Hexadecyltrimethylammonium bromide) in potassium phosphate (pH 6.0) to 50μl of PMNs sample. The mixture was frozen and thawed 3 times on dry ice, and then centrifuged at high speed for 15 min. The supernatant was transferred to a clean microcentrifuge tube. MPO activity was measured in a microtiter plate. Thirty μl

of citrate buffer (pH 4.2), 20 µl of supernatant sample, and 50 µl of 1 mM ABTS (2,2'-azido-di-(3-ethyl dithiazoline sulfonic acid) containing 0.03 % H_2O_2 in citrate buffer) were mixed in a well. The plate was incubated at room temperature from 15 min to 1 h and the reaction was stopped with 10 µl of 1% SDS. The absorbance was measured at 405 nm (Gallati, 1979; Porstman et al., 1981). A reaction mixture without MPO or sample was used as a reference. To determine MPO activity, a standard curve using different concentrations of purified MPO was performed, and the extinction coefficient was estimated. The MPO units present in the experimental samples were determined by the standard curve.

Results

I- Cloning and characterization of the gonococcal catalase gene

A. Cloning of the GC catalase gene

The catalase gene from GC strain 2821 was previously cloned by Johnson et al. (1996). Comparison between the published strain 2821 *kat* sequence and the GC strain FA1090 genome database, revealed a contig of approximately 12 kb that contained a 1.5 kb sequence homologous to the GC 2821 bacterial sequence. Three open reading frames near the gonococcal catalase sequence were identified by BLAST search analysis of the regions outside of the *kat* open reading frame (**Fig. 11**). Southern blot analysis of restricted gonococcal genomic DNA showed the presence of only one copy of the catalase gene based on the hybridization of discrete bands to a labeled internal *kat* fragment probe generated by PCR (**Fig. 12**). A single species of the gonococcal catalase was observed in a native catalase activity gel, supporting the fact that catalase in FA1090 is encoded solely by one gene (**Fig. 13**). Catalase as well as SOD and peroxidase are detoxifying enzymes utilized by microorganisms as defense mechanism against toxic oxygen radical encountered in different niches. The absence of other detoxifying enzymes such as SOD and peroxidase was confirmed by native protein activity gels stained specifically for each enzyme. Neither SOD nor peroxidase activity were detected in the activity gels suggesting the absence of both activities in GC strain FA1090 (**Fig. 14 A** and **B**). In contrast, both activities were observed in *E. coli* HS-4 (**Fig. 14 A**, lane 2; **14 B**, lane 3).

-

Figure 11. Open reading frames identified in 11,958 bp contig from FA1090 genome data base containing the catalase gene. ORF were identified by submitting the contig sequence to BLAST search analysis (NCBI.nih.nlm.gov). ORF-1 encodes for an unknown protein and *fabF*, which overlaps ORF-1, is homologous to 3-oxoacyl-ACP-synthase. The *pglA* gene encodes for a glycosyltransferase and exhibits similarity with *rfpB* of *Shigella dysenteriae* (Gohmann et al., 1994). The *fabF* and *pglA* genes are found in *Neisseria meningitidis* (Jennings et al., 1998). In *N. meningitidis* the *pglA* gene is involved in pilus glycosylation.The RLS (*rpoN*- like sequence), is similar to the alternative sigma factor σ^{54} of *E. coli*. It has been determined that RLS in GC strain MS11 does not contain a single ORF capable of encoding a fuctional protein (Laskos et al., 1998).

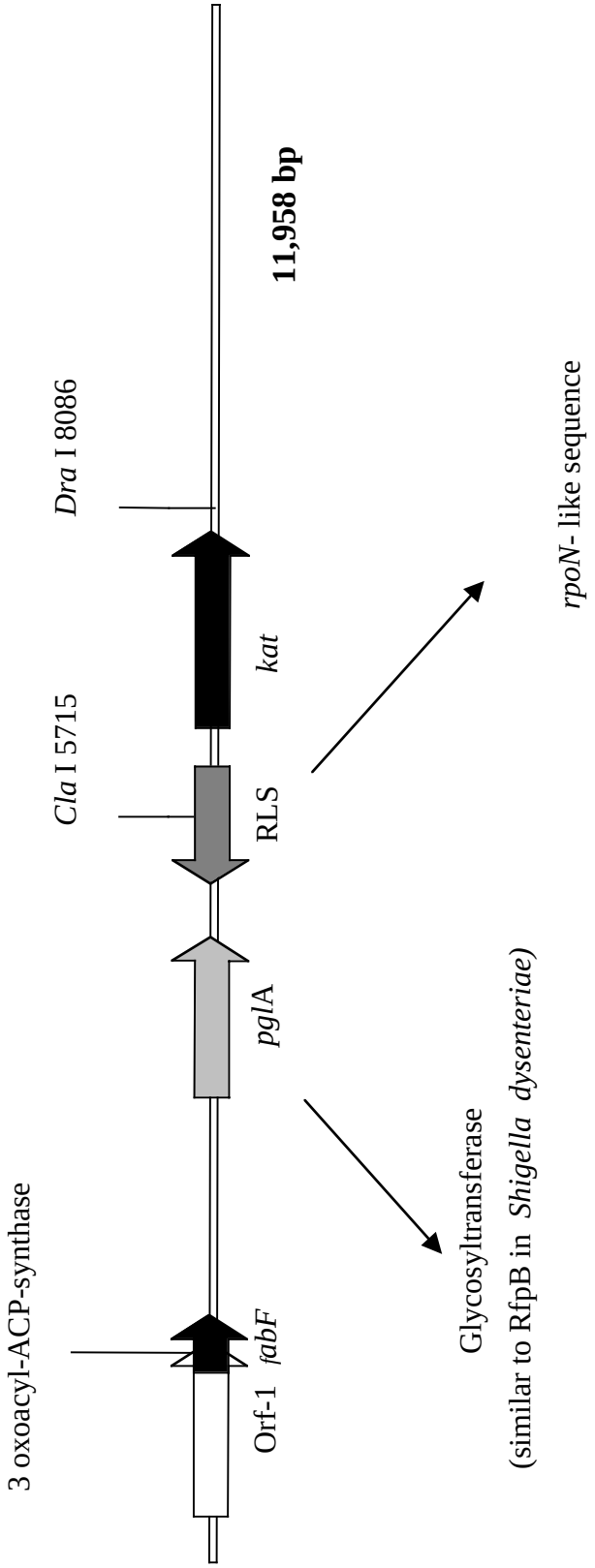
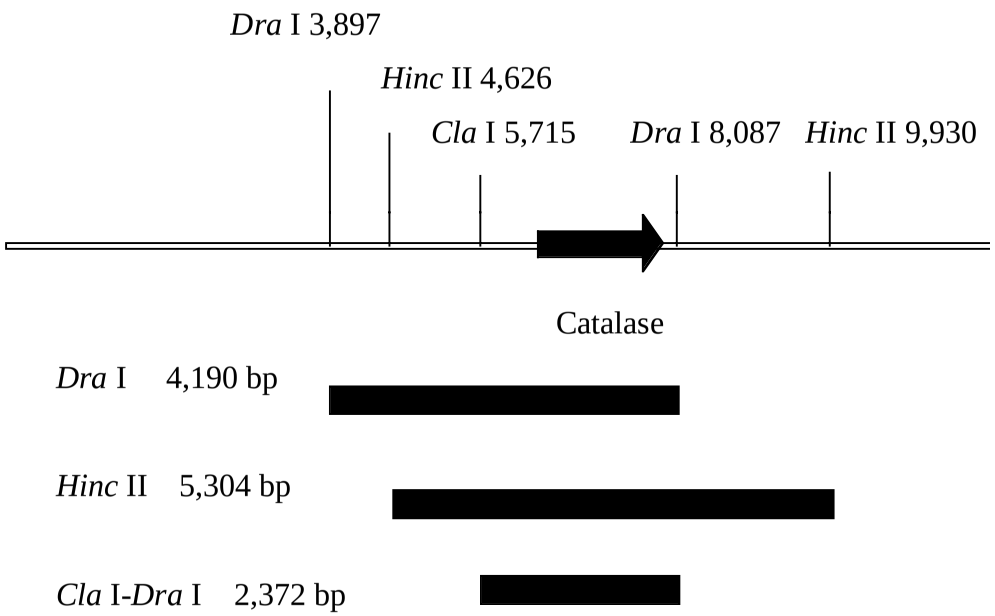

3 oxoacyl-ACP-synthase
Orf-1
fabF
pglA
Glycosyltransferase
(similar to RfpB in Shigella dysenteriae)
Cla I 5715
RLS
kat
Dra I 8086
11,958 bp
rpoN- like sequence

Figure 12. (**A**) Restriction map of contig containing the GC catalase gene and (**B**) genomic Southern blot of FA1090 with internal 700 bp catalase PCR-digoxigenin labeled probe. Lanes correspond to restriction digestion with the following enzymes: **1**) *Cla* I, **2**) *Dra* I, **3**) *Hinc* II, **4**) *Cla* I –*Dra* I, **5**) no sample, and **6**) VII ladder (Ladder consists of 15 digoxigenin pre-labeled fragments of SSP1 DNA cleaved with *Eco*RI. Linear DNA ranges from 8,000 to 370 bp), and **7**) λ ladder (Ladder consists of 13 digoxigenin pre-labeled fragments of *Eco*RI and *Hind* III from λ DNA. Linear fragments range from 21,226 to 125 bp). Digestion with the above enzymes yielded discrete bands of the expected sizes, which hybridized with the *kat* probe.

A


Dra I 3,897
Hinc II 4,626
Cla I 5,715
Dra I 8,087
Hinc II 9,930
Catalase
Dra I 4,190 bp
Hinc II 5,304 bp
Cla I-*Dra* I 2,372 bp

B

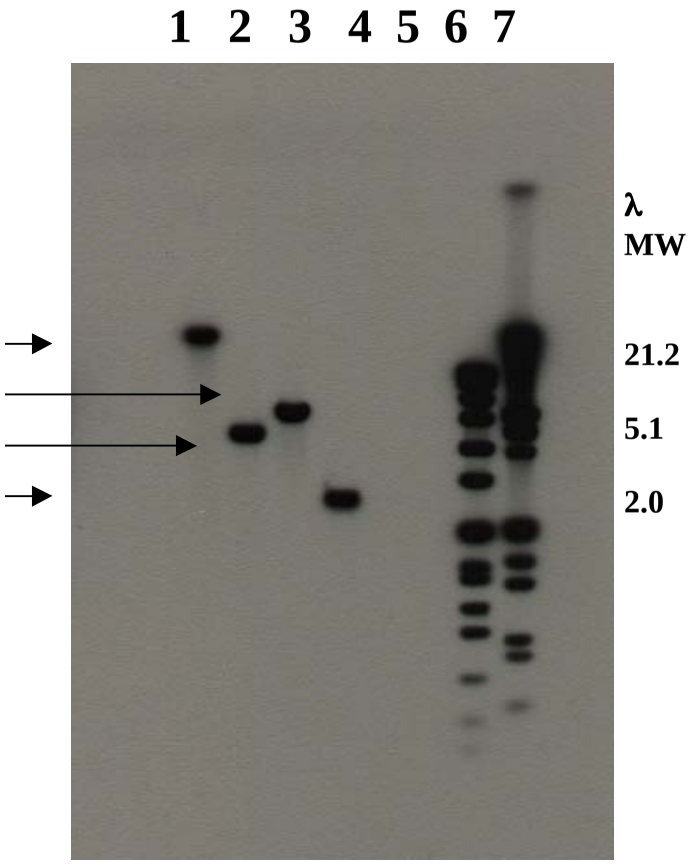
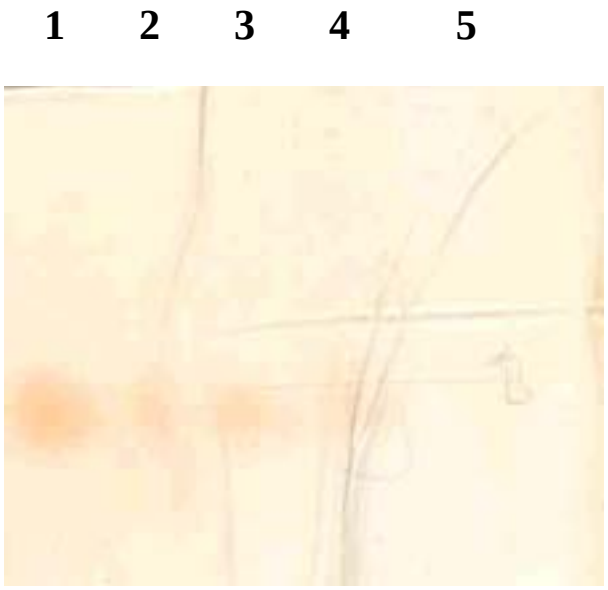

1 2 3 4 5 6 7
λ
MW
21.2
5.1
2.0

Figure 13. Catalase activity gel of FA1090 whole cell lysate. Catalase positive bands are observed as colorless bands against a dark background. Lanes: **1**) one unit of purified liver bovine catalase and **2**) 25 μg of FA1090 whole cell lysate.

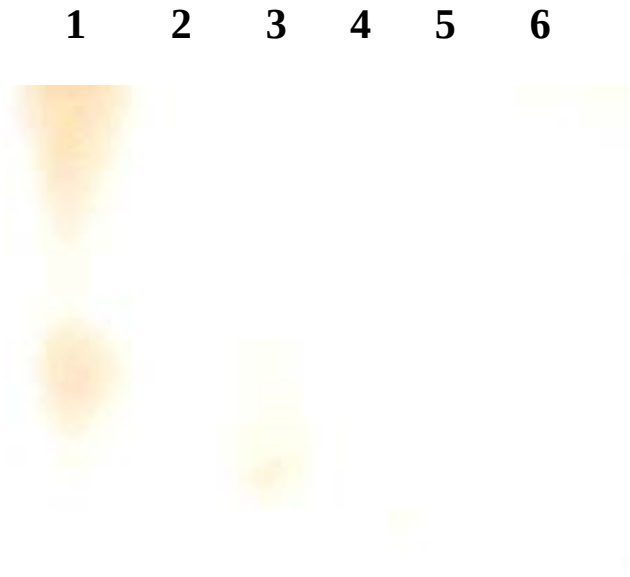
Figure 14. Superoxide dismutase (SOD) and peroxidase activity gels of whole cell lysates. (**A**) SOD and (**B**) peroxidase activity gels. Arrows indicate the relative migration of SOD and peroxidase activities in whole cell lysates of GC and *E. coli* strains. SOD activity was observed exclusively in *E. coli* (lanes **1**-**4**). Peroxidase activity was observed only in lane **1**, purified HRP; and lane **3**, *E. coli* HS-4. Only one protein band with peroxidase activity is observed in *E. coli* HS-4. The relative migration of the protein with peroxidase activity corresponds to HP-1 (hydroxiperoxidase 1). Twenty-five μg of total cell lysate and 1U of HRP were loaded in the SOD and peroxidase activity gels, respectively.

A



1) *E. coli* UM255
2) *E. coli* HS-4
3) *E. coli* UM255 (pACYC184)
4) *E. coli* UM255 (pAAS184-*kat* 30)
5) FA1090

B



1) Purified HRP
2) *E. coli* UM255
3) *E. coli* HS-4
4) *E. coli* UM255 (pACYC184)
5) *E. coli* UM255 (pAAS184-*kat* 30)
6) FA1090

The 1.5 kb ORF from FA1090 that was homologous to GC catalase was cloned into the pACYC184 plasmid vector on a 2.4 kb *Cla* I-*Dra* I fragment, and transformed into *E. coli* UM255 (*kat*G, *kat*F; double catalase mutant). Transformants were selected on LB agar plates containing 50 μg/ml chloramphenicol, and then screened for the formation of bubbles following the addition of a few drops of 3% H_2O_2 directly to the colony. Twenty-two out of 433 transformants obtained after transformation of *E. coli* UM255 exhibited catalase activity by the bubbling assay (**Fig. 15 A**). Interestingly and unexpectedly, a dark color colony phenotype was observed in all 22 of the putative clones expressing gonococcal catalase (**Fig. 15 B**). The reason for the color change in *E. coli* UM255 expressing gonococcal catalase remains unknown. In order to verify the purity of the clones, plasmid preps from each of the catalase positive clones were prepared and transformed to chemically competent *E. coli* UM255. Ten transformants of each of the 22 plasmid preps were analyzed for catalase activity by bubbling assay and colony color phenotype. One-hundred percent of the selected subclones exhibited catalase activity and the dark colony color phenotype. A single clone (pAAS184-*kat*-30; **Fig. 16**) was selected for further characterization.

The entire 2.4 kb *Cla* I-*Dra* I fragment contained in pAAS184-*kat* 30 was sequenced (**Fig. 17**). The *kat* ORF had 98% nucleotide sequence identity to that of the *kat* gene of GC strain 2821 (Johnson et al., 1996). The 10 bp uptake sequence (UPS) required for *Neisseria* DNA transformation was found 80 bp downstream of the *kat* stop site (Elkins et al., 1991) (**Fig. 18**). The presence of the UPS in the cloned *kat* ORF facilitated further genetic manipulations of this DNA. It has been speculated that genes carrying UPS are important for gonococcal virulence.

A

B

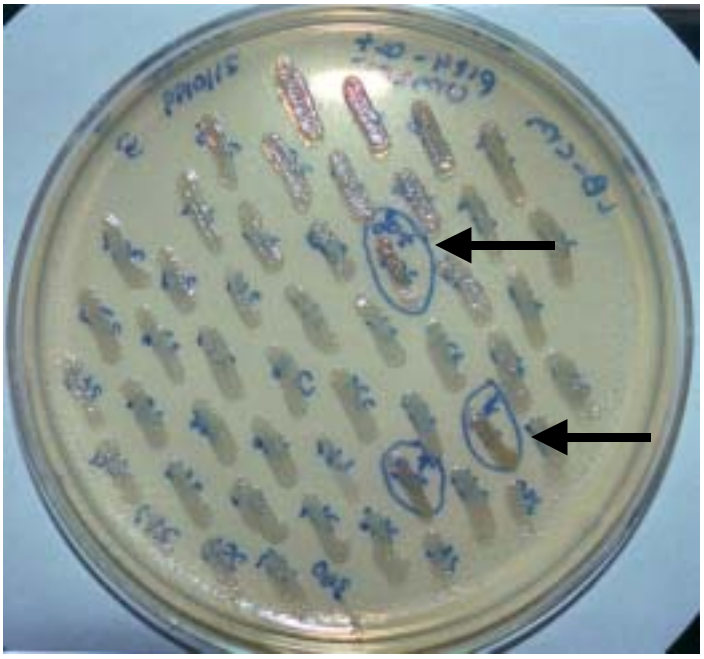


Fig. 15 (**A**), Screening of *kat* $^{+}$ clones in *E. coli* UM255 by bubbling assay. (**B**), Colony phenotype of *E. coli* expressing GC catalase. One-hundred percent of catalase-positive transformants exhibited this unexpected characteristic. Arrows indicate specific transformants exhibiting dark colony phenotype.

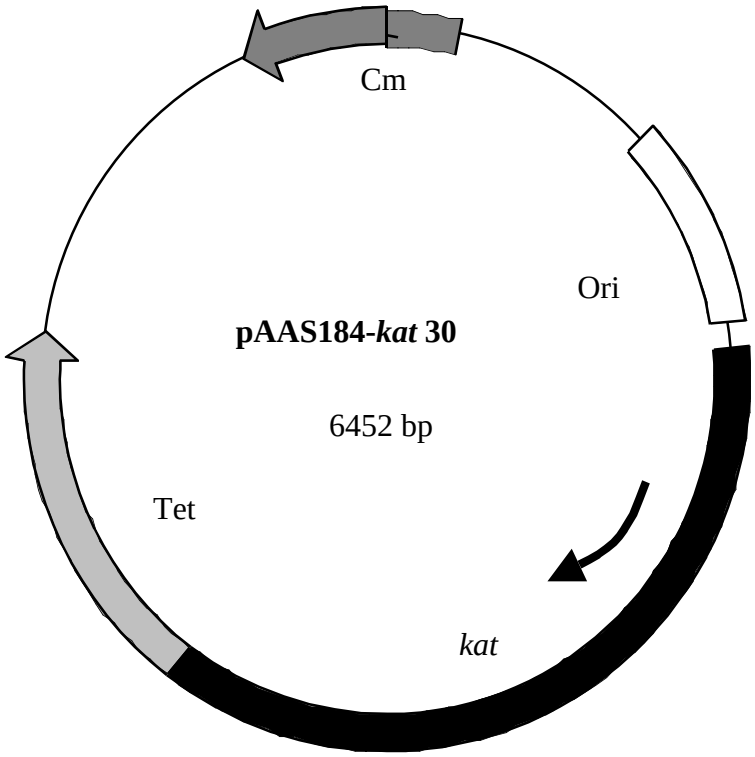


Figure 16. Plasmid pAAS184-*kat* 30

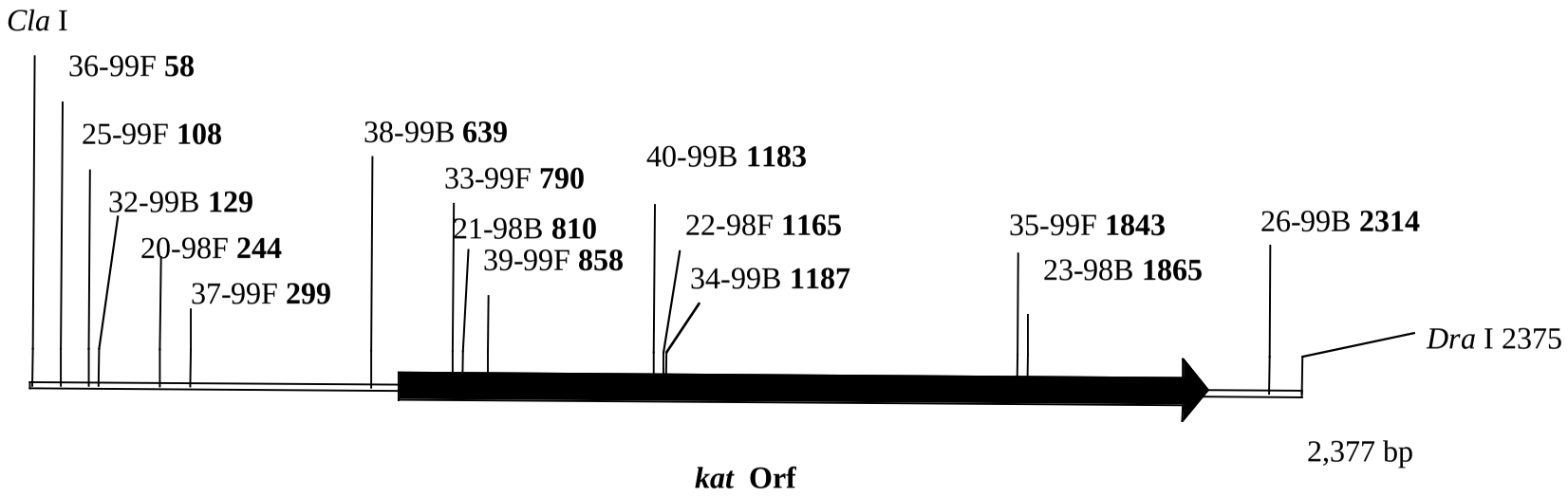


Figure 17. Sequencing strategy used for determination of the nucleotide sequence of the cloned 2.4 kb gonococcal fragment in pAAS184-*kat* 30. Primers used are described in the Material and Methods, **Table 3**.

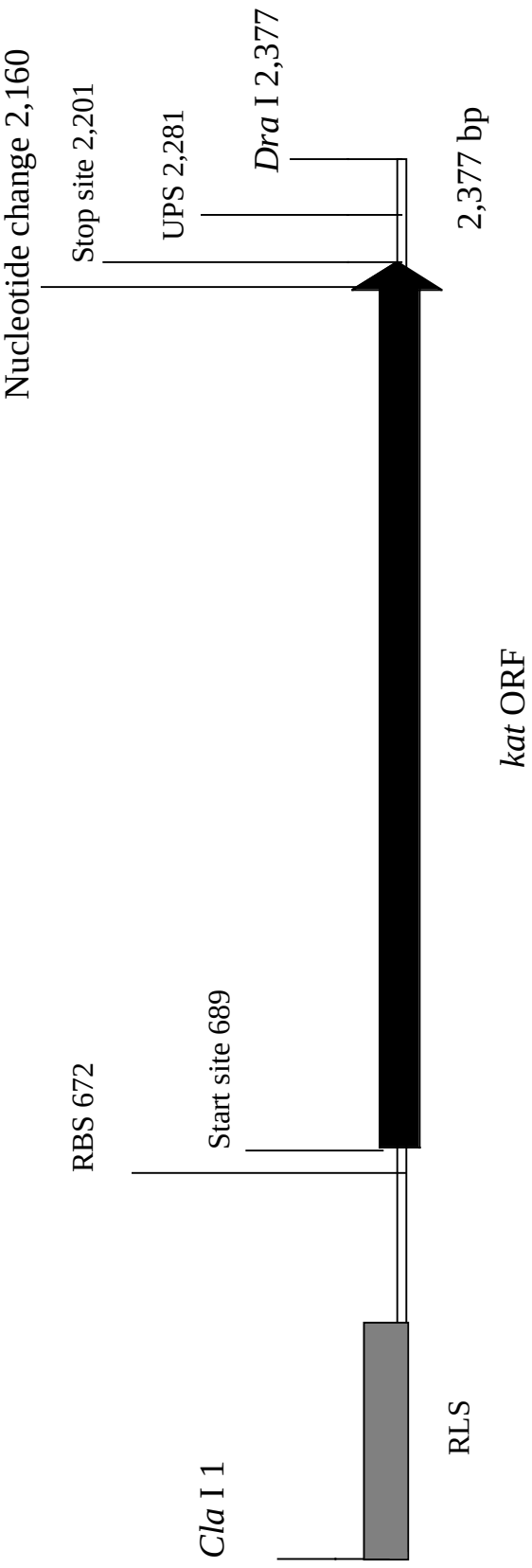


Figure 18. Features of the cloned gonococcal catalase gene as predicted by nucleotide sequence. Position of the nucleotide change is based on the *kat* ORF identified in the FA1090 Genome bank.

Analysis of the DNA sequence showed that the cloned *kat* sequence encodes a protein of 504 amino acids with an estimated molecular weight of 57.3 kDa and theoretical pI of 6.19. No obvious signal sequence was observed in the protein, suggesting that catalase is a cytoplasmic protein. Analysis of the deduced amino acid sequence revealed significant homology to the catalases of *H. influenzae* and GC 2821, among others (**Table 4**).

Protein expression from the selected clone was analyzed by catalase activity gels using *E. coli* UM255 without the plasmid as a negative control. The protein expressed by *E. coli* UM255 (pAAS184-*kat* 30) exhibited identical relative mobility to the catalase expressed by GC FA1090 (**Fig. 19**). Catalase activity expressed from pAAS184-*kat* 30 was also measured by a quantitative spectrophotometric assay. High levels of catalase activity were detected in *E. coli* UM255 (pAAS184-*kat* 30) in contrast to *E. coli* UM255 alone, which had no detectable catalase activity (**Table 5**).

The proximal active site and heme-ligand signatures, two motifs characteristic of catalase, were identified (**Fig. 20**). Alternative start sites that would lead to proteins of different sizes were also identified (**Fig. 21**). To confirm the identity of the recombinant catalase protein expressed in *E. coli* and to establish the correct start site of the protein, the *N*-terminal sequence of the recombinant protein was determined. The resultant peptide sequence was analyzed for similarities through BLAST search. The results confirmed that the protein expressed in *E. coli* is gonococcal catalase and also established that the first methionine in the predicted protein is the start site, as predicted in the ORF analysis (**Fig. 22**). Based on the deduced amino acid sequence, a peptide

Table 4. Comparison of the predicted 504 amino acid sequence of the FA1090 *kat* gene with other bacterial catalases

Microorganisms	Num. Amino acids	Identity	Similarity
H. influenzae Rd KW20	508	91	95
N. gonorrhoeae 2821	500	92	92
H. influenzae	508	91	95
P. fluorescens	506	82	88
B. fragilis	480	73	82
V. fisheri	482	74	83
B. pertussis	482	66	79

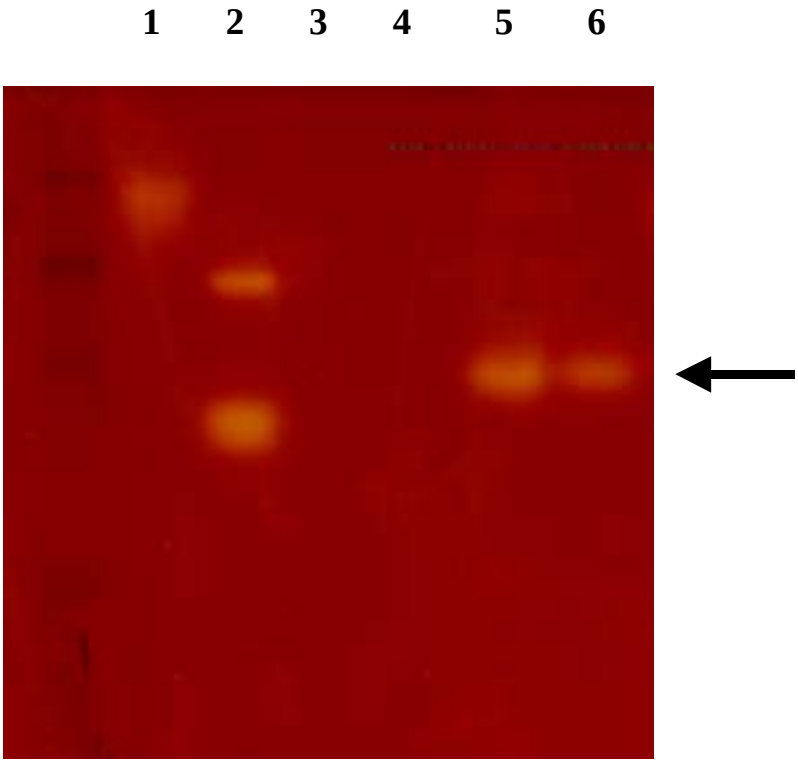


Figure 19. Catalase activity gel. Samples loaded; (**1**) Liver bovine catalase, (**2**) *E. coli* HS-4, (**3**) *E. coli* UM255, (**4**) *E. coli* UM255 (pACYC184), (**5**) *E. coli* UM255 (pAAS184-*kat* 30) and (**6**) FA1090. Twenty-five μg of total cell lysate were loaded in lanes **2**, **3**, **4**, and **6**. One unit and of liver bovine catalase and 0.02 μg of *E. coli* UM255 (pAAS184-*kat* 30) lysate were loaded in lanes 1 and 5, respectively. Arrow indicates the relative mobility of catalase expressed from recombinant clone and GC FA1090.

Table 5. Catalase activity of *E. coli* UM255 with the cloned GC catalase (pAAS184-*kat* 30) or cloning vector (pACYC184) versus that of FA1090 and *E. coli* UM255 alone

Lysate (ID)	**Catalase Activity (U/mg protein)**
1) Liver bovine catalase	42,376± 5,577
2) *E. coli* HS-4	14.6 ± 1.6
3) *E. coli* UM255	0
4) *E. coli* UM255 (pACYC184)	0
5) *E. coli* UM255 (pAAS184-*kat* 30)	25,380 ± 1,410
6) FA1090	30 ± 7

NH-$_2$

MTTSKCPVTHLTMNNGAPVADNQNSLTAGPRGPLLTQDLWLNE

KLADFVREVIPERRMHAKGSGFGTFTVTHDITKYTRAKIFSEVG

KKTEMFARFTTVAGERGAADAERDIRGFALKFYTEEGNWDVVG

NNTPVFFLRDPRKFPDLNKAVKRDPRTNMRSATNNWDFWTLLP

EALHQVTIVMSDRGIPASYRHMHGFGSHTYSFWNEAGERFWVK

FHFRSQQGIKNLTNEEAAKIIADDRESHQRDLYEAIERGEFPKWT

MYIQVMPEADAEKVPYHPFDLTKVWPKKDYPLIEVGEFELNRNP

ENFFADVEQSAFAPSNLVPGIGASPDKMLQARLFNYADAQRYRL

GVNFRQIPVNRPRCPVHSNQRDGQGRADGNYGSLPHYEPNSFGQ

WQQQPDFAEPPLKINGDAAHWDYRQDDDDYFSQPRALFNLMND

AQKQALFDNTAAAMGDAPDFIKYRHIRNCYRCDPAYGEGVAKA

LGLTVEDAQAARATDPALGQAGLL ——— COOH

Figure 20. Deduced amino acid sequence of cloned catalase gene. In light blue, proximal active site signature; in gray, heme-ligand signature; in green, amino acid substitution (alanine for valine) compared to the predicted amino acid sequence of the *Neisseria gonorrhoeae* FA1090 *kat* gene in the genome data base.

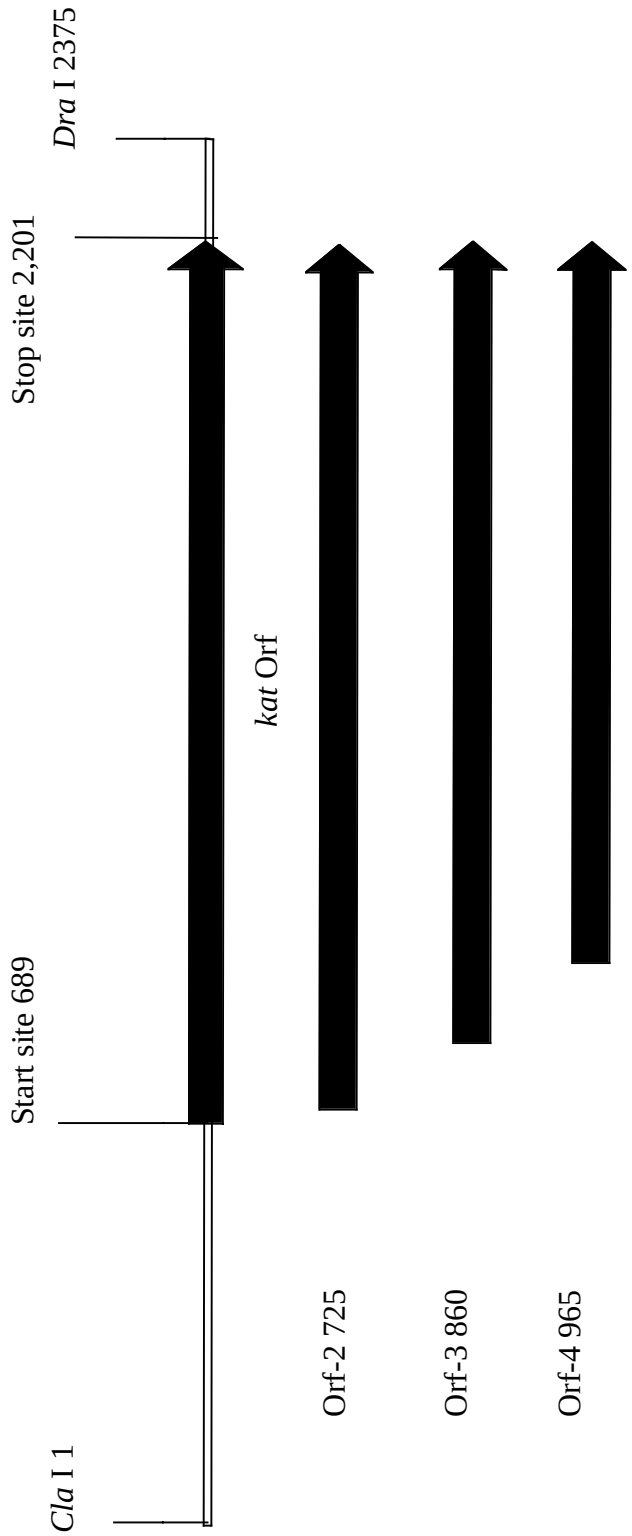


Figure 21. Alternatives start sites in the FA1090 *kat* gene based on nucleotide sequence analysis.

AAC CTG AGA AAA GGA ACA AGA GCG ATG ACT ACC TCC
M T T S

N-terminal sequence M T T S

AAA TGC CCC GTA ACC CAT CTG ACC ATG AAC AAC GGC GCG
K C P V T H L T M N N G A

K V P V T H L T M N N G A

CCC GTT GCC GAC AAT CAA AAC AGC CTG ACC GCC GGC CCG
P V A D N Q N S L T A G P

P V A

CGC GGC CCC CTG CTG ACG CAA GAT TTG TGG CTG AAT GAA

Figure 22. *N*-terminal sequence analysis of the GC catalase protein expressed in *E. coli* UM255. In red, ATG codons; grey, protein translation from first ATG; blue, amino acid sequence obtained from *N*-terminal sequencing. Pink denotes a valine that was obtained instead of the predicted cysteine in the sequence. In order to detect cysteine, a second sequencing reaction must be set up in which the protein of interest is treated with reagents specifically for cysteine determination (Personal communication, Mike Flora; BIC, USUHS).

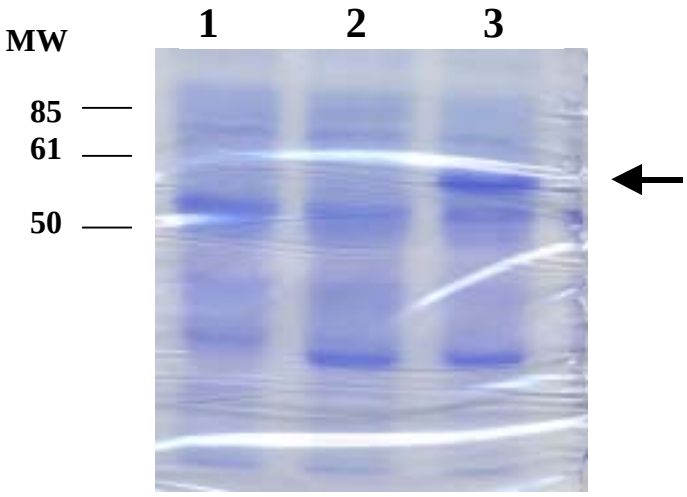
composed of amino acids 140 to 160 was synthesized and used to immunize mice (**Fig. 23 A**). This sequence was selected based on Kyte-Doolittle, Hoop-Woods and surface exposure analysis (**Fig. 23 A**). The resultant antiserum was used in a western blot to probe total lysates of *E. coli* UM255 with and without pAAS184-*kat* 30. A protein band of approximately 58 kDa was recognized by the antiserum. This band corresponds to the most predominant protein in stained gels of lysates of UM255 carrying the pAAS184-*kat* 30, and was absent in lysates of *E. coli* UM255 or UM255 (pACYC184) (**Fig. 23 B**, **C** and **D**).

B. Biochemical and functional characterization of GC catalase

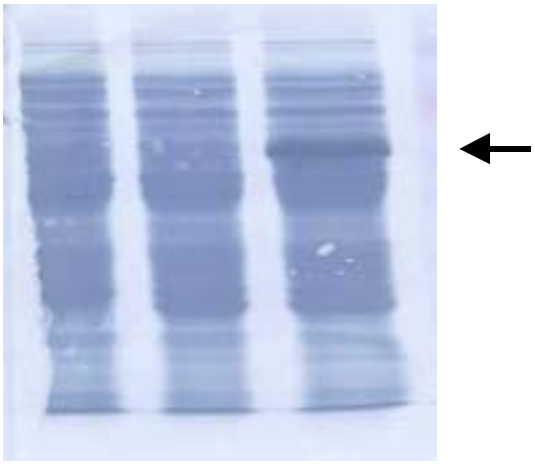
Catalases are classified into three distinct groups: monofunctional catalases, catalase-peroxidases and non-heme or pseudocatalases. Johnson et al. (1993) reported that catalase from GC 2821 was a catalase-peroxidase based on peroxidase activity gels and inhibition of enzymatic activity by 3-amino-1,2,4-triazole. However, in this current study, no peroxidase activity was detected in strain FA1090. This result suggests that gonococcal catalase from this strain is a monofunctional protein. To confirm that GC catalase of FA1090 is not a catalase-peroxidase, total lysate from *E. coli* UM255 carrying GC catalase was assayed for peroxidase activity using 3 different substrates and conditions, and by peroxidase activity gel. No peroxidase activity was detected in any of the 4 enzymatic assays used (**Table 6**), nor was peroxidase activity detected in the peroxidase activity gel. These data are further evidence that catalase from GC strain FA1090 is a monofunctional catalase.

Figure 23. Western blot analysis of GC catalase expressed in *E. coli* UM255. (**A**) Sequence of the peptide used to immunize BALB/c mice. (**B**) Coomassie blue stained PAGE gel: 100 μg of total lysate were loaded in each lane; **1**) *E. coli* UM255, **2**) *E. coli* UM255 (pACYC184) and **3**) *E. coli* UM255 (pAAS184-*kat* 30). (**C**) Amido black stained membrane after transfer of gel and (**D**) Western blot of total protein lysates using anti-gonococcal catalase peptide antibody (1/200 dilution). The anti-catalase peptide antibody reacted specifically with a predominant band expressed in *E. coli* UM255 (pAAS184-*kat* 30). Arrow in (**A**) and (**B**) indicate the intense 58 kDa band observed in stained lysates from *E. coli* UM255 (pAAS184-*kat* 30) and not in *E. coli* UM255 without this plasmid, and (**C**), the 58 kDa band that reacted with catalase peptide-specific antiserum.

A. **NH_2-RDPRKFPDLNKAVKRDPRTNM-COOH**



B. Coomassie blue stained gel



C. Amido black stained membrane

D. Western blot

Table 6- Peroxidase activity in *E. coli* known to possess catalase-peroxidases (*E. coli* HS-4 and *E. coli* HB101) versus *E. coli* UM255 (pAAS184-*kat* 30)

Whole Cell Lysate	**Peroxidase assay***			
	I (U/mg P)	**II (U/ml)**	**III (U/ml)**	**IV (U/mg/ml)**
HRP	599 ± 152	392 ± 62	260 ± 52	3,469 ± 1,326
***E.coli* HS-4**	0	0	274 ± 13	0.5 ±0.08
***E. coli* HB101**	0	0	1,658 ± 79	0.4 ± 0.04
***E. coli* UM255**	0	0	0	0
***E. coli* UM255 (pAAS184-*kat* 30)**	0	0	0	0
FA1090	0	0	0	0

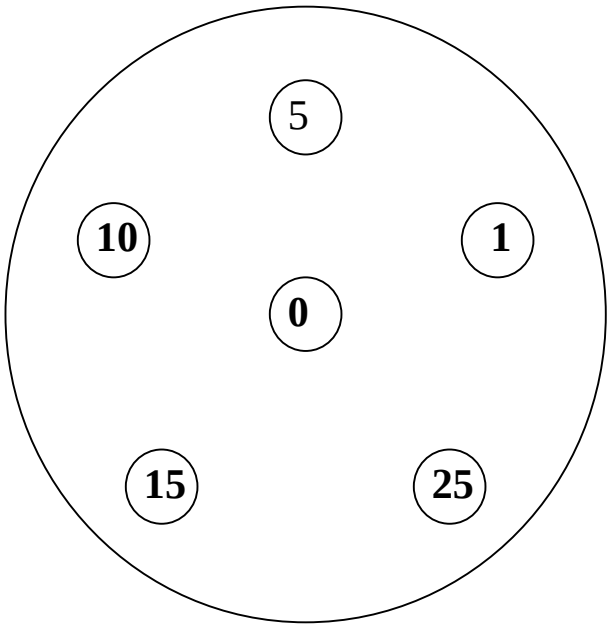
*Peroxidase assays varies on the hydrogen donor used and pH to determine the peroxidase activity. Assay I, 4-aminoantypirine; II, diaminobenzidine at pH 4.5; III, diaminobenzidine at pH 7.0, and IV, o-dianisidine. HRP, horse radish peroxidase.

To further characterize the activity of the recombinant GC catalase expressed in *E. coli* UM255, the H_2O_2 sensitivity of *E. coli* UM255 with and without the cloned GC *kat* gene was tested by disk diffusion assay. As expected, no significant difference in H_2O_2 sensitivity was observed between *E. coli* UM255 and UM255 (pACYC184) ($p = 0.3$ to 0.6). Expression of gonococcal catalase in *E. coli* UM255 conferred resistance to H_2O_2 at concentrations ranging from 5 to 25 mM ($p < 0.05$; **Fig. 24 A and B**). Although expression of gonococcal catalase in *E. coli* UM255 conferred H_2O_2 resistance wild type *E. coli* HS-4, was significantly more resistant to H_2O_2 than *E. coli* UM255 (pAAS184-*kat* 30) at concentrations ranging from 50 to 500 mM ($p > 0.05$ and 0.005). No significant difference was observed among the two bacteria at 1,000 mM H_2O_2 (**Fig. 25**).

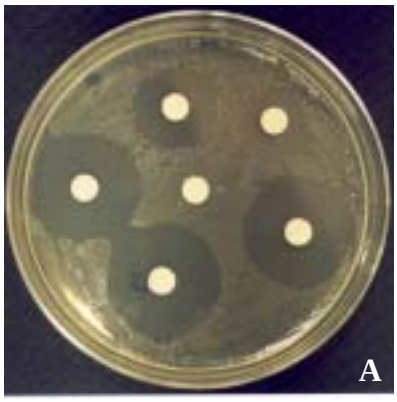
C. Characterization of the *kat* promoter region

The 5' region upstream of the cloned catalase gene contains the putative promoter of the *kat* gene (**Fig. 26**). Computer analysis of this region reveals a motif typical of the anaerobic regulator, fumarate-nitrate regulator (FNR). FNR has been shown to have a role in regulation on the gonococcal *aniA* gene (Householder et al., 1999). To determine the minimum length of the region upstream of the *kat* structural gene that is required to direct the expression of the GC *kat* gene in *E. coli*, deletion mutants containing progressively less of the 5' end region were constructed by restriction digestion followed by religation and testing for catalase activity in *E. coli* UM255 (**Fig. 7, 26 A and B**). This approach revealed that the first 427 bp of the cloned 2.4 kb are not necessary for catalase activity in *E. coli* given that clones lacking this region exhibited both phenotypes observed in *E. coli* UM255 expressing gonococcal catalase, specifically, positive catalase activity in the bubbling assay and dark colony color (**Table 7**).

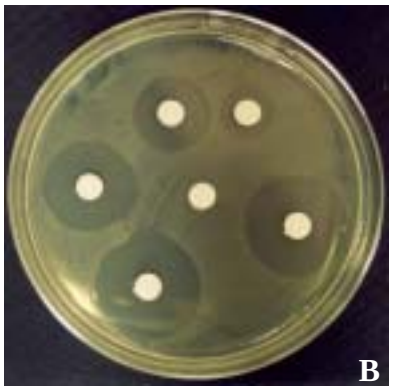
Figure 24. H_2O_2 sensitivity of *E. coli* UM255 expressing gonococcal catalase. Bacteria were exposed to H_2O_2 concentrations ranging from 1-25 mM. Below, schematic organization of the sensitivity plate. Expression of gonococcal catalase by *E. coli* UM255 (pAAS184-*kat* 30) (**E**) more resistant to increasing concentrations of H_2O_2 than *E. coli* with and without the cloning vector (**A** and **B**). The experiment was performed 3 times in duplicate. Each bar represent the average of the 3 experiments and the error bars correspond to the standard deviation of the mean. (*) $p < 0.05$ and (**) $p < 0.005$ (**E**).



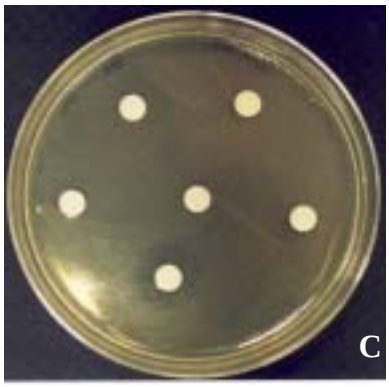
***E. coli* UM255**



***E. coli* UM255 pACYC184**



***E. coli* UM255 (pAAS184-*kat* 30)**



***E. coli* HS-4**

E

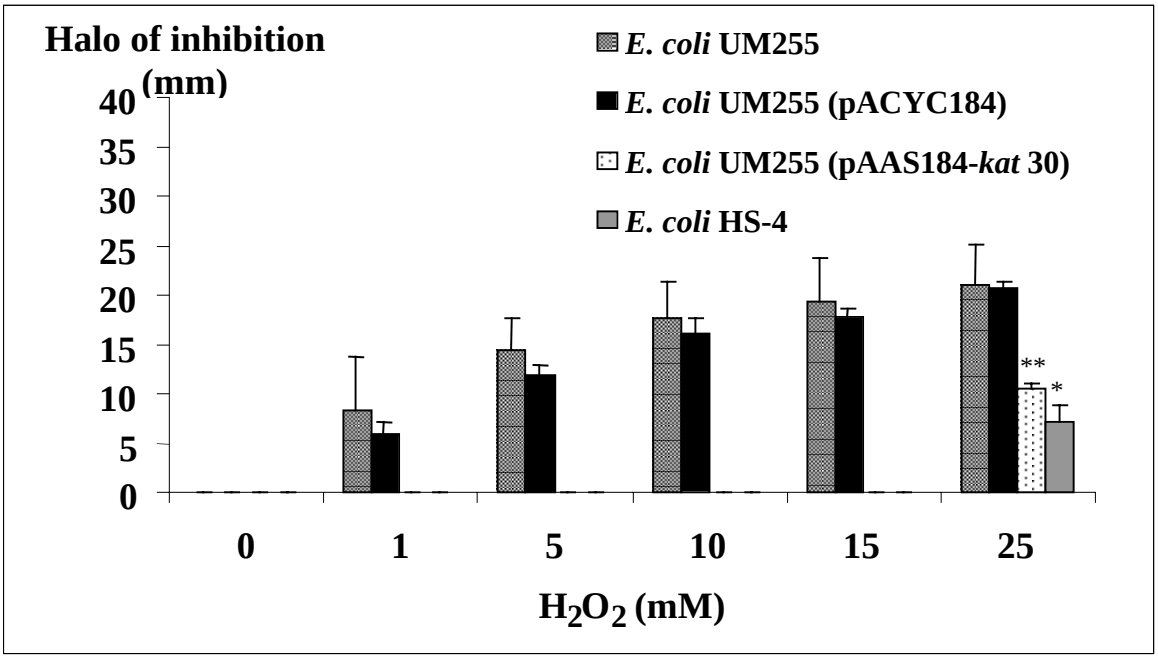
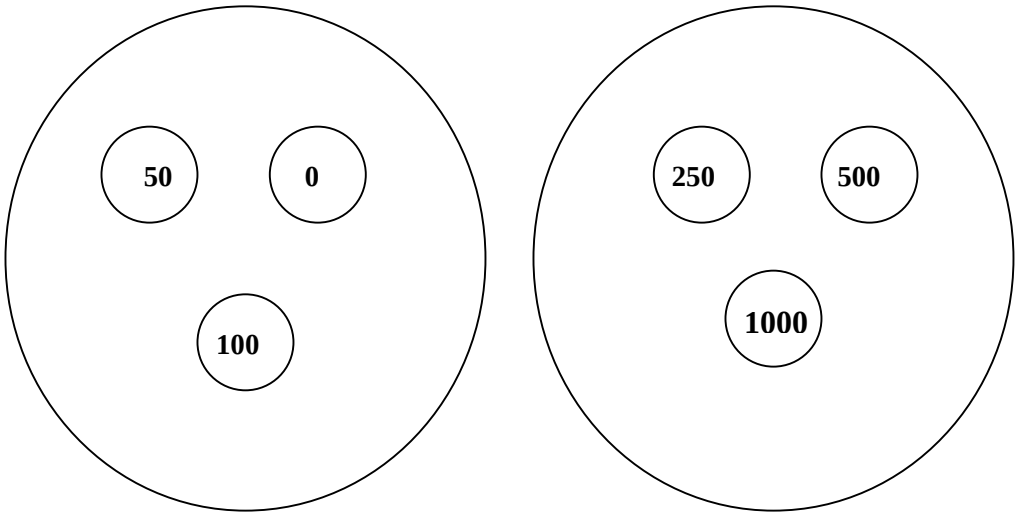
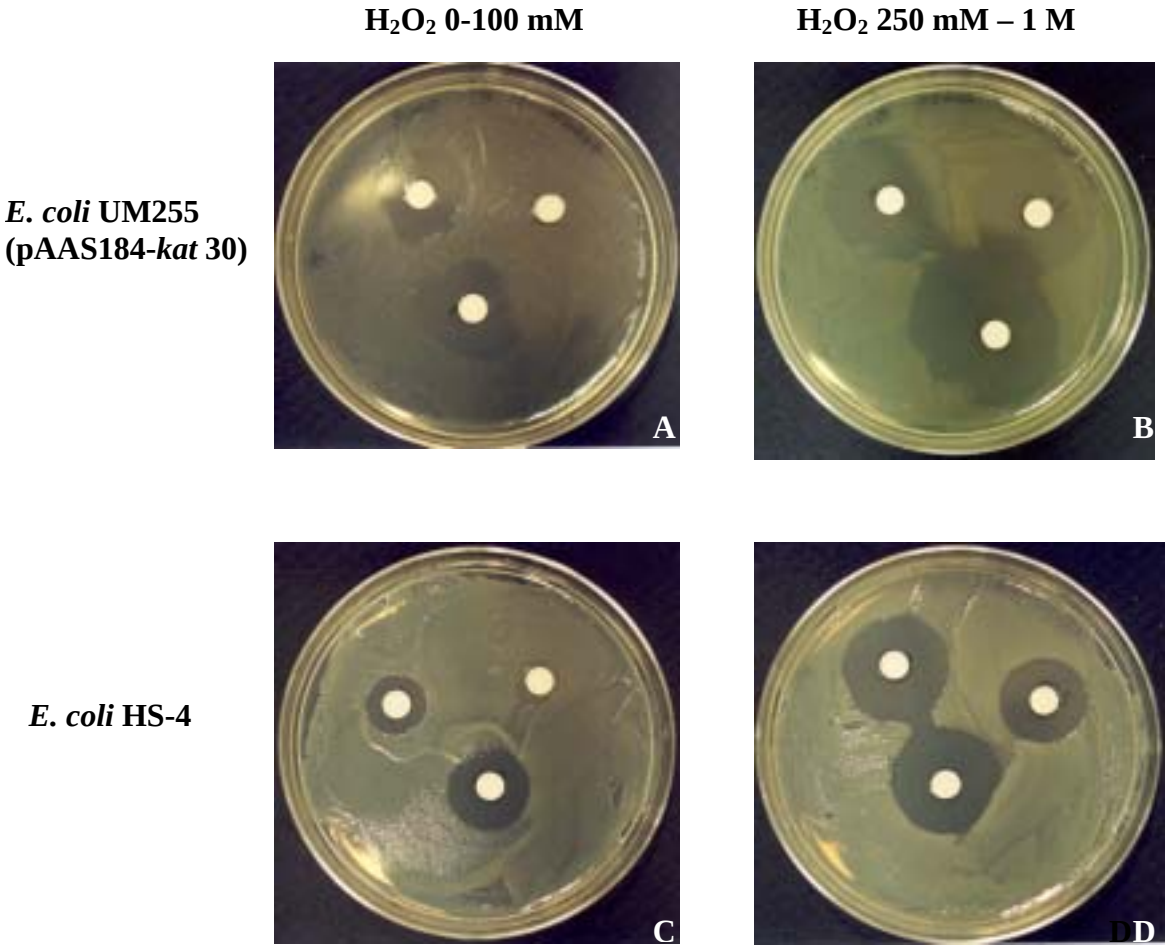


Figure 25. H_2O_2 sensitivity of *E. coli* UM255 (pAAS184-*kat* 30) and *E. coli* HS-4. Bacteria were exposed to H_2O_2 concentrations ranging from 0-1,000 mM. Below, schematic organization of sensitivity plates. Expression of gonococcal catalase in *E. coli* UM255 conferred levels of H_2O_2 resistance (**A** and **B**) equal to that expressed by the commensal *E. coli* strain HS-4 (**C** and **D**). The experiment was performed 3 times in duplicate. Each bar represent the average of the 3 experiments and the error bars correspond to the standard deviation of the mean. (*) $p < 0.05$ and (**) $p < 0.005$ (**E**).




H_2O_2 0-100 mM
H_2O_2 250 mM – 1 M
E. coli UM255 (pAAS184-*kat* 30)
E. coli HS-4
A
B
C
DD

E

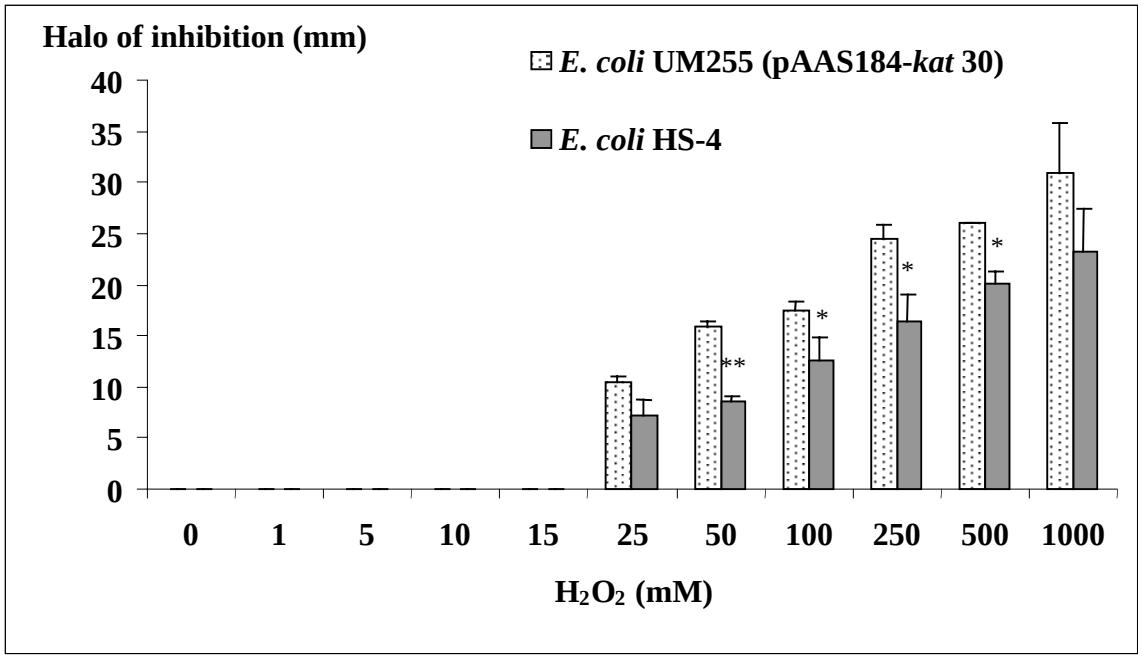

Halo of inhibition (mm)
E. coli UM255 (pAAS184-*kat* 30)
E. coli HS-4
40
35
30
25
20
15
10
5
0
0
1
5
10
15
25
50
100
250
500
1000
H_2O_2 (mM)
*
**
*
*
*

Figure 26. Nucleotide motifs (**A**) and restriction map (**B**) of the putative catalase promoter region. (**A**) Three fumarate and nitrite reductase (FNR) regulator protein binding sites were found in the putative catalase promoter region. The consensus FNR binding site consists of the following sequences TTGAT and ATCAA. FNR, in conjunction with σ^{70}, is required for anaerobic gene expression of the gonococcal *ani*A gene (Householder et al., 1999).

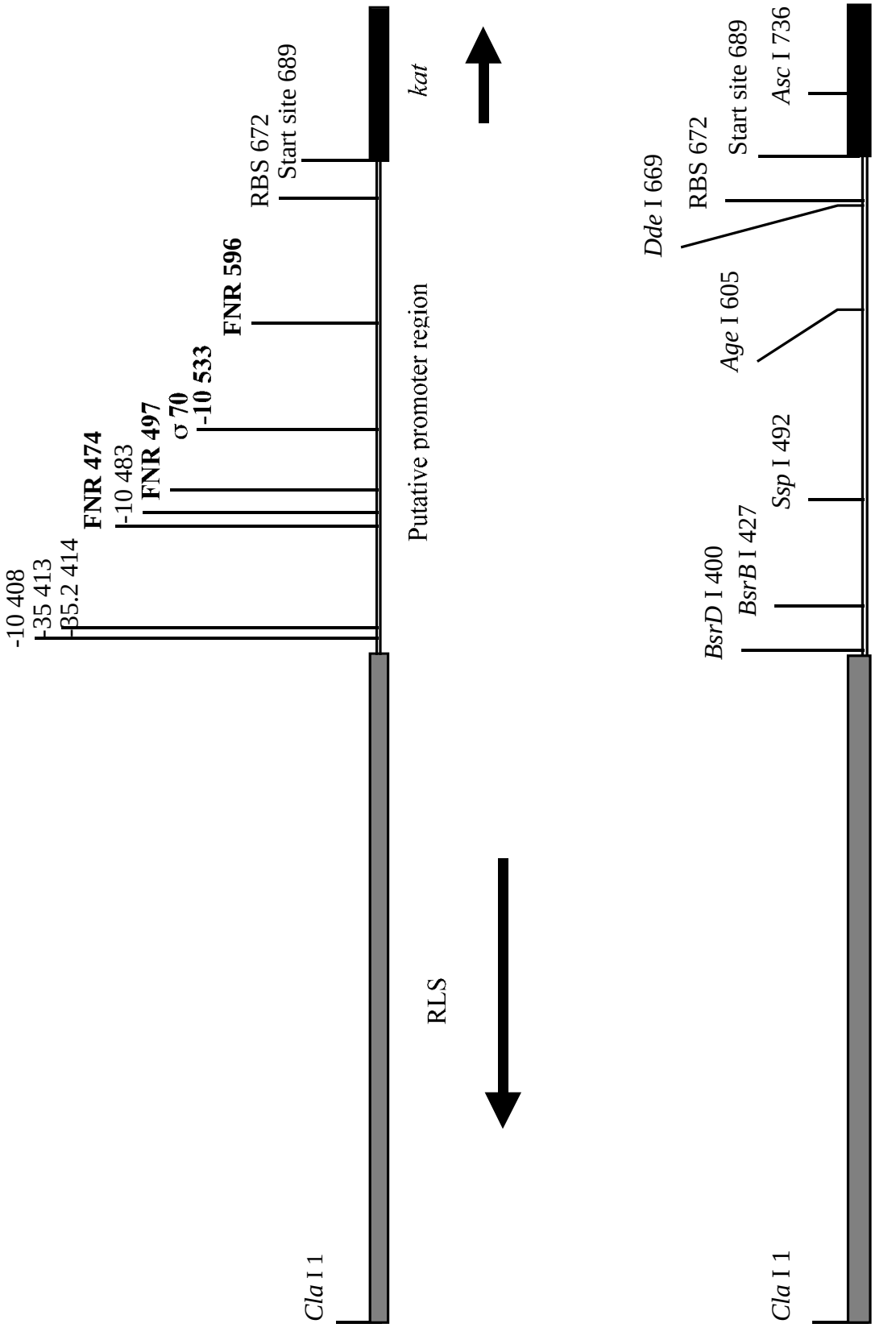

-10 408
-35 413
-35.2 414
FNR 474
-10 483
FNR 497
σ 70
-10 533
FNR 596
RBS 672
Start site 689
kat
Putative promoter region
RLS
Cla I 1
Dde I 669
RBS 672
Start site 689
Asc I 736
Age I 605
Ssp I 492
BsrD I 400
BsrB I 427
Cla I 1

Table 7. Catalase phenotype of transformants carrying subcloned deletions of the *kat* promoter region

Construct (plasmids)	Restriction enzyme	Position	Catalase activity	Colony color phenotype
pAAS184-*kat* 30	-	-	+	+
pAAS184-*kat* $_{\Delta (-) 400}$*	*Bsr* DI	(-) 400	+	+
pAAS184-*kat* $_{\Delta (-) 427}$*	*Bsr* BI	(-) 427	+	+
pAAS184-*kat* $_{\Delta (-) 492}$*	*Ssp* I	(-) 492	-	-
pAAS184-*kat* $_{\Delta (-) 605}$*	*Age* I	(-) 605	-	-
pAAS184-$_{kat \Delta (-) 669}$*	*Dde* I	(-) 669	-	-
pAAS184-*kat* $_{\Delta (+) 47}$*	*Asc* I	(+) 47	-	-

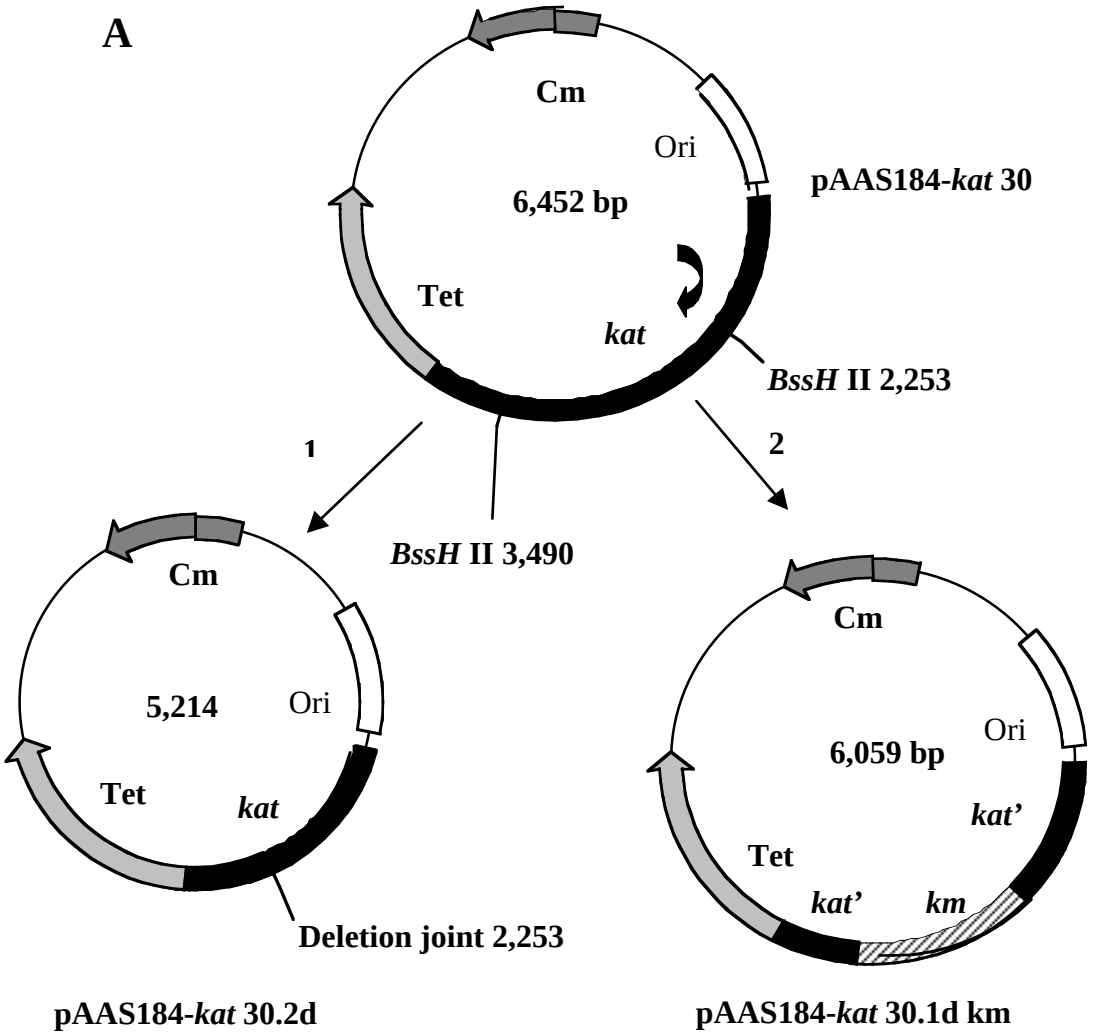
*Deletions in the putative catalase promoter were made by restriction digestion. Truncated DNA fragments were then cloned into pACYC184 and transformed into *E. coli* UM255. Catalase activity was detected by bubbling assay. The presence of truncated forms of the catalase gene were checked by PCR and sequencing. Numbers refers to the number of nucleotides deleted upstream (-) and downstream (+) of the ATG of the cloned GC *kat* gene.

II-Construction and characterization of a GC catalase mutant

A. Construction of a GC catalase mutant

Two approaches were used to construct the catalase mutant (**Fig. 27 A**). In both approaches a 1.2 kb *BssH* II fragment internal to the *kat* gene was removed by restriction digestion. The DNA was then religated on itself to create pAAS184-*kat* 30.2d, or blunt ended and then ligated with a nonpolar km resistance cassette (*aph*A-3)(Ménard et al., 1993) to create pAAS184-*kat* 30.1d km. The modifications in the catalase gene and the orientation of the km^R cassette were verified by PCR and nucleotide sequencing (**Fig. 27 B**, **28 A** and **B**). To confirm that the gonococcal catalase gene was successfully mutated, both modified plasmids were transformed into *E. coli* UM255. The resultant transformants were negative by bubbling assay and did not exhibit a dark color colony phenotype. The mutated catalase genes were released from the plasmid backbones as *Cla* I-*Nhe* I fragments and transformed as linear DNA into GC FA1090 by spot dilution transformation as described in the Materials and Methods. Introduction of the mutated catalase gene carrying the *aph*A-3 cassette into the chromosome of wild-type GC to create mutant NGAAS-500 was relatively easy compared to introduction of the *kat* gene carrying only the internal deletion. After several unsuccessful attempts, mutant NGAAS-501 was constructed by transformation of mutant NGAAS-500 with linear DNA from pAAS184-*kat* 30 (*kat* Δ 1.2 kb). The desired mutant, NGAAS-501, which carried an internal deletion in the *kat* gene without a selectable marker was isolated by screening for catalase negative and kanamycin susceptibility phenotype.

Figure 27. Mutation of the catalase gene in pAAS184-*kat* 30. (**A**) Modifications to plasmid pAAS184-*kat* 30 resulted in a *kat* gene containing an internal deletion or a *kat* gene carrying an *aph*A-3 non-polar cassette within the deleted gene region. (**B**) Agarose gel of PCR-screened modifications in the cloned catalase gene. Lanes **1** and **12**), 1 kb ladder; **2** and **11**), 100 bp ladder; **3**) pACYC184; **4**) pAAS184-*kat* 30; **5** and **9**) pAAS184-*kat* 30.2d; **6** and **10**) pAAS184-*kat* 30.1d km; **7**) pUC18 and **8**) pUC18 km. Lanes 3 to 6 represent PCR products amplified with primers 43-99 F and 44-99 B (to amplify insert in pACYC184). PCR products in lanes **7** to **10** were amplified with PCR primers 45-99 F and 46-99 B (to amplify km^R cassette, **Fig. 8**). All the PCR products showed the expected sizes.


A
Cm
Ori
6,452 bp
Tet
kat
pAAS184-kat 30
BssH II 2,253
1
2
BssH II 3,490
Cm
5,214
Ori
Tet
kat
Deletion joint 2,253
pAAS184-kat 30.2d
Cm
Ori
6,059 bp
kat'
Tet
kat'
km
pAAS184-kat 30.1d km

B

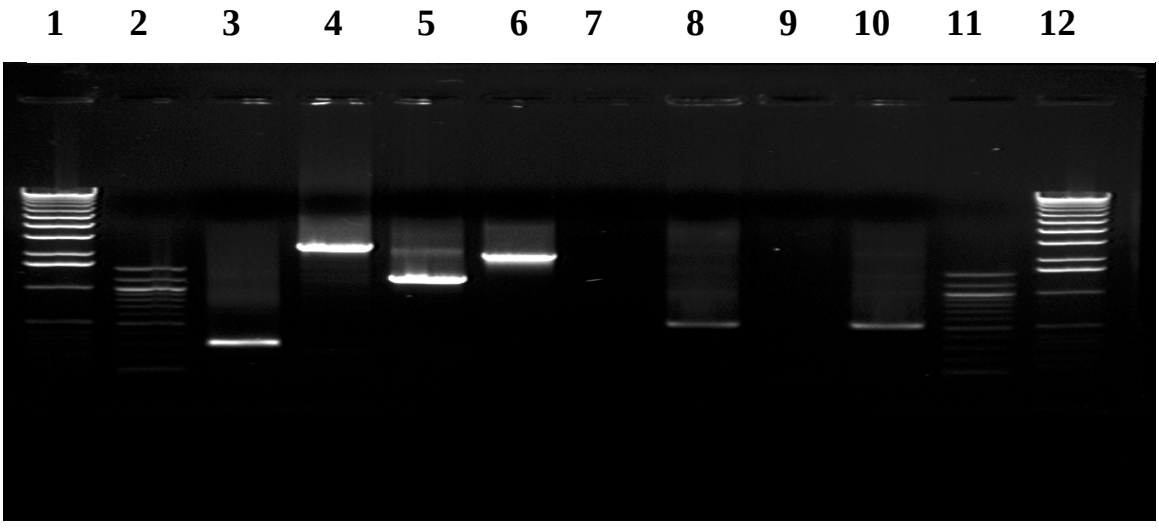
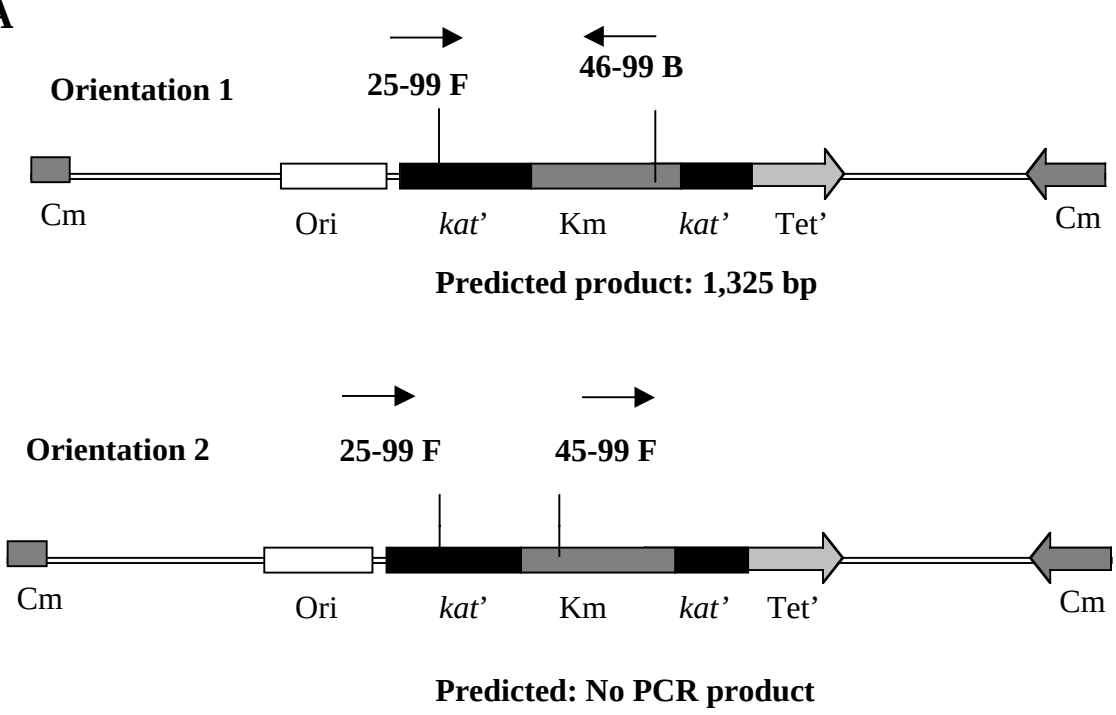
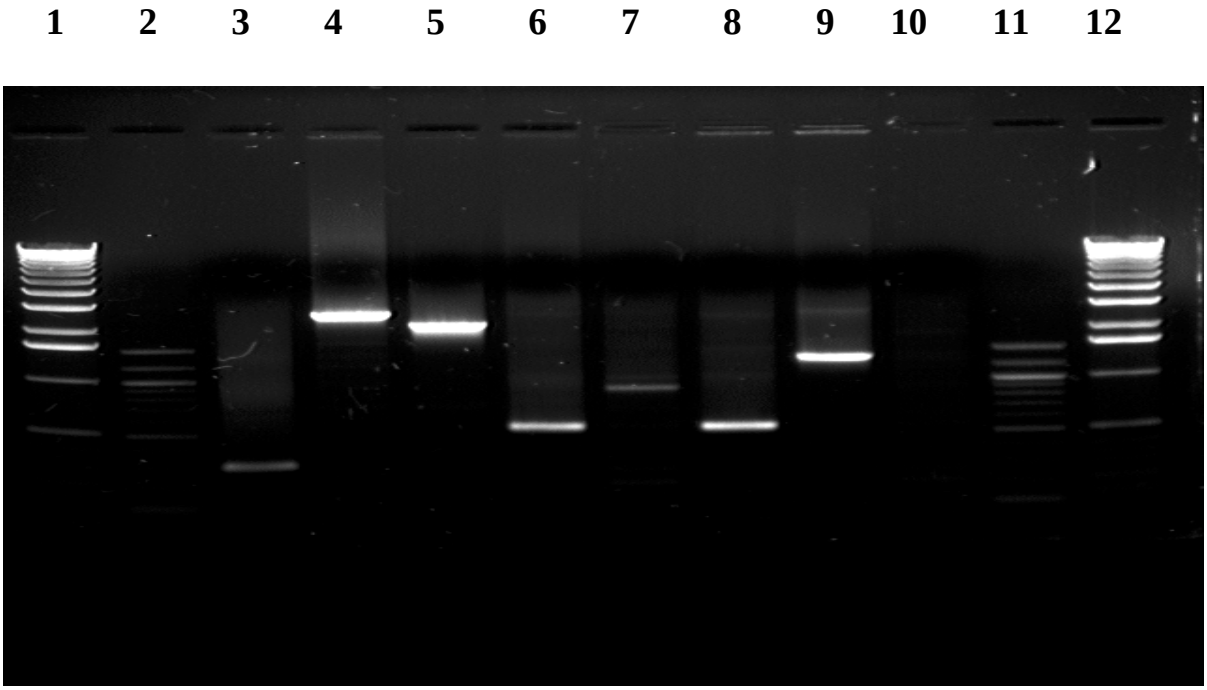

1 2 3 4 5 6 7 8 9 10 11 12

Figure 28. Determination of the *aph*A-3 cassette orientation in pAAS184-*kat* 30.1d km. (**A**) Schematic illustration of predicted PCR products, which will reveal the orientation of the *aph*A-3 cassette in pAAS184-*kat* 30.1d km. (**B**) Agarose gel analysis of PCR screening. Lanes **1** and **12**), 1 kb ladder; **2** and **11**), 100 bp ladder; **3**) pACYC184; **4** and **7**) pAAS184-*kat* 30; **5**, **8**-**10**) pAAS184-*kat* 30.1d km; **6**) pUC18 km. Lanes **3** to **5** represent PCR products amplified with primers 43-99 F and 44-99 B. This pair of primers annealed in the vector pACYC184 upstream to the *Cla* I site and downstream of the *Eco* RV site in the plasmid. PCR products in these lanes show a shift in size due to the deletion of an internal region of the catalase gene and insertion of the *aph*A-3 cassette (**Fig. 8**). PCR products in lanes **6** and **8** were amplified with primers 45-99 F and 46-99 B (to amplify *aph*A-3 cassette). These lanes show the presence of the *aph*A-3 cassette in the mutated catalase gene. Lane **7**, product amplified with primers 25-99 F and 21-98 B (to amplify *kat* gene putative promoter). Lanes **9** and **10** represent the PCR products from primer combinations shown in (**A**) orientation 1 and 2, respectively. PCR screening shows the expected results. No PCR product was observed in lane 10 (orientation 2), indicating that the *aph*A-3 cassette is found in the same direction as the gonococcal catalase promoter. As a consequence, expression of the Km^R gene is driven by the *kat* promoter, creating a non-polar insertion for the downstream genes once it is recombined into the GC chromosome.

A


Orientation 1
25-99 F
46-99 B
Cm
Ori
kat'
Km
kat'
Tet'
Cm
Predicted product: 1,325 bp
Orientation 2
25-99 F
45-99 F
Cm
Ori
kat'
Km
kat'
Tet'
Cm
Predicted: No PCR product

B


1 2 3 4 5 6 7 8 9 10 11 12

B. Genetic characterization of the GC catalase mutant

Figures 29 and **30** show the predicted sizes of bands that hybridized to the full length catalase and *aphA*-3 probes in genomic DNA from NGAAS-500. GC catalase mutants NGAAS-500 and NGAAS-501 were characterized by Southern blot (**Fig. 31 A**, **B**, **C** and **D**). A shift in the size of the band that hybridized with the full length *kat* probe, corresponding to the integration of the *aph*A-3 cassette, was observed (**Fig. 31 B**). The introduction of an internal deletion into the *kat* gene in both mutants was confirmed by probing with the internal 700 bp catalase fragment (**Fig. 31 C**). The insertion of the *aph*A-3 cassette in the *kat* gene of the mutant NGAAS-500 was confirmed by using a *aph*A-3 cassette probe consisting of 500 bp (**Fig. 31 D**).

C. Complementation of GC catalase mutants

Loss of catalase activity was tested by catalase activity gel and enzymatic assay (**Fig. 32** and **Table 8**). As expected, the two catalase mutants did not show any catalase activity in either assay. Restoration of the catalase deficiency in strains NGAAS-500 and 501 was attempted by transferring a wild-type copy of the catalase gene by conjugation into the mutants using the shuttle vector pLEE20 (**Fig. 6 C**). Conjugation was performed by using *E. coli* strain S-17 carrying the plasmid pAAS20-*kat* 30 (**Fig. 33**) as a donor and the catalase mutants as recipients. Transconjugants were selected on GC agar plates in the presence of erythromycin (the antibiotic resistance marker of the complementing plasmid) and by the bubbling assay. Mutants NGAAS-500 and NGAAS-501 carrying pAAS20-*kat* 30 were isolated (NGAAS-502 and NGAAS-503, respectively) and analyzed for restoration of catalase activity.

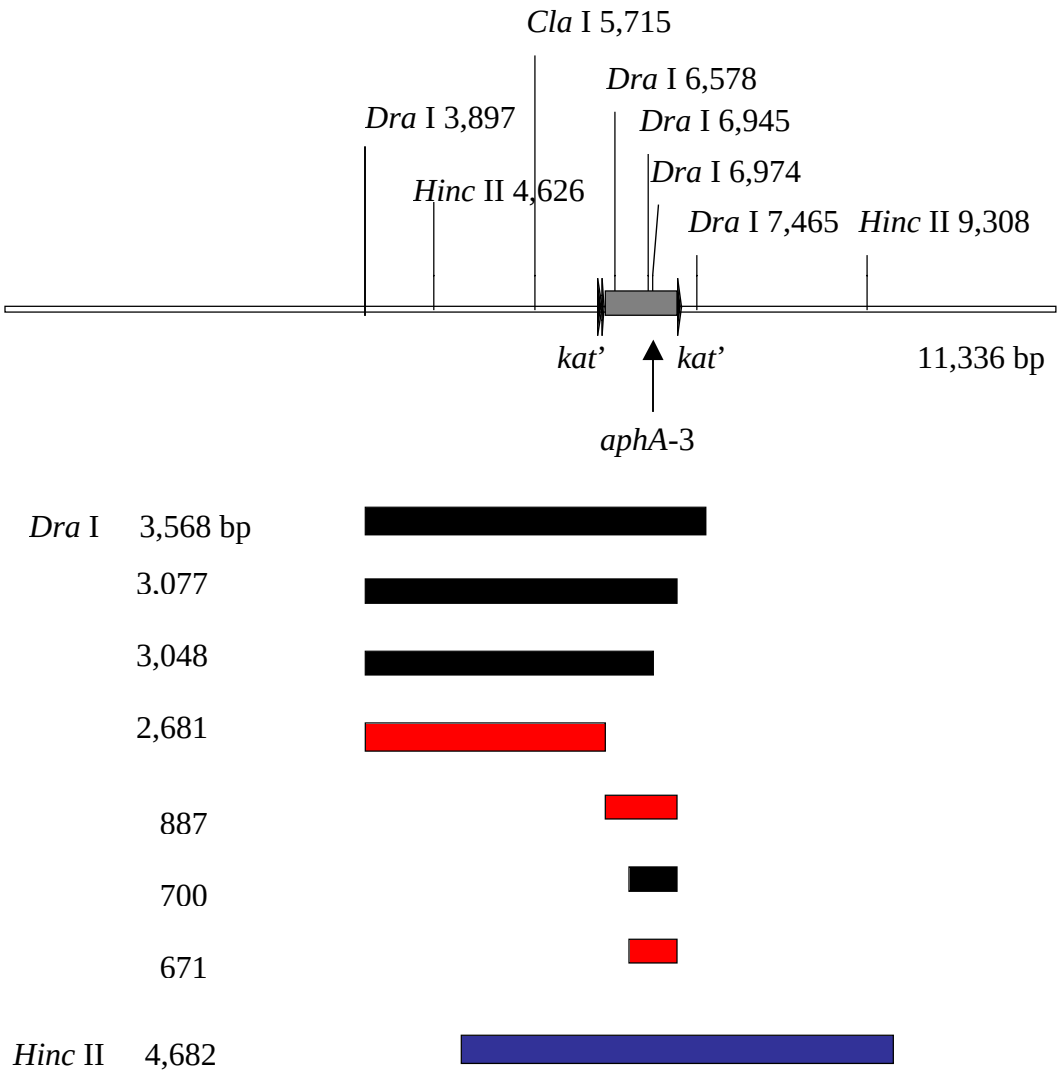


Figure 29. Restriction map of mutated region in NGAAS-500 and expected sizes to hybridize with entire GC *kat* gene probe. Expected restriction fragments after genomic DNA digestion with *Dra* I and *Hinc* II. *Cla* I digestion is not presented in the diagram given that fragment generated experimentally is bigger than that of the contig. Only one *Cla* I site was found in the contig. Fragments in red and blue correspond to the *Dra* I and *Hinc* II discrete bands that hybridized with entire *kat* gene as a probe, respectively (**Fig. 31 B**). Restriction digestion of genomic DNA with *Cla* I and *Dra* I simultaneously generates fragments 887 and 671 bp fragment.

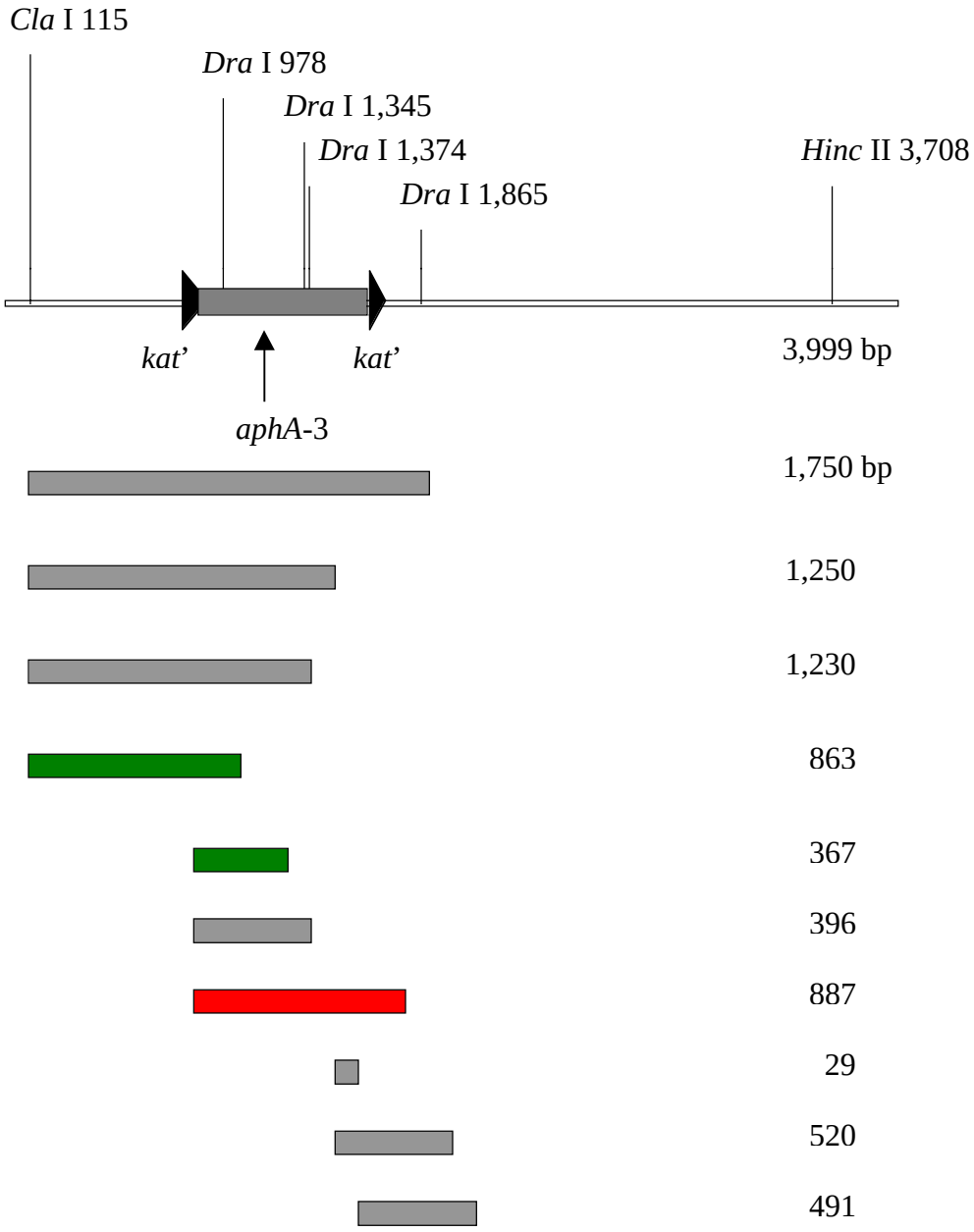


Figure 30. Restriction map of mutated region in NGAAS-500 and expected sizes to hybridize with *aph*A-3 cassette probe. Expected restriction fragments after digestion of genomic DNA with *Cla* I and *Dra* I. Fragments in green and red represent the bands that hybridized with *aph*A-3 cassette probe (**Fig. 31 D**).

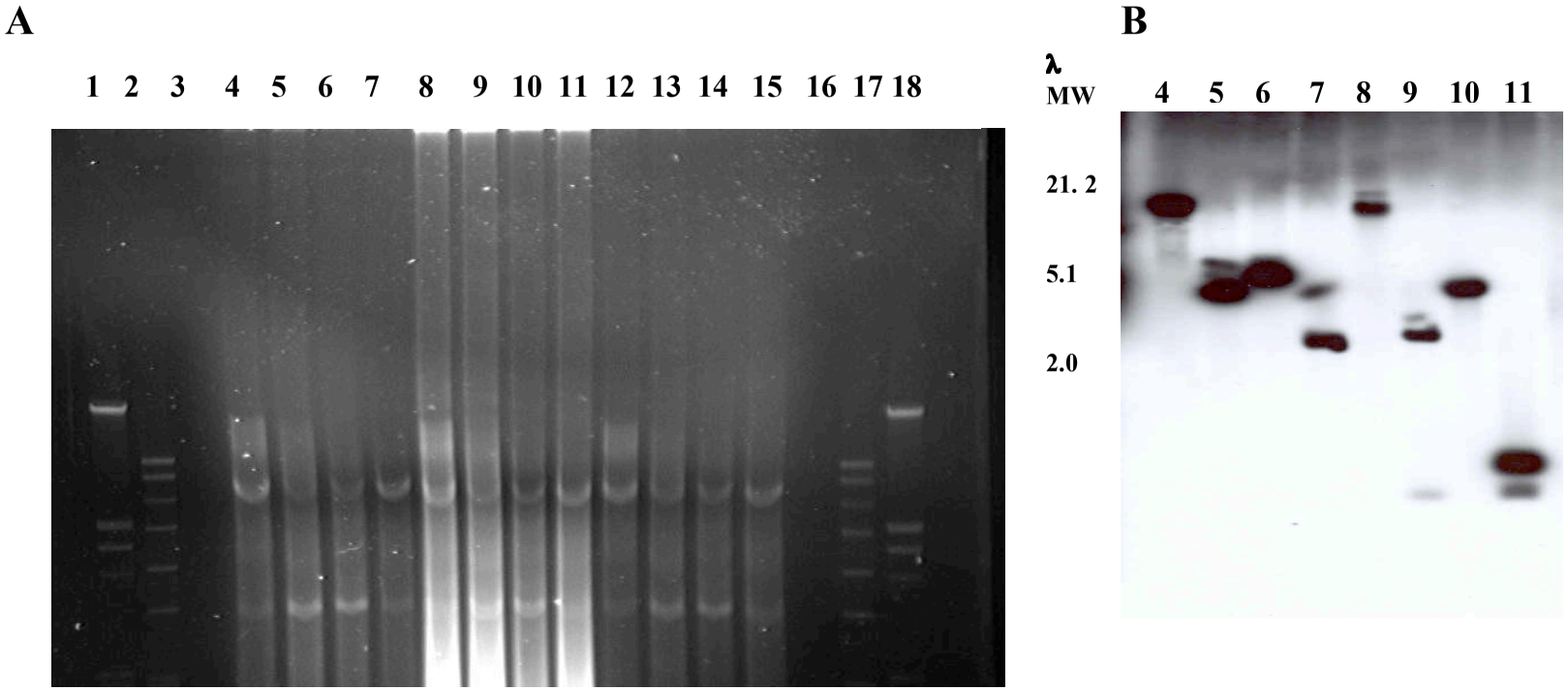


Figure 31. (**A**) Ethidium bromide agarose stained gel used in Southern blot shown in panel **B**. Lanes **1** and **18**) λ ladder; **2** and **17**) VII ladder; **4-7**FA1090; **8-11**) NGAAS-500 and **12-15**) NGAAS-501. In lanes **4**, **8** and **12** genomic DNA cut with *Cla* I; **5**,**9** and **13** *Dra* I; **6**, **10** and **14** *Hinc* II and **7**, **11** and **15** *Dra* I- *Cla* I. (**B**) Southern blot of restricted genomic DNA form FA1090 (**4-7**) and NGAAS-500 (**8-11**). Probe used was the entire catalase gene labeled with digoxigenin.

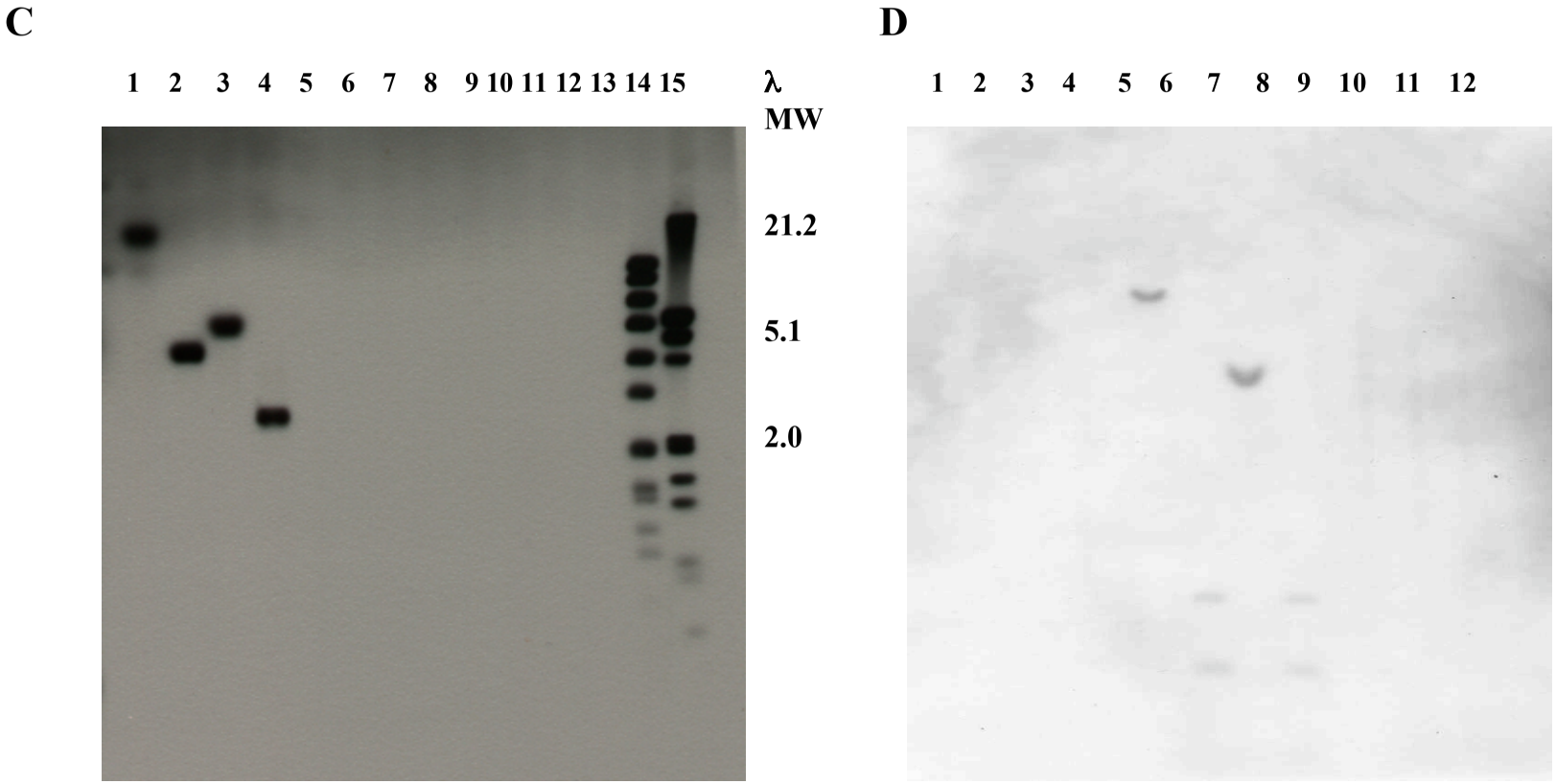


Figure 31. (**C**) and (**D**) Southern blot of restricted genomic DNA from FA1090 (**1-4**) NGAAS-500 (**5-8**) and NGAAS-501 (**9-12**). Lanes **14** and **15**) VII and λ ladder, respectively. In lanes **1**, **5** and **9** genomic DNA was cut with *Cla* I; **2**, **6** and **10** *Dra* I; **3**, **7** and **11** *Hinc* II and **4**, **8** and **12** *Dra* I- *Cla* I. (**C**) Hybridization with the700 bp internal catalase probe. (**D**) Hybridization with the *aph*A-3 cassette probe. Smaller bands observed in panel (**D**),lanes **6** and **8** correspond to digestion of DNA by *Dra* I, which cuts inside the *aph*A-3 cassette. There are 3 *Dra* I sites in the *aph*A-3 cassette. The two observed bands correspond to the expected 863 and 367 bp fragments, respectively.

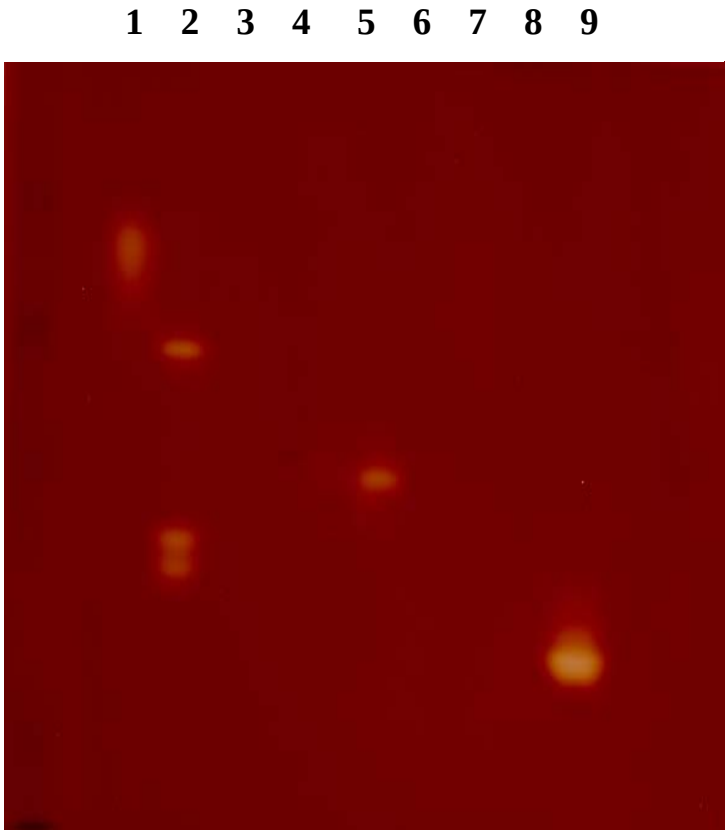


Figure 32. Biochemical characterization of NGAAS-500 and NGAAS-501 catalase mutants by catalase activity gel. Sample loaded; (**1**) Liver bovine catalase, (**2**) *E. coli* HS-4, (**3**) *E. coli* UM255, (**4**) *E. coli* UM255 (pACYC184), (**5**) *E. coli* UM255 (pAAS154-*kat* 30), (**6**) NGAAS-500 (GC FA1090 Δ 1.2 kb::*aphA*-3), (**7**) NGAAS-502 [NGAAS-500 (pAAS20-*kat* 30), (**8**) NGAAS-501 (GC FA1090 Δ 1.2 kb), and NGAAS-503 [NGAAS-501 (pAAS20-*kat* 30). Twenty-five μg of total lysate and 1 unit of liver bovine catalase were loaded in the native gel. Catalase mutants did not show catalase activity in the assays. Complementation of *kat* mutants *in trans* using pAAS20-*kat* 30 restored the catalase activity (sample **10**, **Table 8**) and the presence as a protein with catalase activity in the catalase activity gel (lane **9**) in mutant NGAAS-501 only. The catalase expressed in complemented mutant NGAAS-503 exhibited a different mobility pattern compared to the wild-type protein (lane **5**). In contrast, *E. coli* UM255 carrying pAAS184-*kat* 30, produced a recombinant protein with identical relative mobility as catalase expressed from GC FA1090 (lane **5**).

Table 8. Quantitation of catalase enzymatic activity in catalase mutant and complemented clones.

Lysate (ID)	Catalase activity U/mgP
1) Liver bovine catalase	42,367 ± 5,577
2) *E. coli* HS-4	14.6 ± 1.6
3) *E. coli* UM255	0
4) *E. coli* UM255 (pACYC184)	0
5) *E. coli* UM255 (pAAS184- *kat* 30)	25,380 ± 1,410
6) *E. coli* UM255 (pAAS20-*kat* 30)	1.5 ± 0.2
7) NGAAS-500	0
8) NGAAS-502	0
9) NGAAS-501	0
10) NGAAS-503	47 ± 5.5
11) FA1090	30 ± 7

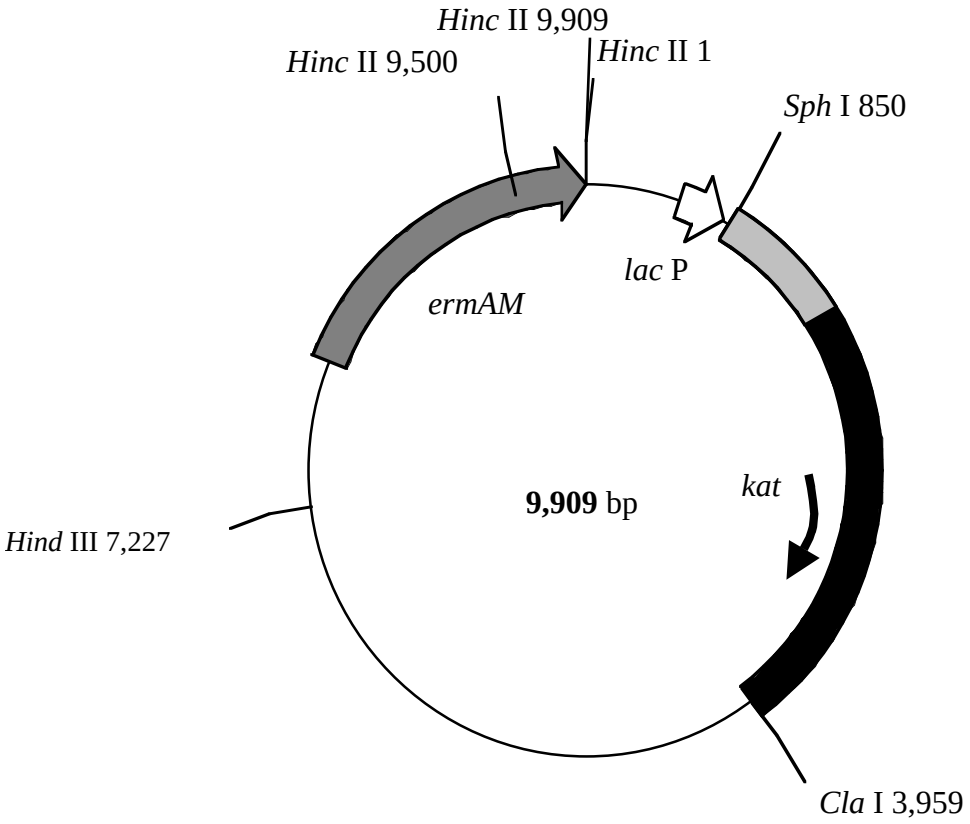


Figure 33. Complementing plasmid pAAS20-*kat* 30. Arrow indicates direction of *kat* gene transcription and translation. Diagram shows specific restriction sites and their positions. Numbers indicate base pairs.

Strain NGAAS-502, despite being catalase positive in the bubbling assay, did not exhibit detectable catalase activity by activity gel or spectrophotometric enzymatic assay (**Fig. 32** and **Table 8**). On the other hand, complemented strain NGAAS-503 produced almost twice as much catalase activity (47 U) as FA1090 (30 U). Interestingly, NGAAS-503 produced a protein with catalase activity as detected by catalase activity gel; however the relative mobility of the protein compared to that of the FA1090 was different, suggesting that the recombinant catalase protein, when expressed in GC was different in size.

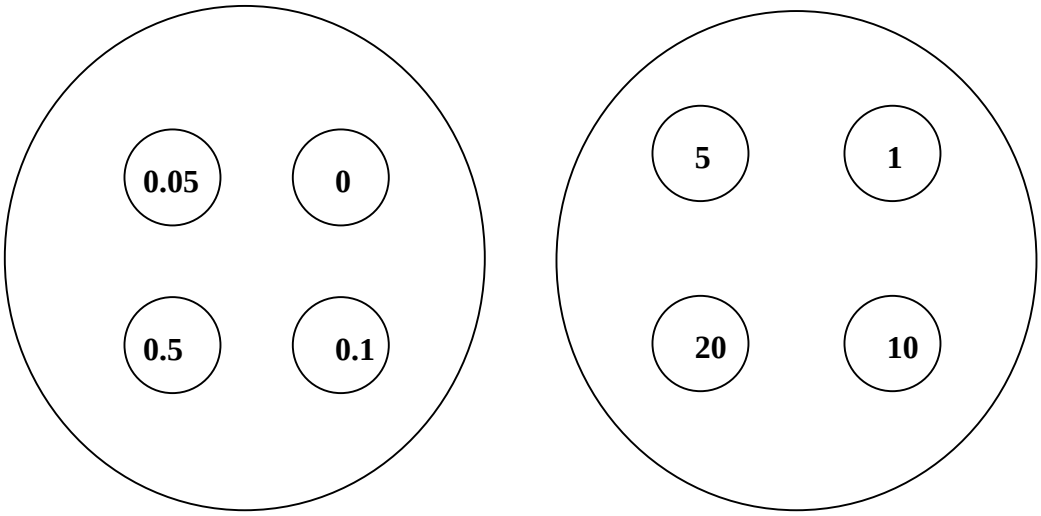
To elucidate the differences observed in the protein produced by wild-type GC and the faster migrating species produced by NGAAS-503, *N*-terminal sequencing of the 2 predominant proteins present in whole cell lysate from NGAAS-503 was performed. Unfortunately, attempts to determine the *N*-terminal sequence were unsuccessful. One of the selected bands appeared to be modified at the *N*-terminal end. The following sequence NH_2-XVKVAINGFGRIGRLAFRRIGXV-COOH was obtained from the second protein band. BLAST search analysis identified the protein as glyceraldehyde-3-phosphate dehydrogenase, an enzyme involved in glycolysis, among other functions (Sirover, 1996). It can be concluded that the band with catalase activity was not isolated, and therefore, the modification responsible for the difference in relative mobility of the protein expressed in NGAAS-503 remains unknown. In summary, it was concluded that mutant NGAAS-501 was partially complemented as evidenced by the detection of catalase activity in the bubbling and quantitative enzymatic assays, and the fact that 2 out of 3 other phenotypes were restored (see below). We believed that the inability of

NGAAS-503 to restore the third phenotype (resistance to H_2O_2; see below) may be due to the altered size of the catalase protein expressed from the plasmid in GC.

D. Characterization of catalase mutant for sensitivity to toxic oxygen radicals *in vitro*.

The sensitivity of the catalase deficient mutant NGAAS-501 to toxic oxygen radicals or inducers of toxic oxygen radicals such as H_2O_2, paraquat, streptonigrin, tetracycline, gentamicin and SIN-1 was tested using the sensitivity disk assay. H_2O_2 is a small compound that can diffuse across membranes, damaging DNA and proteins. Mutant NGAAS-501 was significantly more sensitive than wild-type FA1090 at concentrations of H_2O_2 ranging from 0.1 to 20mM ($p > 0.05$ and 0.005) (**Fig. 34 A** to **E**). Inducers of toxic oxygen radicals such as paraquat significantly inhibited NGAAS-501 at high concentrations ranging from 20-60 mM ($p < 0.05$ and 0.005) (**Fig. 35**). In contrast, streptonigrin significantly inhibited NGAAS-501 at the lowest concentration tested 0.25 mg/ml ($p = 0.042$) (**Fig. 36**). Streptonigrin is an antitumor antibiotic that cause strand breakes in DNA *in vivo* (White, 1977). The activity of this antibiotic requires intracellular reduction of the antibiotic and the presence of oxygen. In the presence of iron its lethality is enhanced due to the formation of hydroxyl radical (Hassett et al., 1989; White and Yeowell, 1982). Hassett et al. (1987) reported that 0.025 μg/ml effectively killed wild-type GC. In our study 10-fold more streptonigrin was needed to see a significant inhibition of *kat* mutant GC. The differences in the amount of streptonigrin required to inhibit GC growth can be attributed to the different methods used to test GC sensitivity to streptonigrin. Differences in GC sensitivity to toxic oxygen

Figure 34. H_2O_2 sensitivity of FA1090 (**A** and **B**) and NGAAS-501 catalase mutant (**C** and **D**). Bacteria was exposed to increasing concentrations of H_2O_2 ranging from 0.05-10 mM. Below, schematic organization of sensitivity plates. The experiment was performed 3 times in duplicate. Each bar represent the average of the 3 experiments and the error bars correspond to the standard deviation of the mean. (*) $p < 0.05$ and (**) $p < 0.005$ (**E**).



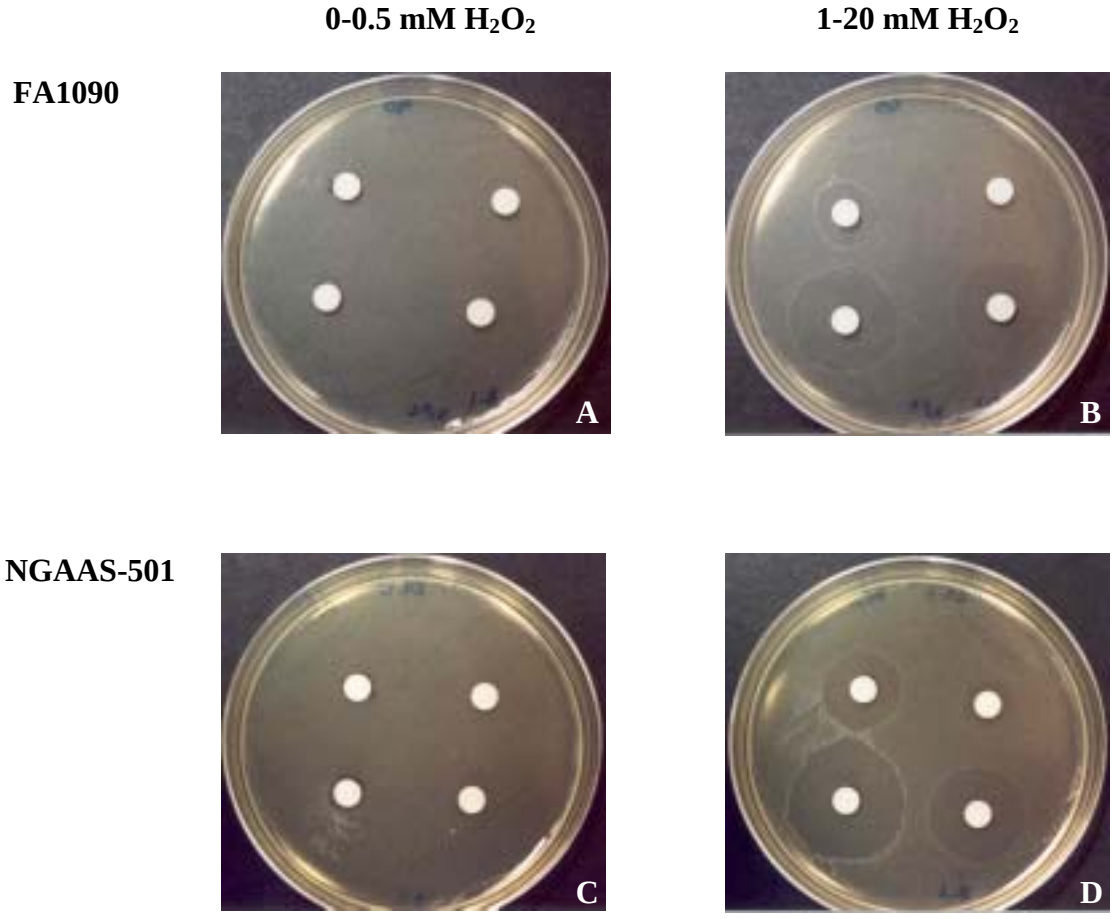
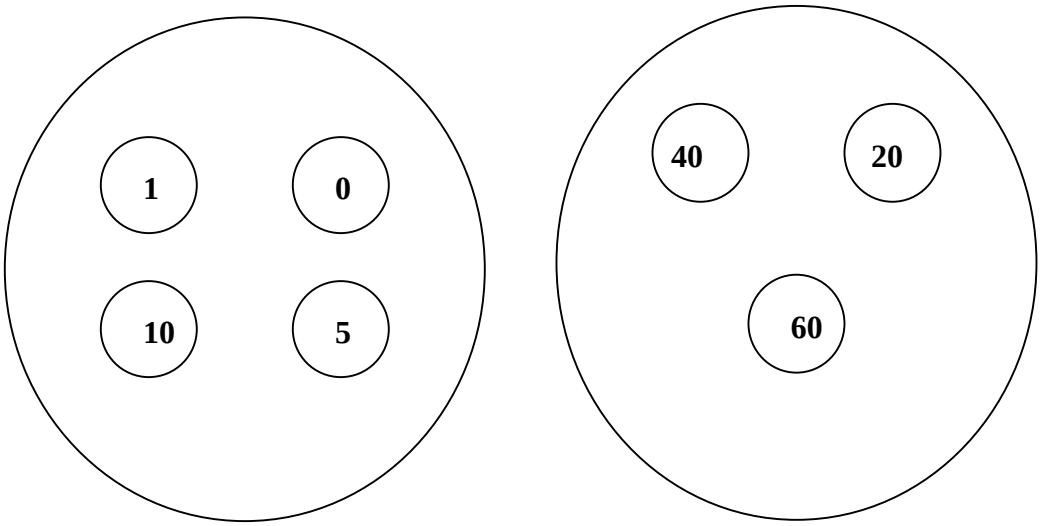
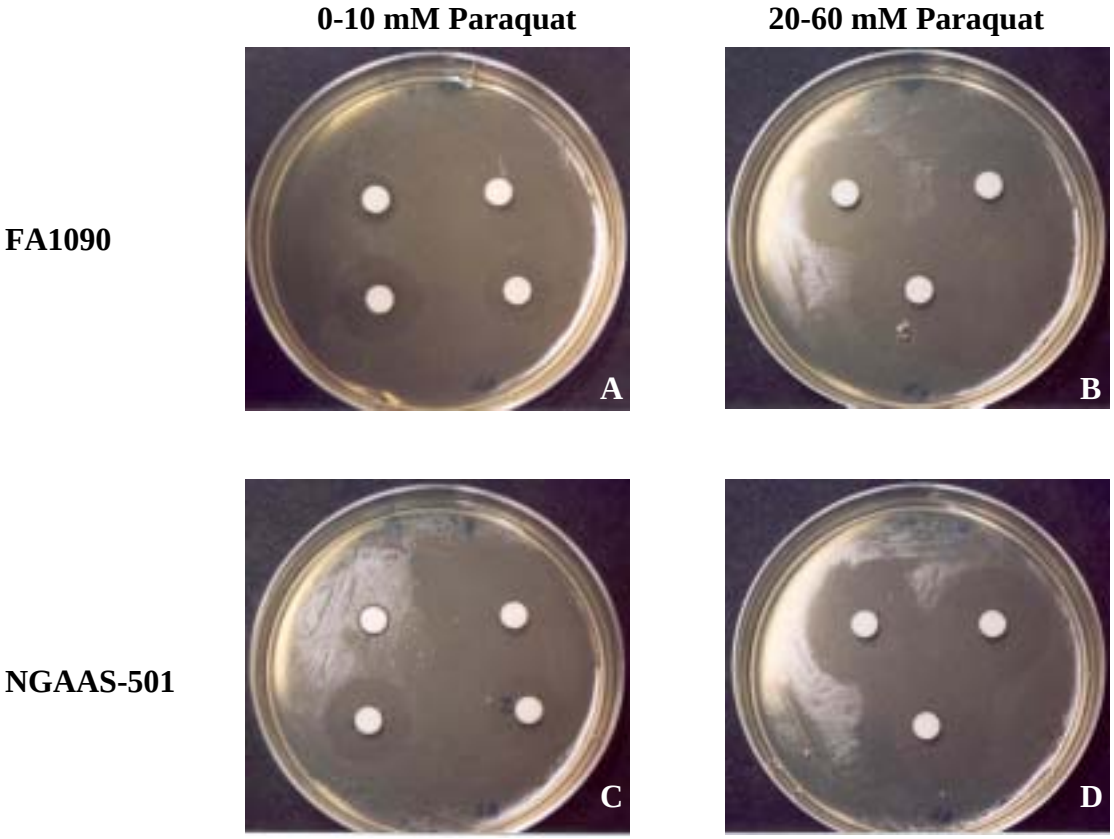

0-0.5 mM H_2O_2
1-20 mM H_2O_2
FA1090
NGAAS-501
A
B
C
D
E
Halo of inhibition (mm)
35
30
25
20
15
10
5
0
*
**
*
*
*
*
*
0
0.05
0.1
0.5
1
5
10
20
H_2O_2 (mM)
FA1090
NGAAS-501

Figure 35. Paraquat sensitivity of FA1090 (**A** and **B**) and NGAAS-501 catalase mutant (**C** and **D**). Bacteria were exposed to increasing concentrations of paraquat ranging form 1-60 mM. Below, schematic organization of sensitivity plates. The experiment was performed 3 times in duplicate. Each bar represent the average of the 3 experiments and the error bars correspond to the standard deviation of the mean. (*) $p < 0.05$ and (**) $p < 0.005$ (**E**).




0-10 mM Paraquat
20-60 mM Paraquat
FA1090
NGAAS-501
A
B
C
D

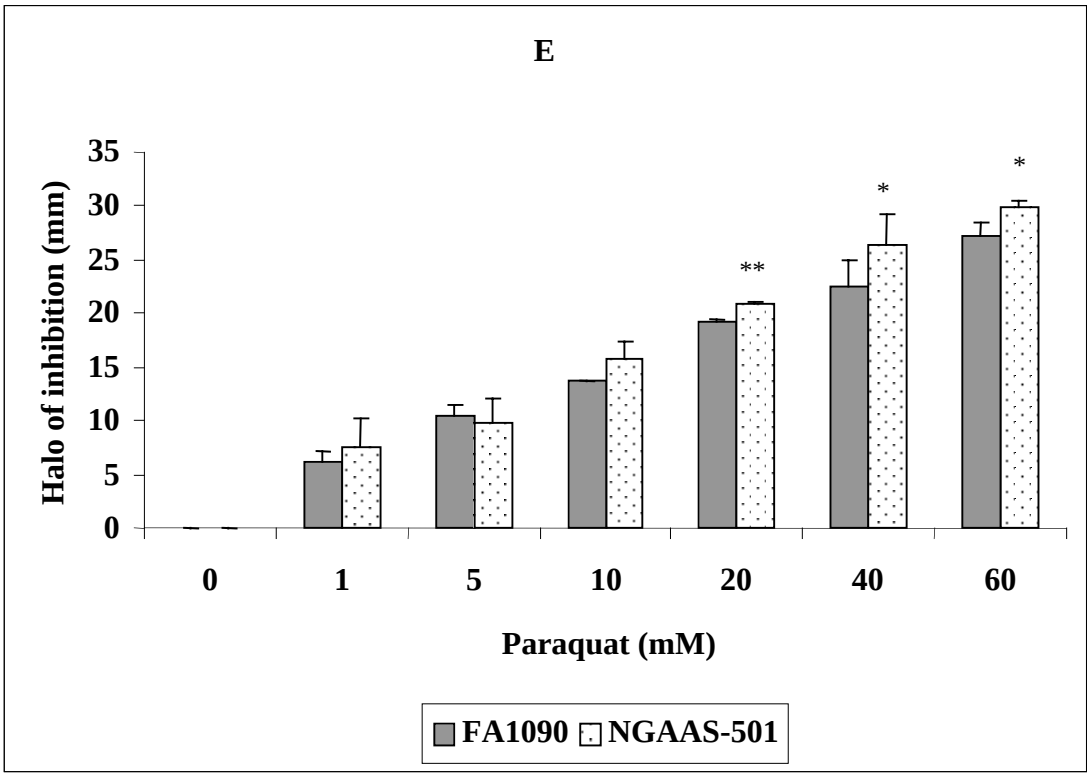
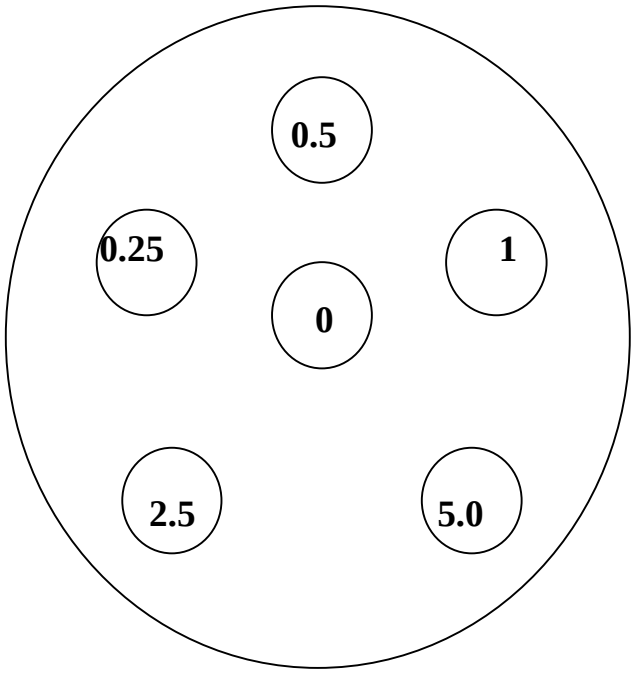
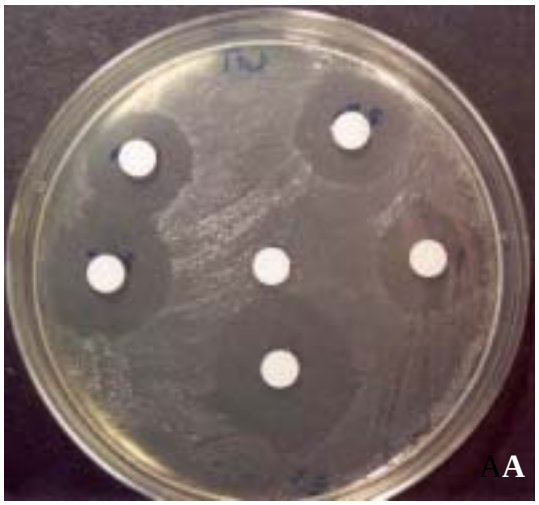
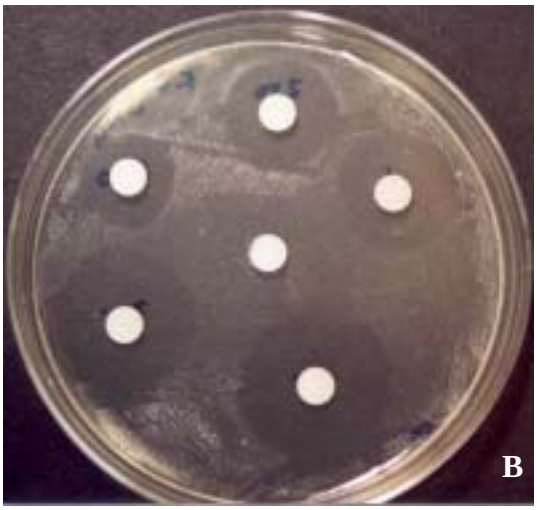

E
Halo of inhibition (mm)
35
30
25
20
15
10
5
0
**
*
*
0
1
5
10
20
40
60
Paraquat (mM)
FA1090
NGAAS-501

Figure 36. Streptonigrin (SNG) sensitivity of FA1090 (**A**) and NGAAS-501 catalase mutant (**B**). Bacteria were exposed to increasing concentrations of streptonigrin ranging from 0.5-5 μg/ml. Below, schematic organization of sensitivity plates. The experiment was performed 3 times in duplicate. Each bar represent the average of the 3 experiments and the error bars correspond to the standard deviation of the mean. (*) $p < 0.05$ (**C**).

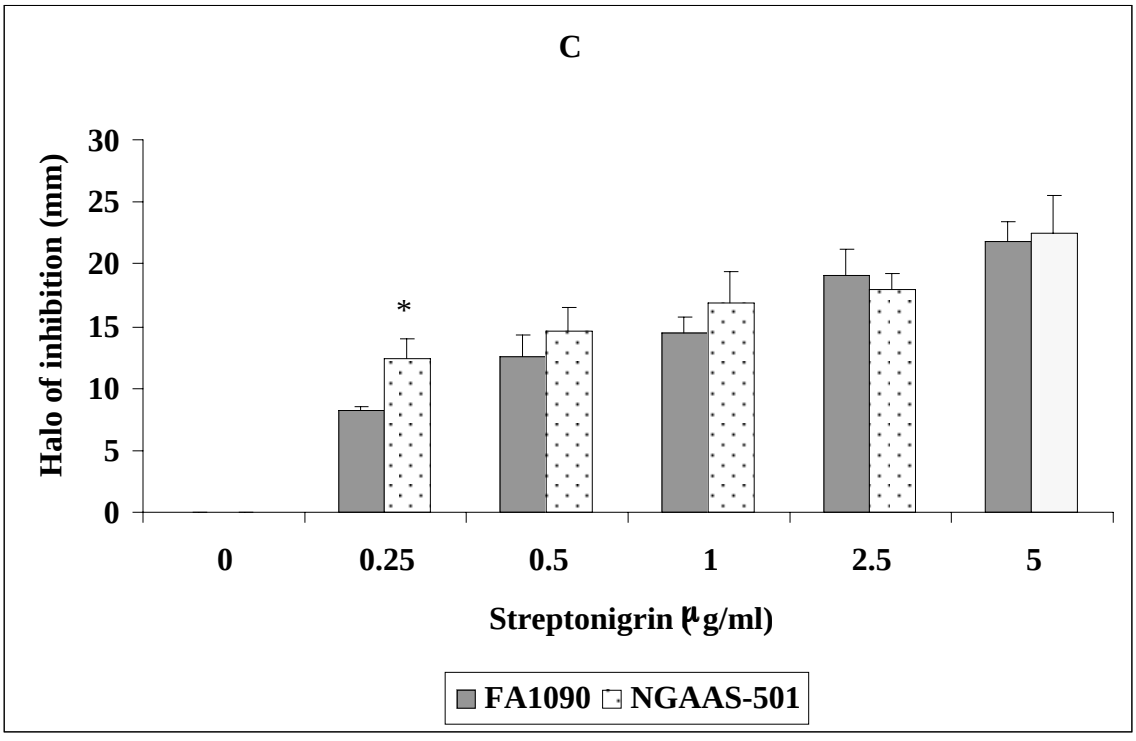




FA1090



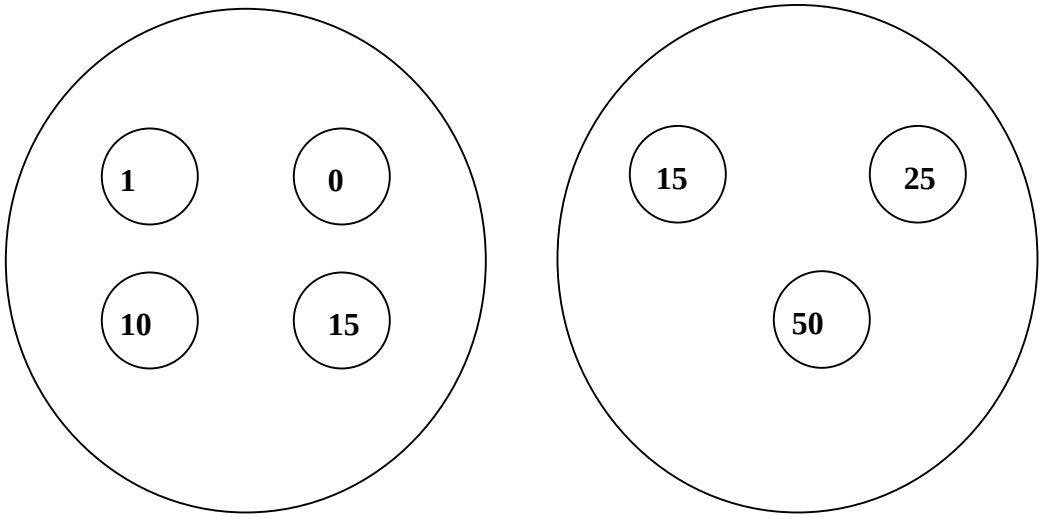
NGAAS-501

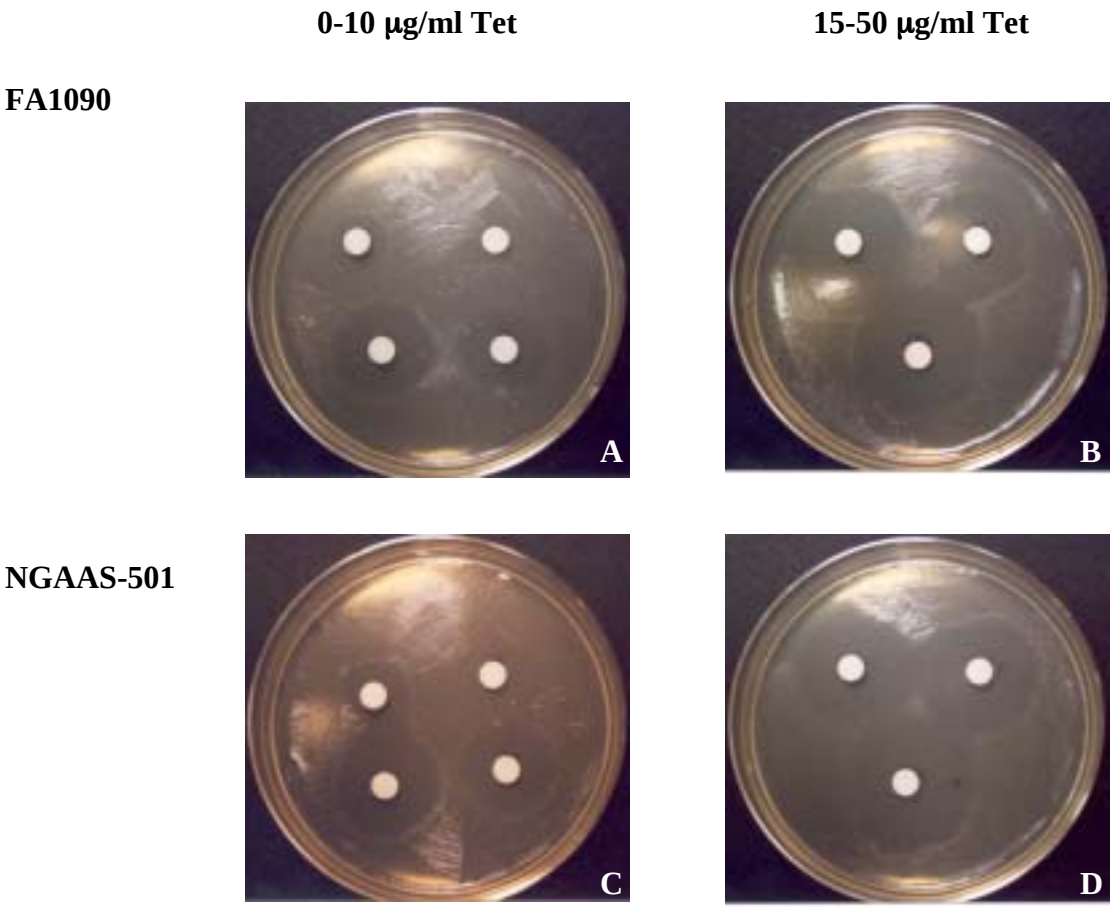


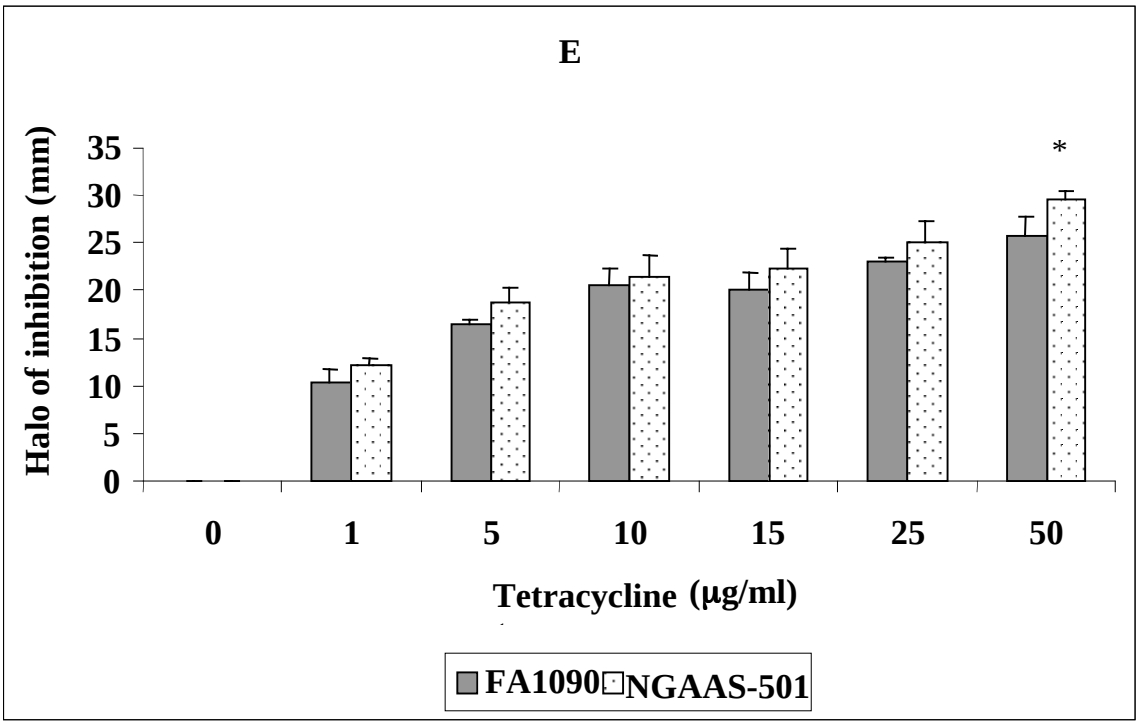
radicals due to the use of different methods have been reported before (Alcorn et al., 1994). Susceptibility of bacteria to tetracycline was believed to depend on the ability of this antibiotic to bind to the 30S ribosomal subunit, impairing protein synthesis by inhibiting translation (Chopra and Roberts, 2001). Tetracycline antibiotics cause the degradation of carbohydrates due to the formation of hydroxyl radical and by the stimulation of lipid peroxidation in the presence of iron and copper salts (Quinlan and Gutteridge, 1988). NGAAS-501 was only significantly more sensitive than FA1090 at a concentration of 50 μg/ml ($p = 0.05$) (**Fig. 37**) SIN-1 induce the formation of peroxynitrite, a compound formed by the interaction of superoxide ion and nitric oxide (Groves, 1999; Motohashi and Saito, 2002). Gentamicin promotes the generation of toxic oxygen radicals and an enhanced lipid peroxidation in the presence of iron (Sha and Schacht, 1999). No inhibition of either mutant or wild-type GC was observed at any of the concentrations of gentamicin and SIN-1 tested (**Fig. 38** and **39**). The lack of inhibition observed in the case of SIN-1 may be attributed to the lack of nitric oxide necessary to form the peroxynitrite, although this agent has been utilized to test susceptibility of Salmonella to peroxynitrite *in vitro* (Motohashi and Saito, 2002).

The complemented strain NGAAS-503 was exposed to increasing concentrations of H_2O_2 and paraquat (an inducer of superoxide) using the disk sensitivity assay described before. Expression of GC catalase in trans from pAAS20-*kat* 30 did not rescue the catalase deficiency in NGAAS-501 in the case of H_2O_2 exposure, suggesting that some properties of the catalase protein expressed are impaired (**Fig. 40**). Expression of GC catalase in trans from pAAS20-*kat* 30, however rendered the bacteria more resistant to paraquat (**Fig. 41**). It is not known why expression of GC catalase from pAAS20-*kat*

Figure 37. Tetracycline sensitivity of FA1090 (**A** and **B**) and NGAAS-501 catalase mutant (**C** and **D**). Bacteria were exposed to increasing concentrations of tetracycline ranging from 1-50 μg/ml. Below, organization of sensitivity plates. The experiment was performed 3 times in duplicate. Each bar represent the average of the 3 experiments and the error bars correspond to the standard deviation of the mean. (*) $p < 0.05$ (**E**).




0-10 µg/ml Tet
15-50 µg/ml Tet
FA1090
NGAAS-501
A
B
C
D


E
Halo of inhibition (mm)
35
30
25
20
15
10
5
0
*
0
1
5
10
15
25
50
Tetracycline (µg/ml)
FA1090
NGAAS-501

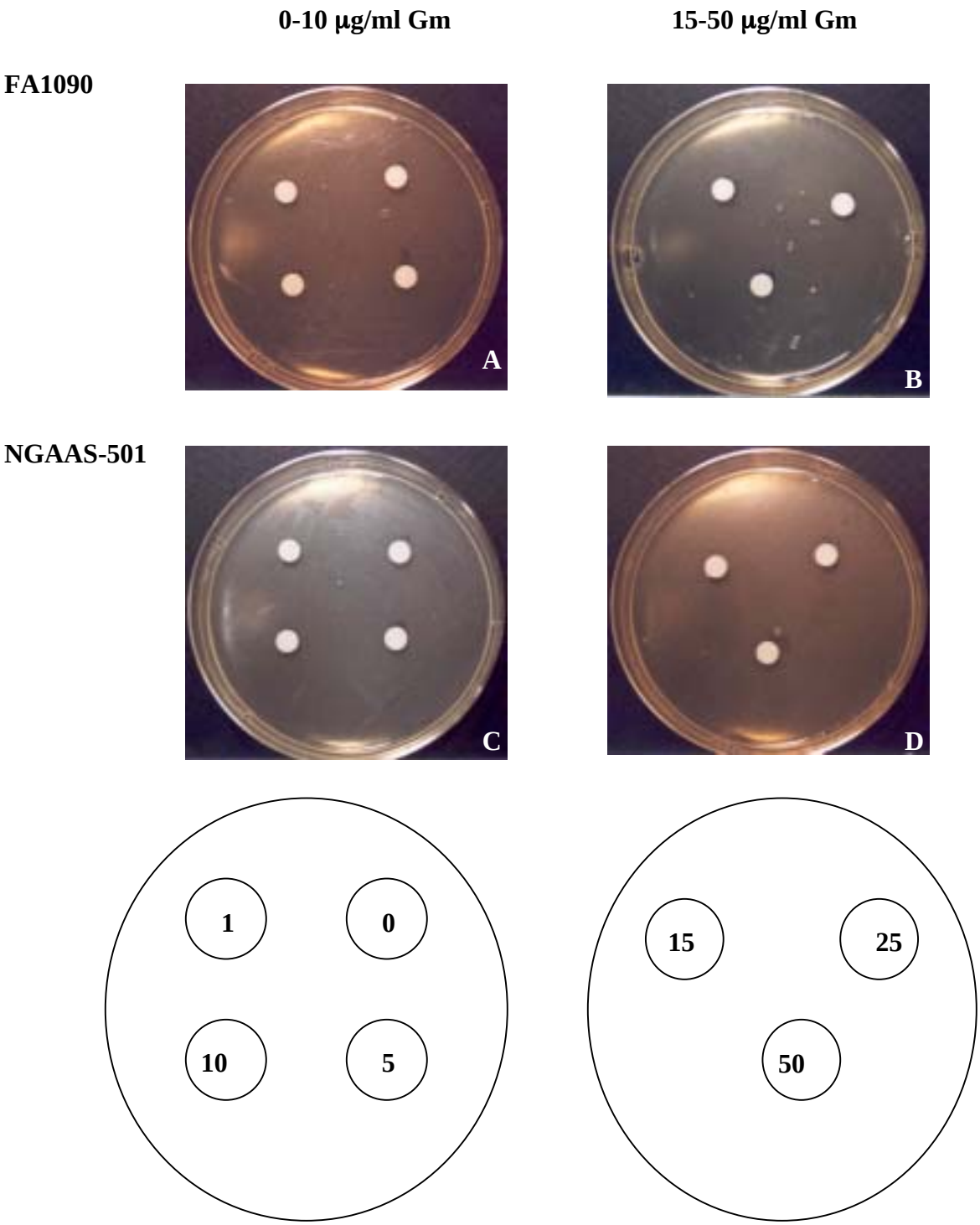


Figure 38. Gentamicin susceptibility of FA1090 (**A** and **B**) and NGAAS-501 catalase mutant (**C** and **D**). Bacteria were exposed to increasing concentrations of gentamicin ranging from 1-50 µg/ml. Above, schematic organization of sensitivity plates. The experiment was performed 3 times in duplicate. No inhibition of the wild-type or the *kat* mutant was observed at any concentration.

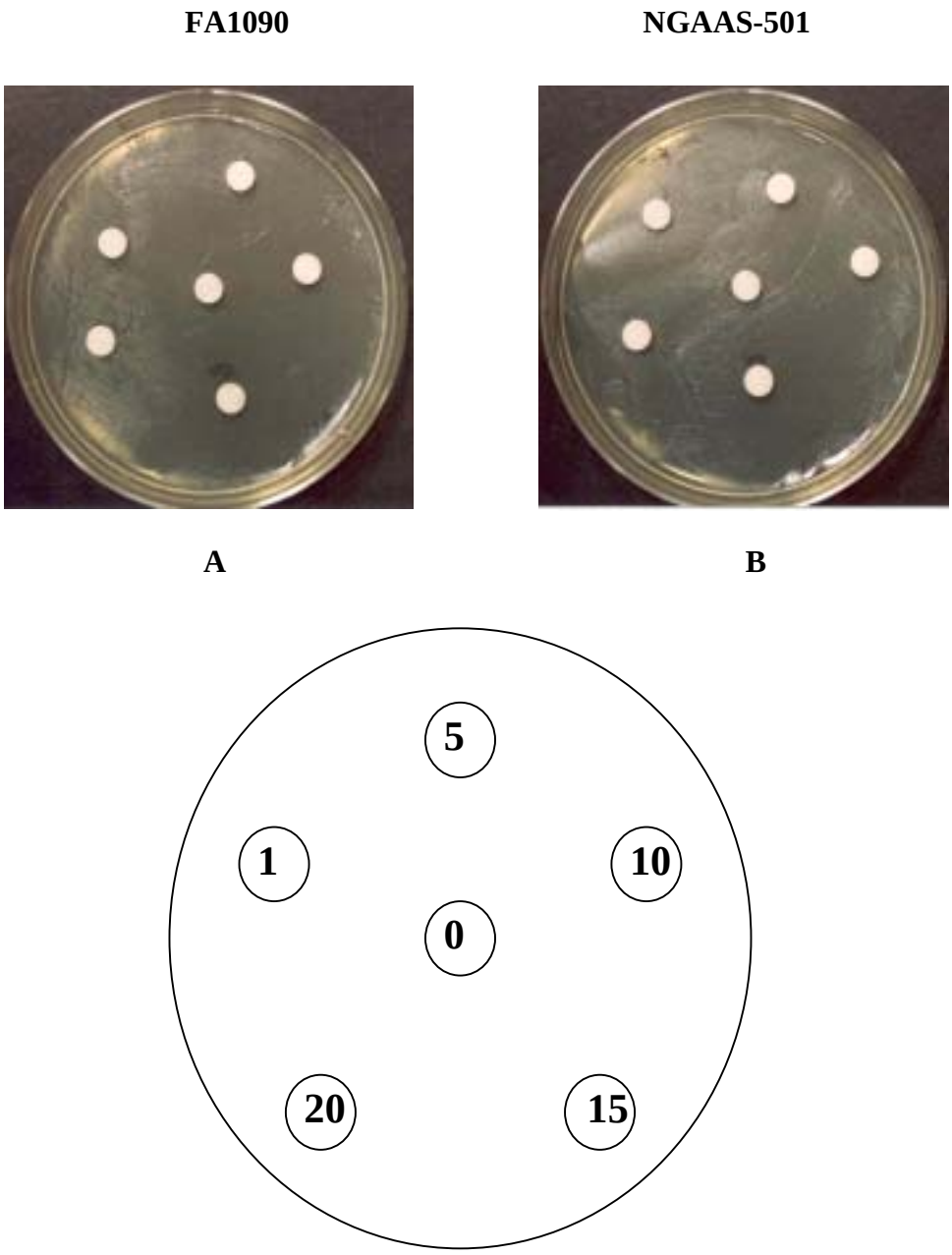


Figure 39. SIN-1 susceptibility of FA1090 (**A**) and NGAAS-501 catalase mutant (**B**). Bacteria were exposed to increasing concentrations of SIN-1 ranging form 1 to 20 mM. Above, schematic organization of sensitivity plates. The experiment was performed 3 times in duplicate. No inhibition of the wild-type or the *kat* mutant was observed at any concentration tested.

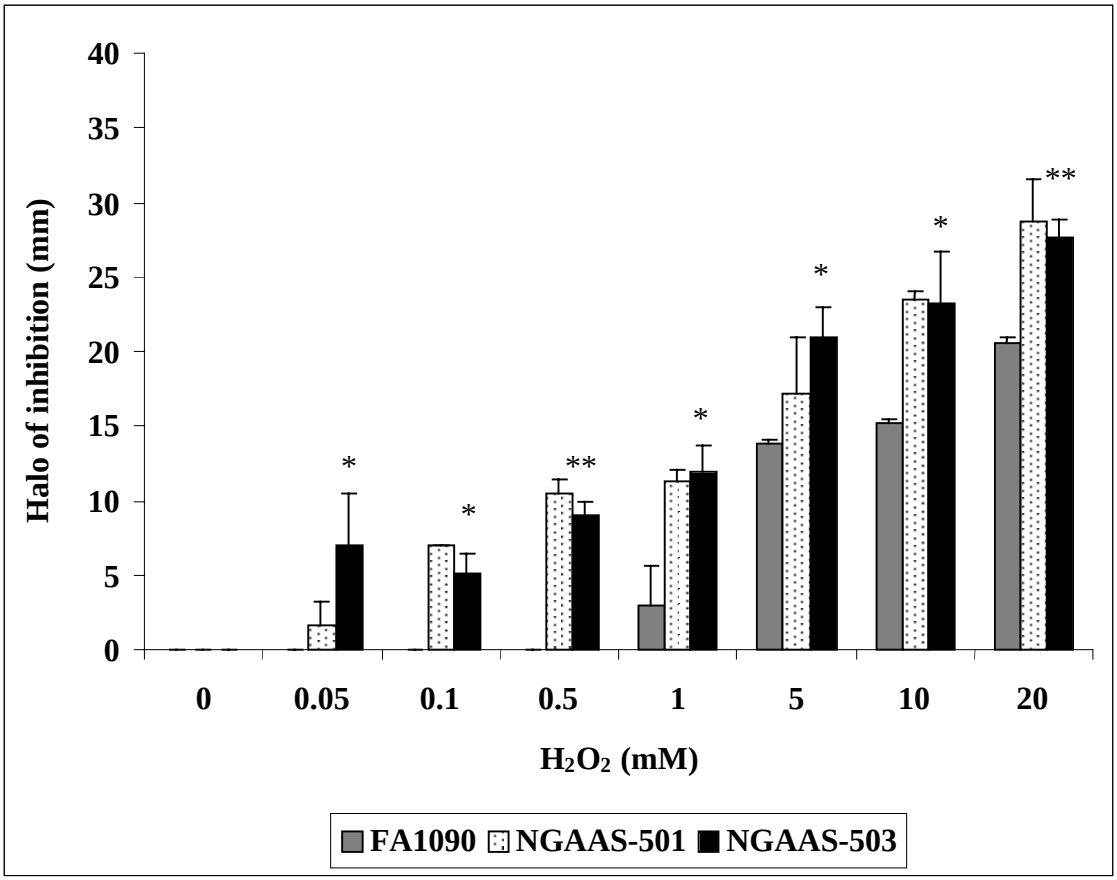


Figure 40. H_2O_2 sensitivity of FA1090, NGAAS-501 and NGAAS-503. The experiment was performed 3 times in duplicate. Each bar represent the average of the 3 experiments and the error bars correspond to the standard deviation of the mean. (*) $p < 0.05$ and (**) $p < 0.005$.

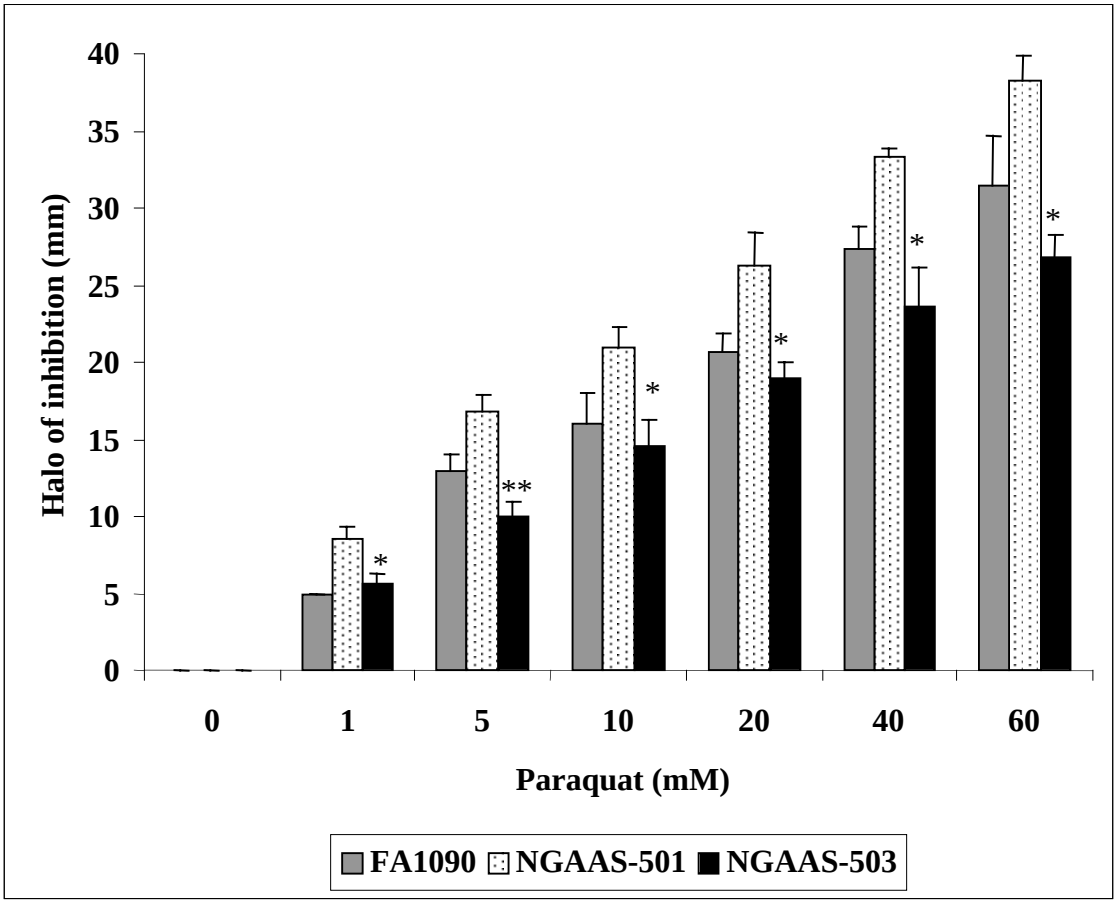
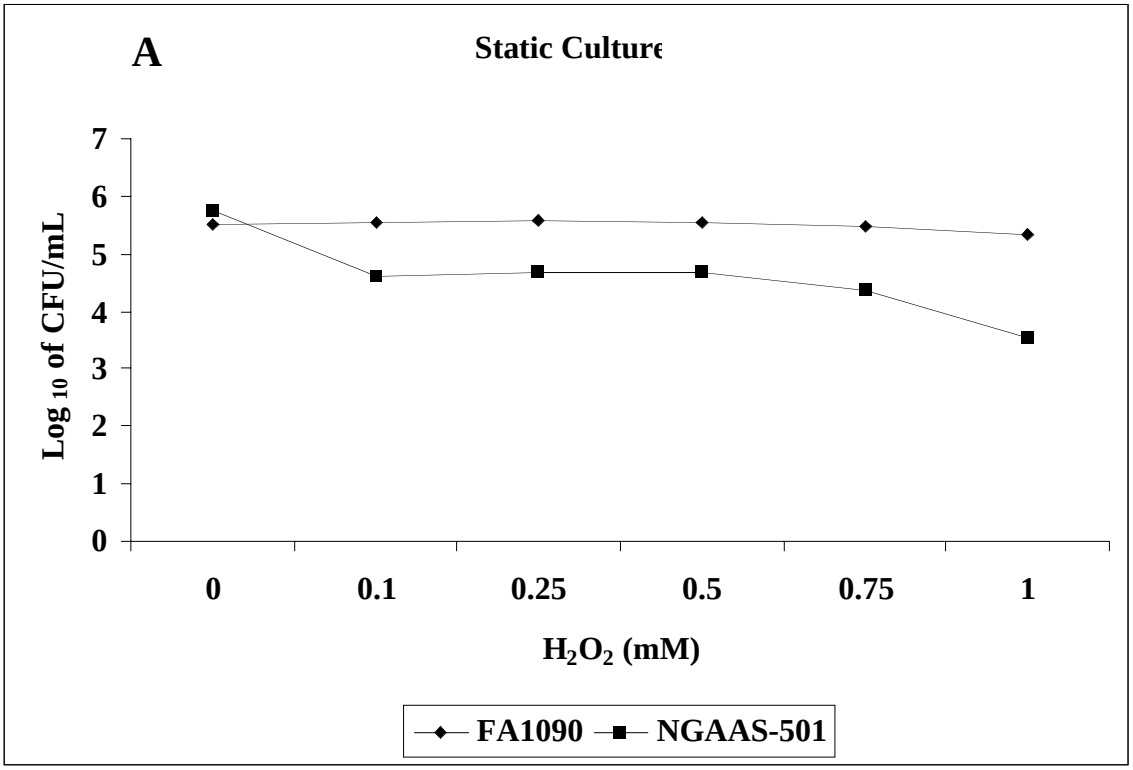


Figure 41. Paraquat sensitivity of FA1090, NGAAS-501 and NGAAS-503. The experiment was performed 3 times in duplicate. Each bar represent the average of the 3 experiments and the error bars correspond to the standard deviation of the mean. (*) $p < 0.05$.

30 restores paraquat resistance but not resistance to H_2O_2. The apparent difference in size of the recombinant *kat* protein expressed in NGAAS-503 may be responsible. The difference in size perhaps is due to the lack of a critical domain for resistance to H_2O_2 but not paraquat, that is not present in the recombinant *kat* protein expressed in NGAAS-503.

As mentioned before, H_2O_2 can diffuse across bacterial membranes to target several microbial components. The effects of this small molecule can be enhanced by modifying the culture conditions. As an example, diffusion of H_2O_2 across the membrane is increased in vigorously agitated cultures. Given the differences observed in sensitivity to H_2O_2 between the wild-type FA1090 and mutant NGAAS-501, the effects of H_2O_2 in static and shaking culture conditions were studied. Both strains were exposed for 30 min to increasing concentrations of H_2O_2 in GC broth, after which the number of viable CFU was determined. Little change in the recovery of the wild-type was observed at concentrations of 0 to 1 mM. In contrast, and as expected, a decrease in the number of mutant CFU recovered was observed with increasing concentrations of H_2O_2 (**Fig. 42 A** and **B**). A more dramatic decrease in the viability of the catalase mutant was observed under shaking culture conditions (**Fig. 42 B**). Analysis of DNA damage revealed an association between increasing concentrations of H_2O_2 in shaking cultures and degree of DNA damage, suggesting that injury to DNA was responsible for the decline in the NGAAS-501 upon exposure to H_2O_2 (**Fig. 43** and **44**). During static culture conditions, limited DNA damage was observed as evidenced in the alkaline agarose gel (**Fig. 43 B**). This low degree of DNA damage can be attributed to the limited diffusion of H_2O_2 under these conditions. To further demonstrate the H_2O_2 sensitivity of the NGAAS-501, mixed cultures containing a 1:1 ratio of wild-type and mutant were exposed to increasing

Figure 42. H_2O_2 sensitivity of FA1090 and NGAAS-501 in static (**A**) and shaking (**B**) broth cultures.


A
Static Culture
Log 10 of CFU/mL
7
6
5
4
3
2
1
0
0
0.1
0.25
0.5
0.75
1
H2O2 (mM)
FA1090
NGAAS-501

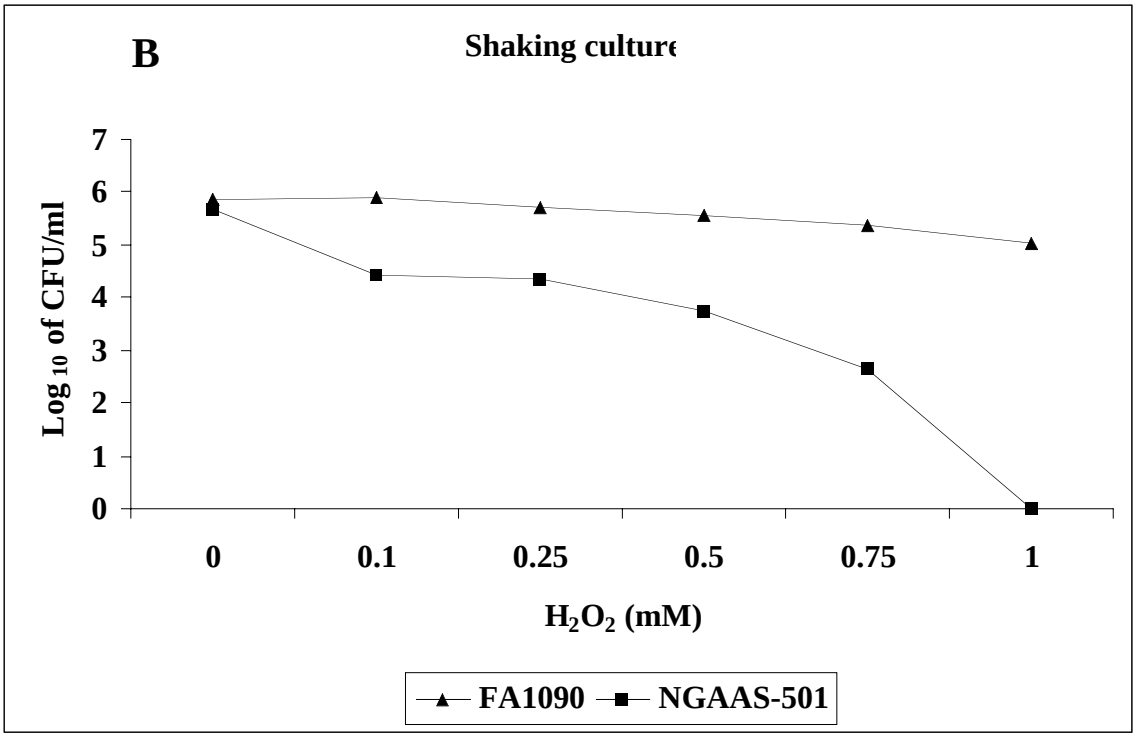
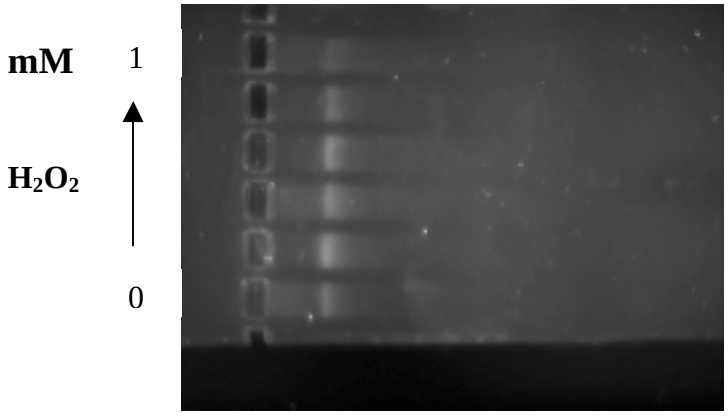
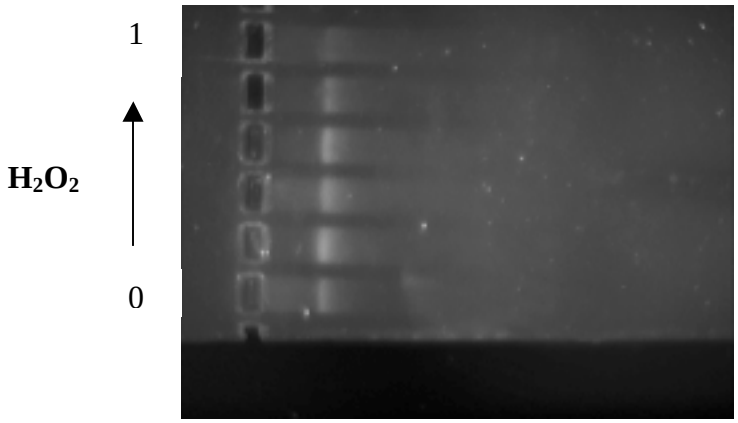

B
Shaking culture
Log 10 of CFU/ml
7
6
5
4
3
2
1
0
0
0.1
0.25
0.5
0.75
1
H2O2 (mM)
FA1090
NGAAS-501

Figure 43. Visualization of DNA damage by alkaline agarose gel analysis of FA1090 (**A**), NGAAS-501 (**B**) and a 1:1mixture of wild-type and catalase mutant (**C**) under static culture. Bacteria were exposed to increasing concentrations of H_2O_2 ranging from 0.1-1 mM (0, 0.1, 0.25, 0.5, 0.75 and 1 mM).

A. FA1090



B. NGAAS-501



C. Mixture 1:1

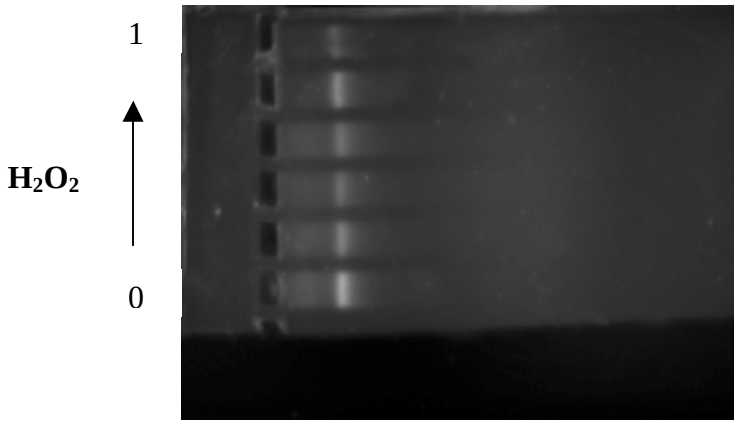
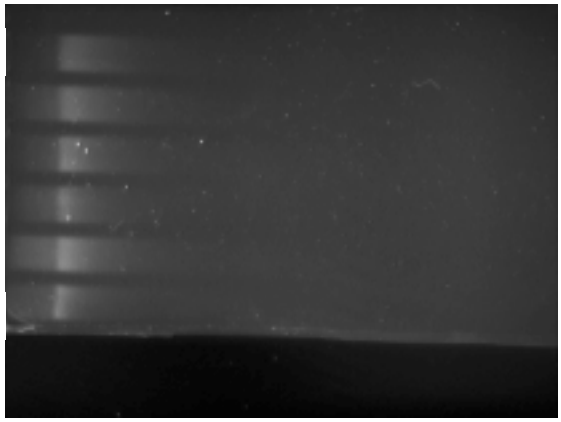
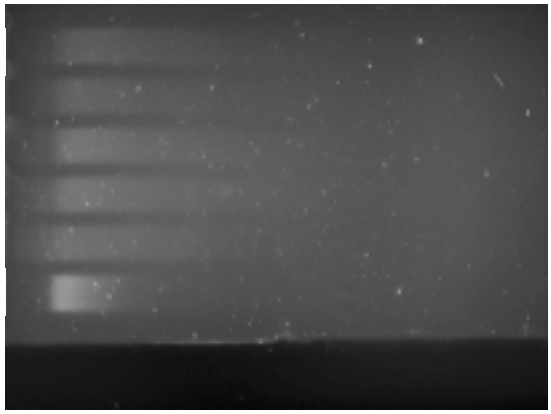


Figure 44. Visualization of DNA damage by alkaline agarose gel analysis of FA1090 (**A**), NGAAS-501 (**B**) and a 1:1mixture of wild-type and catalase mutant (**C**) under shaking culture. Bacteria and mixture were exposed to increasing concentrations of H_2O_2 ranging from 0.1-1 mM (0, 0.1, 0.25, 0.5, 0.75 and 1 mM). DNA damage was observed predominantly in NGAAS-501 (**B**) as compared to FA1090 (**A**).

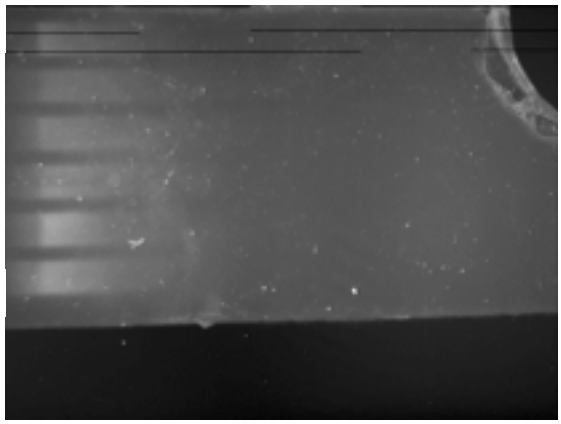
A. FA1090



B. NGAAS-501



C. Mixture

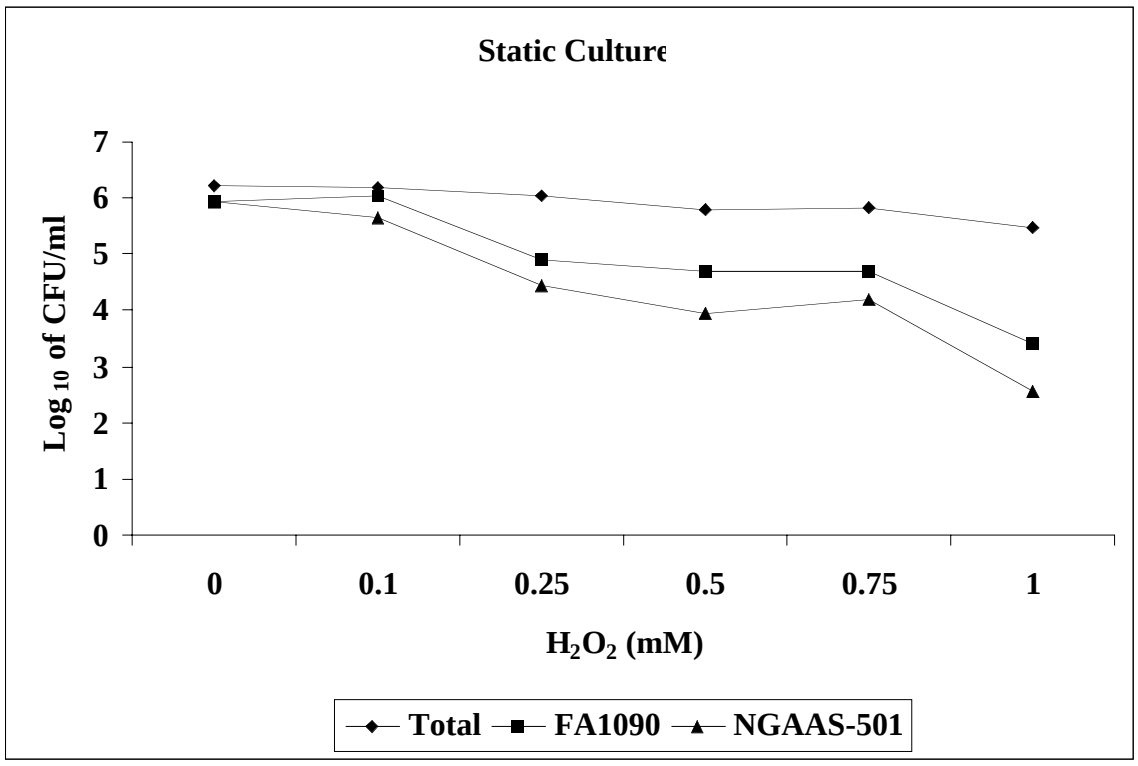


concentration of H_2O_2 as before. Again, the catalase deficient mutant appeared to be more sensitive than the wild-type strain in both conditions tested (**Fig. 45**). The DNA damage observed in alkaline agarose gels loaded with samples from the shaking mixed culture can be attributed to strain NGAAS-501 (**Fig. 43 C** and **44 C**).

E. Effect of H_2O_2-producing lactobacilli

H_2O_2-producing lactobacilli have been proposed as a biological method to control genital tract infections such as gonorrhea (McBride et al., 1978; Saigh et al., 1978). To study the possible role of gonococcal catalase as a mechanism of defense against H_2O_2-producing microflora, FA1090, NGAAS-501 and NGAAS-503 were exposed to different species of H_2O_2-producing lactobacilli grown at pH 5.8 and 7.0 using the overlay technique (Saigh et al., 1978). NGAAS-501 was inhibited more by *L. jensenii* and *L. crispatus* compared to FA1090 at both pHs tested (**Fig. 46 A** and **B**). Greater zones of inhibition were observed at pH 5.8 compared to pH 7.0 suggesting that the production of H_2O_2 is enhanced at low pH or other factors are produced that probably act synergistically or in parallel with H_2O_2. NGAAS-503 demonstrated the same level of resistance against *L. crispatus* as the wild-type strain. In contrast, wild-type levels of resistance were not restored in the presence of *L. jensenii.* Therefore, it can be concluded that the *kat* mutation in NGAAS-501 is responsible for the increased sensitivity to lactobacilli. These data suggest that gonococcal catalase helps defend gonococci against H_2O_2-producing microflora.

Figure 45. H_2O_2 sensitivity of FA1090 and NGAAS-501 in mixed static and shaking broth cultures. Mixtures were exposed to increasing concentrations of H_2O_2 ranging from 0.1-1 mM (0, 0.1, 0.25, 0.5, 0.75 and 1 mM).


Static Culture
Log 10 of CFU/ml
7
6
5
4
3
2
1
0
0
0.1
0.25
0.5
0.75
1
H2O2 (mM)
Total
FA1090
NGAAS-501

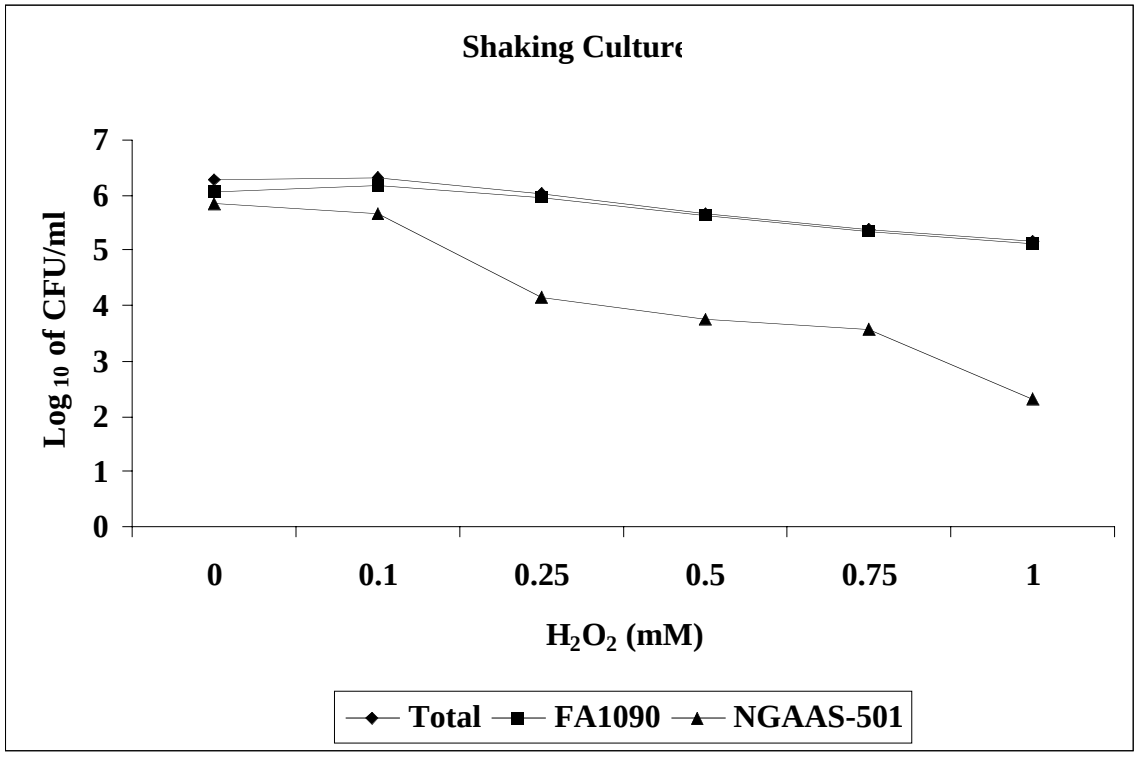
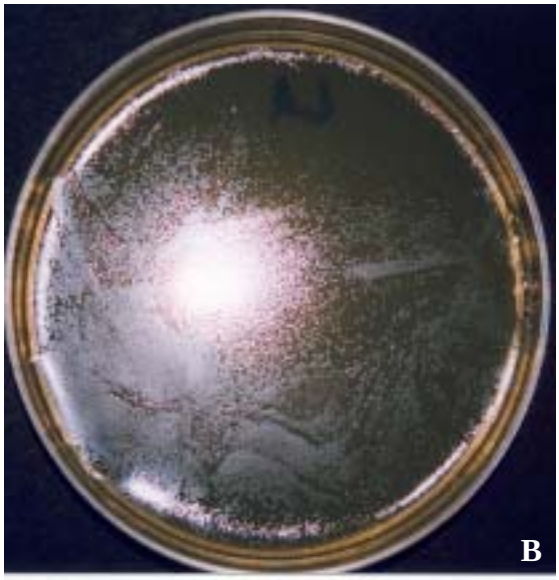

Shaking Culture
Log 10 of CFU/ml
7
6
5
4
3
2
1
0
0
0.1
0.25
0.5
0.75
1
H2O2 (mM)
Total
FA1090
NGAAS-501

Figure 46. Effect of H_2O_2-producing lactobacilli on growth of NGAAS-501. (**A**) Qualitative measure of H_2O_2 production by Lactobacillus species (McGroarty et al., 1992). Lactobacilli were grown on MRS plates containing TMB (0.25 mg/ml of tetramethylbenzidine) and HRP (0.01 mg/ml). The plates were incubated under anaerobic conditions at 37^{o}C for 2-3 days. After incubation, plates were exposed to ambient air. A pigment is formed due to oxidation of TMB by HRP in the presence of H_2O_2 produced by lactobacilli. **1**, *Lactobacillus jensenii*; **2**, empty; **3**, *Lactobacillus murinus* (mouse isolate, non H_2O_2-producer); and **4**, *Lactobacillus crispatus*. (**B**), (**C**) and (**D**) show the agar overlay assay. Spots with different dilutions of lactobacilli were incubated onto HIA plates (pH 7.0) and overlayed with GC agar. One-hundred μl of NGAAS-501 suspension (0.1 OD_{600}) were spreaded over the GC agar layer. Plates were incubated for 24 hrs at 37^{o}C in CO_2. (**B**), *L acidophilus* (no inhibition); (**C**), *L. jensenii* (clear zones of inhibition at all dilutions) and (**D**), *L. crispatus* (smaller zones of inhibition compared to *L. jensenii*).


2
3
1
4
A


B


C

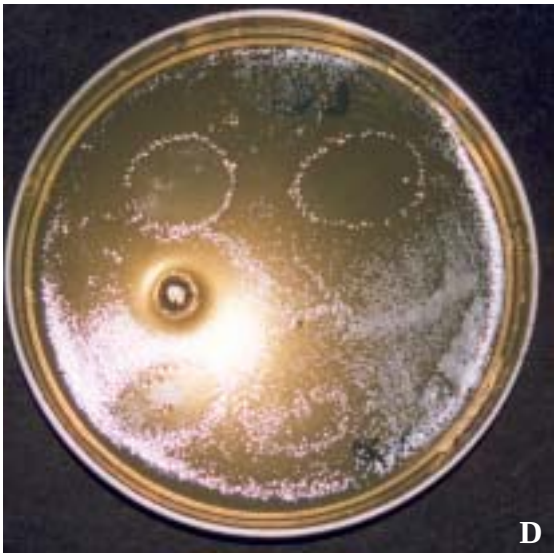
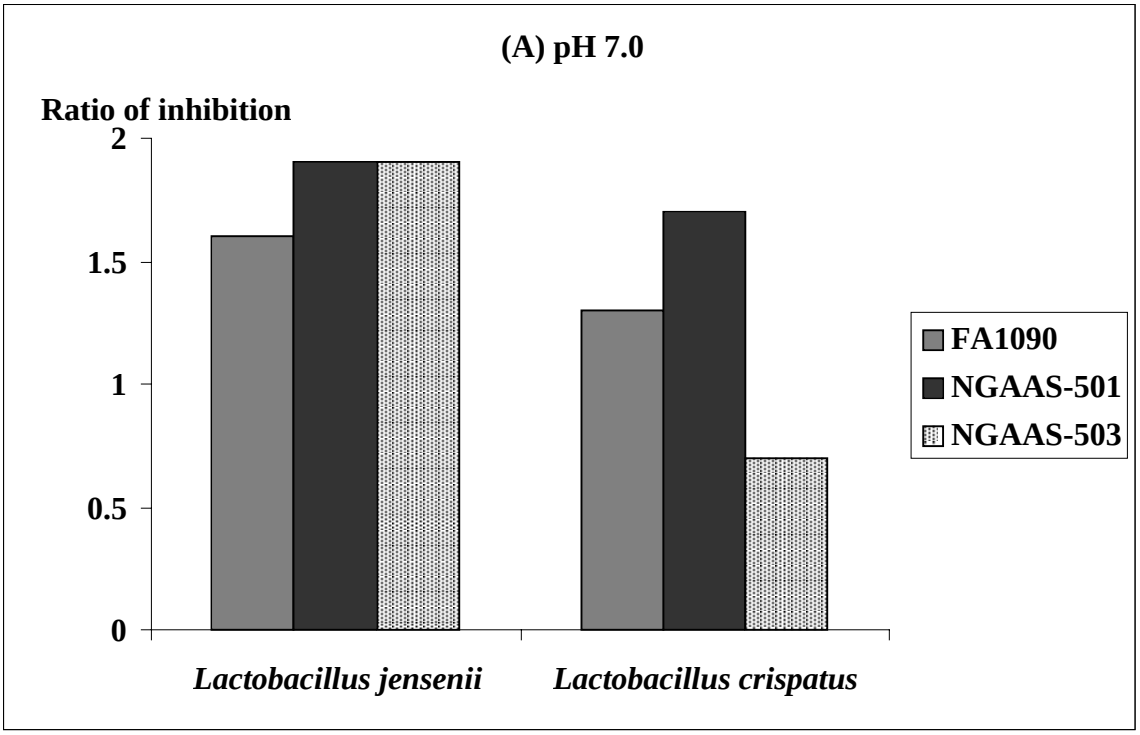
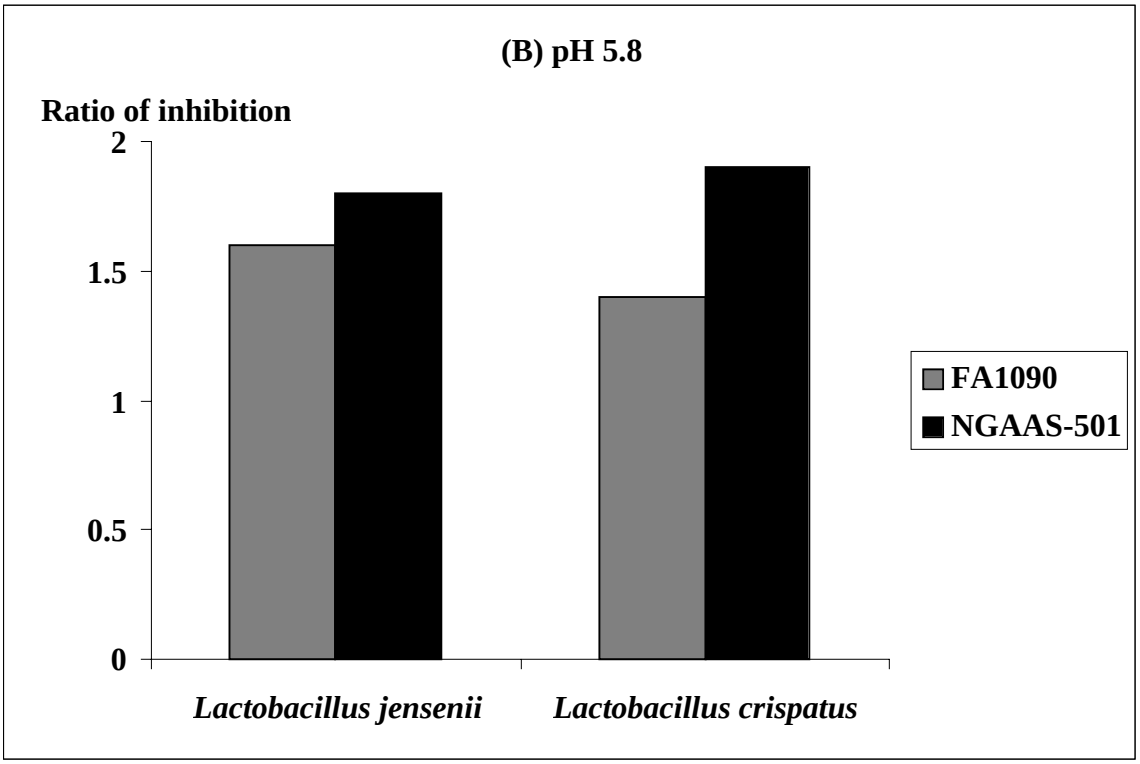

D

Figure 47. Inhibition of FA1090, NGAAS-501, and NGAAS-503 [NGAAS-501 (pAAS20-*kat* 30) gonococci by H_2O_2-producing lactobacilli. (**A**) Overlay performed at pH 7.0 and (**B**) pH 5.8. Bars represent an average of 2 (NGAAS-503) and 3 experiments (FA1090 and NGAAS-501).


(A) pH 7.0
Ratio of inhibition
2
1.5
1
0.5
0
FA1090
NGAAS-501
NGAAS-503
Lactobacillus jensenii
Lactobacillus crispatus


(B) pH 5.8
Ratio of inhibition
2
1.5
1
0.5
0
FA1090
NGAAS-501
Lactobacillus jensenii
Lactobacillus crispatus

III- Role of gonococcal catalase *in vivo*

A. Single infection experiments

The hallmark of a symptomatic gonococcal infection is a high influx of neutrophils to the site of infection (Shafer, 1989). Gonococcal catalase has been suggested to have a role during the inflammatory response as a defense mechanism against oxidative killing utilized by PMNs. Zheng et al. (1992) performed several *in vitro* experiments that support this hypothesis. First, exposure of GC to 1 mM H_2O_2 caused a 3-fold increase in catalase activity. Second, the increase in gonococcal catalase activity was accompanied by new protein synthesis as suggested by the demonstration that in the presence of chloramphenicol (an inhibitor of protein synthesis), catalase activity did not increase upon exposure of GC to H_2O_2. Third, exposure of GC to PMA-treated human PMNs (activated) led to a 2-fold increase in gonococcal catalase activity; this increase was abrogated by the addition of exogenous catalase, but not by SOD or desferrioxamine (iron chelator). These results suggest that the oxidative species responsible for inducing GC catalase activity is the H_2O_2 produced by the PMNs. Fourth, previous exposure of GC to 1 mM H_2O_2 rendered GC more resistant to subsequent exposure to higher concentrations of H_2O_2. All these lines of evidence support the hypothesis that gonococcal catalase plays a role in GC pathogenesis.

To assess directly the role of gonococcal catalase in survival of GC *in vivo*, the estradiol-treated female mouse model of genital tract infection was used (Jerse, 1999). Estradiol-treated BALB/c mice were inoculated intravaginally with 6.1×10^5 to 1.0×10^6 CFU ($\log_{10}$ CFU 5.8 to 6.0) of FA1090 and NGAAS-501. The infection was monitored daily for 12 days by quantitative culture of vaginal swab suspensions. In 2 out of 3

experiments no significant difference in the duration of recovery of either strain was observed ($p = 0.107$, 0.544 and 0.013) (**Fig. 48**).

In the three single infection experiments there were slight differences in the number of wild-type and catalase deficient strains used to inoculate the mice (**Fig. 48**). In order to rule out the possibility that slight differences in dose might affect the outcome of the results, a titration of the inocula was performed to create three different doses of wild-type and *kat* mutant GC (2.5×10^5, 5.0×10^5 and 1.0×10^6). Each dose was inoculated into mice as before ($n = 6$). No significant difference in the duration of recovery of either bacteria was observed at any dose tested ($p > 0.3$, **Fig. 49**). These results suggest that NGAAS-501, the catalase deficient mutant, can survive in the genital tract of female mice as well as the wild-type strain.

To see if the catalase mutant survived in the presence of an inflammatory response, the percent of PMNs in vaginal smears from mice inoculated with either wild-type or *kat* mutant GC was compared to that of the placebo controls. Although both the wild-type and *kat* mutant were associated with murine PMNs as observed by light microscopy (**Fig. 50 A, B** and **C**), a correlation between the degree of PMN influx and clearance of infection could not be established (**Tables 9 A-B**, **Tables 10 A-B**, **and Tables 11 A-B)**. Although a higher average number of CFUs was recovered from mice infected with FA1090 (**Fig. 51 A**, **52 A and 53 A**), in 2 out of the 3 experiments, GC NGAAS-501 elicited more PMNs than the wild-type (**Fig. 51 B**, **52 B and 53 B**). In 2 outof the 3 single infection experiments, the *kat* mutant also elicited a higher percent of PMNs than the wild-type (**Fig. 54 A**, **B** and **C**). Although none of these differences was

Figure 48. Comparison of the duration of recovery of FA1090 and NGAAS-501 in estradiol-treated mice individually infected with either bacteria. The black horizontal solid bar represents the average recovery in days.

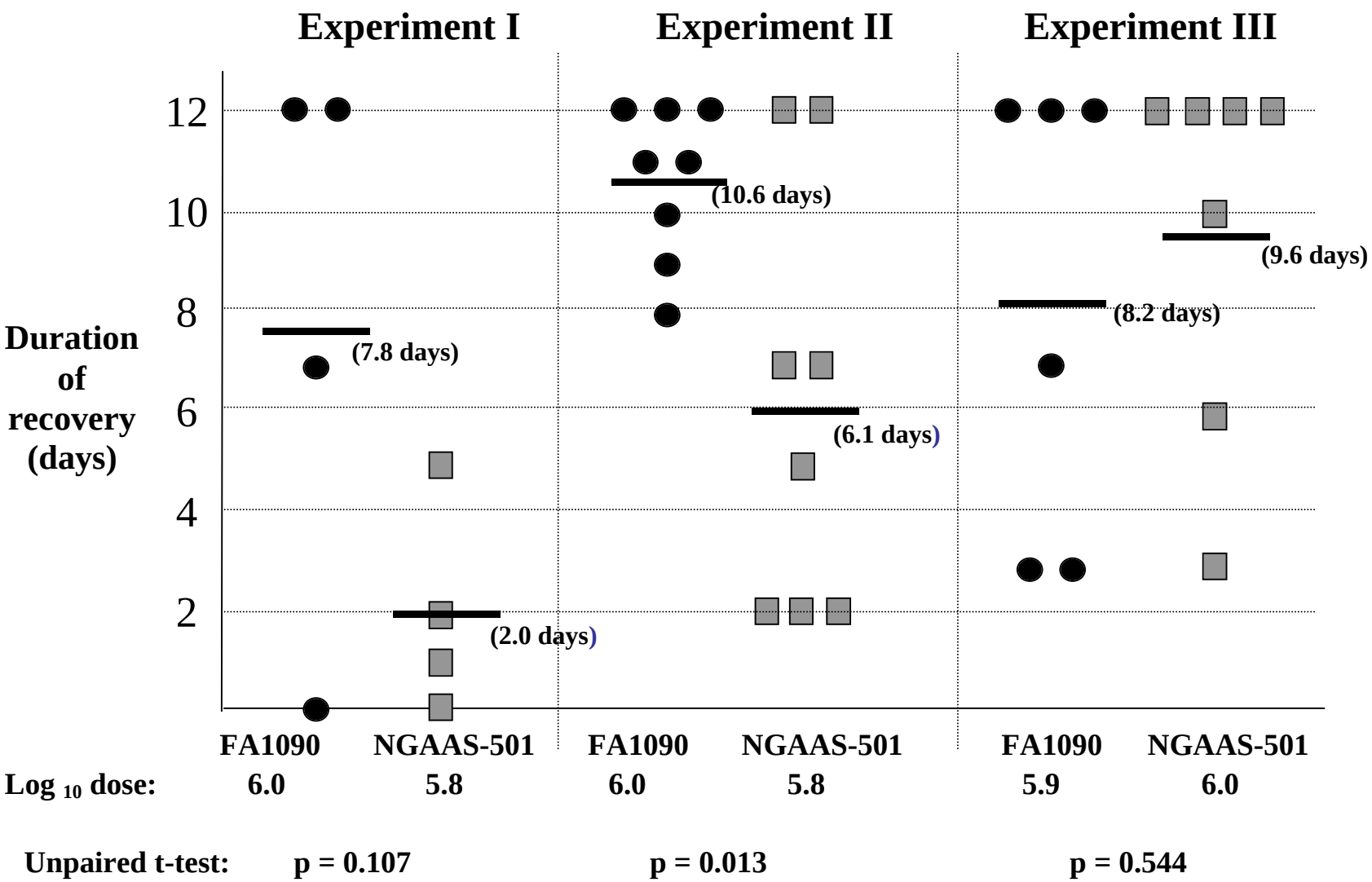
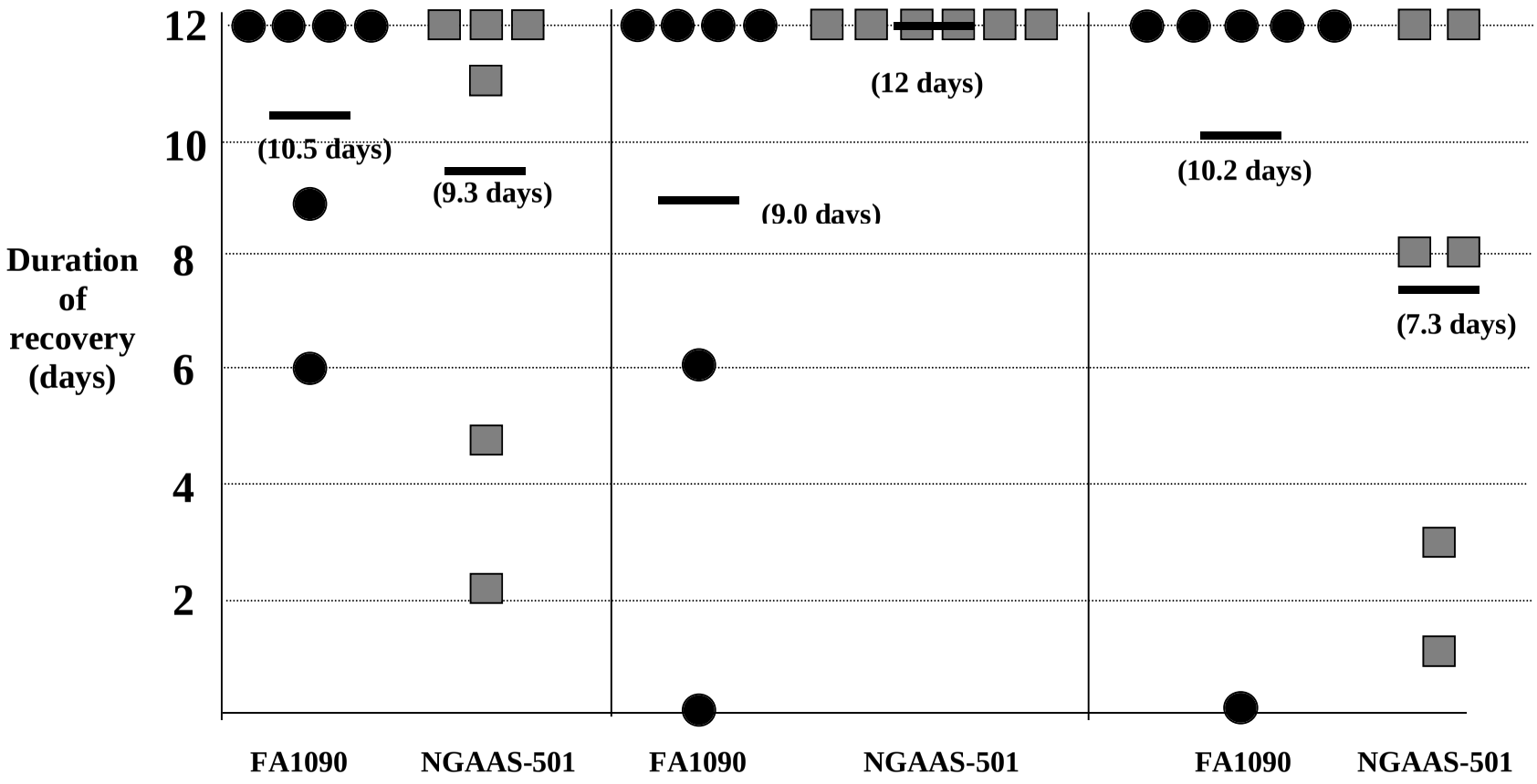

Experiment I
Experiment II
Experiment III
Duration of recovery (days)
12
10
8
6
4
2
(7.8 days)
(10.6 days)
(6.1 days)
(2.0 days)
(8.2 days)
(9.6 days)
FA1090
NGAAS-501
FA1090
NGAAS-501
FA1090
NGAAS-501
Log 10 dose:
6.0
5.8
6.0
5.8
5.9
6.0
Unpaired t-test:
p = 0.107
p = 0.013
p = 0.544

Figure 49. Duration of recovery of FA1090 and NGAAS-501 from estradiol-treated mice individually infected with either bacteria at doses ranging from 10^5 to 10^6 CFU/mouse (Log_{10} CFU 5.4 to 6.0). The black horizontal solid bars represent the average recovery in days.



Log $_{10}$ dose: 5.4 5.7 6.0

Unpaired t-test: p = > 0.30 for all doses tested

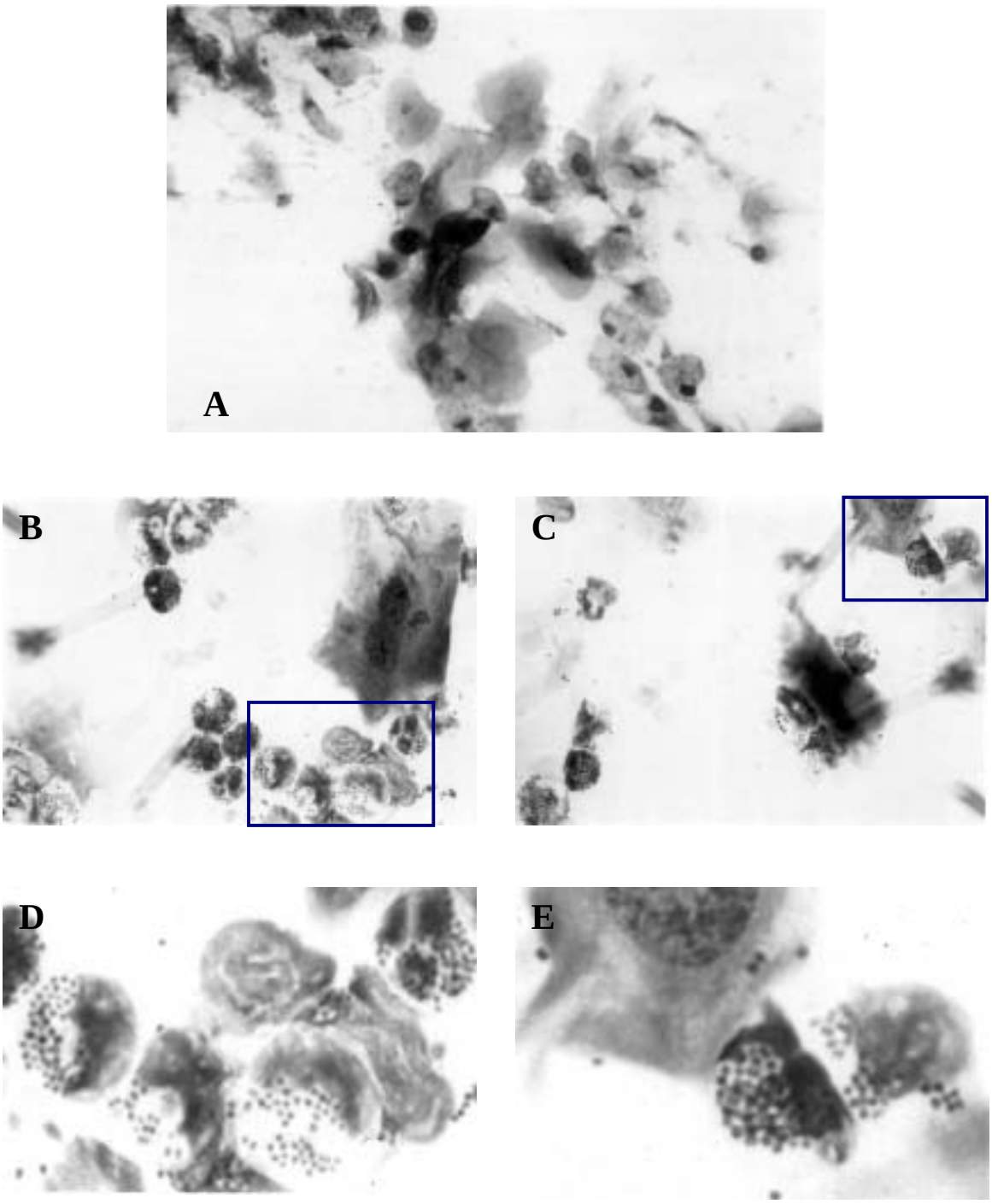


Figure 50. Stained vaginal smears from estradiol-treated mice infected with FA1090 and NGAAS-501 after 8 days post-inoculation. (**A**) Vaginal smear of uninfected mouse prior to intravaginal inoculation with gonococci. (**B** and **D**) Mouse # 20 and (**C** and **E**) Mouse # 14 inoculated with wild-type *N. gonorrhoeae* and catalase deficient NGAAS-501, respectively. High numbers of wild-type (**D**) and *kat* mutant (**E**) GC are seen associated with PMNs. Magnification 400X

Table 9. Duration of infection and occurrence of PMN influx in mice infected with FA1090 and NGAAS-501 (Exp. I)

A. Mice infected with FA1090

Mouse #	Duration of colonization (days)	Period of inflammation*	Mean % PMNs per peak (range)
5	0	day 5 day 7-day 12	2 33 (13-56)
6	7	day 7-day 9	45 (41-52)
7	12	day 4 day 8-day 12	11 33 (10-58)
8	12	day 8 day 10 day 12	10 32 50
X (n = 4)	7.8 (Range = 1-12)	_	_

B. Mice infected with *kat*$^{-}$ GC NGAAS-501

Mouse #	Duration of colonization (days)	Period of inflammation*	Mean % PMNs per peak (range)
9	0	day 7	46
10	1	day 1-day 12	35 (2-59)
11	5	day 6-day 12	50 (35-67)
12	2	day 7-day 12	47 (26-64)
X (n = 4)	2.0 (Range = 1-12)	_	_

*** Inflammation was defined as the presence of a higher percent of PMNs than the baseline percent. Baseline % PMNs = Daily PMN average in placebo control + 1 standard deviation.**

Table 10. Duration of infection and occurrence of PMN influx in mice infected with FA1090 and NGAAS-501 (Exp.II)

A. Mice infected with WT GC FA1090

Mouse #	Duration of colonization (days)	Period of inflammation*	Mean % PMNs per peak (range)
6	8	day 6 day 10-day12	24 57-71
7	9	day 4	7
8	10	day 2 day 4	13 10
9	12	day 1-day 2 day 4	15-23 10
10	12	day 2 day 4	8 13
16	11	0	0
17	12	0	0
18	9	day 2-day 4 day 11	12-25 60
X (n = 8)	10.4 (Range = 1-12)	_	_

B. Mice infected with *kat*$^{-}$ GC NGAAS-501

Mouse #	Duration of colonization (days)	Period of inflammation*	Mean % PMNs per peak (range)
11	6	day 4-day 8	36 -64
12	7	day 6-day 12	29-94
13	12	day 2-day 4 day 12	10-18 67
14	6	day 2-day 5	24-56
15	2	0	0
19	2	day 1-day 7 day 11-day12	21-78 58-75
20	5	day 6-day 8	82-90
21	2	0	0
X (+/- S. D.) (n = 7)	5.3 (Range = 1-12)	_	_

*** Inflammation was defined as the presence of a higher percent of PMNs than the baseline percent. Baseline % PMNs = Daily PMN average in placebo control + 1 standard deviation.**

Table 11. Duration of infection and occurrence of PMN influx in mice infected with FA1090 and NGAAS-501 (Exp. III)

A. Mice infected with FA1090

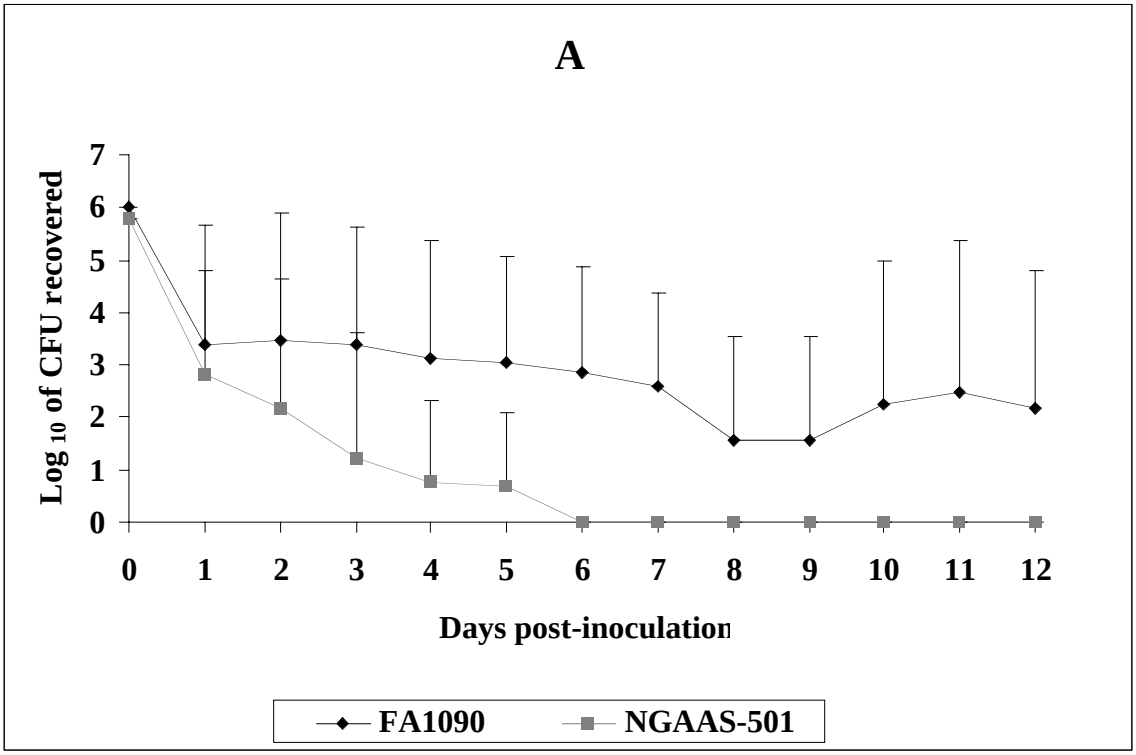
Mouse #	Duration of colonization (days)	Period of inflammation*	Mean % PMNs per peak (range)
1	12	day 1-day 4 day 8	23 (6-33) 25
3	7	day 2	13
6	12	day 6	18
20	12	0	0
24	3	day 4	2
29	3	day 2	1
X (n = 6)	8.2 (Range = 1-12)	–	–

B. Mice infected with *kat*$^{-}$ GC NGAAS-501

Mouse #	Duration of colonization (days)	Period of inflammation*	Mean % PMNs per peak (range)
9	12	day 6-day 10	36 (6-53)
11	3	day 1-day 5	30 (6-62)
13	12	day 4 day 6 day 8	2 4 20
7	12	day 2-day 3	1
8	6	day 6-day 10	30 (10-59)
14	12	day 1- day 10	51 (14-72)
15	10	day 8	19
X (n = 7)	9.6 (Range = 1-12)	–	–

*** Inflammation was defined as the presence of a higher percent of PMNs than the baseline percent. Baseline % PMNs = Daily PMN average in placebo control + 1 standard deviation.**

Figure 51. Average log $_{10}$ of CFU recovered (**A**) and percent of PMNs (**B**) in vaginal smears from mice infected either with FA1090 or NGAAS-501 (individually infected mice with either wild-type or the mutant, experiment I)


A
Log 10 of CFU recovered
0
1
2
3
4
5
6
7
0
1
2
3
4
5
6
7
8
9
10
11
12
Days post-inoculation
FA1090
NGAAS-501

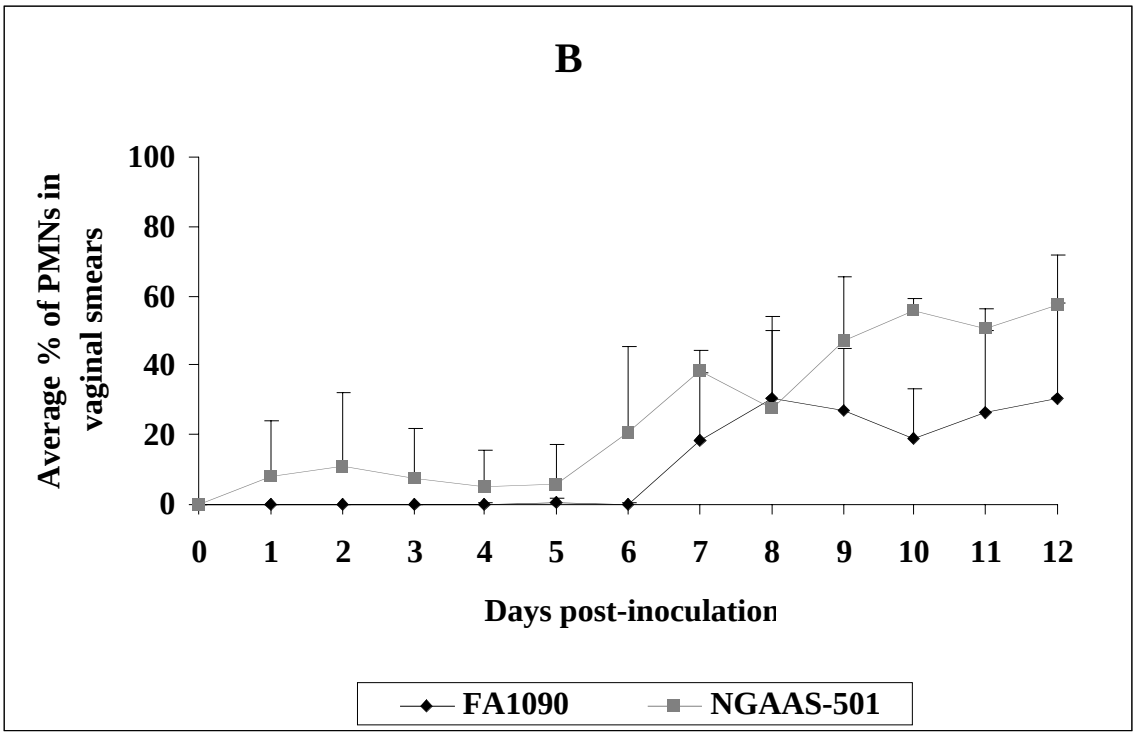
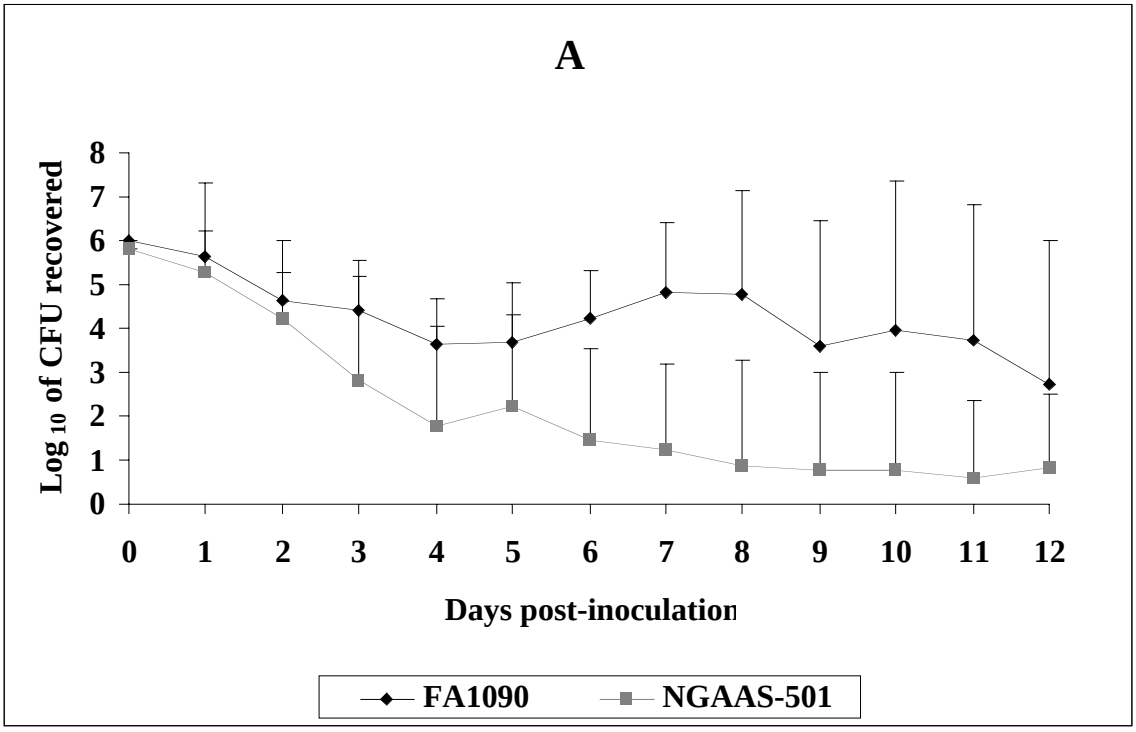

B
Average % of PMNs in vaginal smears
0
20
40
60
80
100
0
1
2
3
4
5
6
7
8
9
10
11
12
Days post-inoculation
FA1090
NGAAS-501

Figure 52. Average log $_{10}$ of CFU recovered (**A**) and percent of PMNs (**B**) in vaginal smears from mice infected either with FA1090 or NGAAS-501 (individually infected mice with either wild-type or the mutant, experiment II).


A
Log 10 of CFU recovered
0
1
2
3
4
5
6
7
8
0 1 2 3 4 5 6 7 8 9 10 11 12
Days post-inoculation
FA1090
NGAAS-501

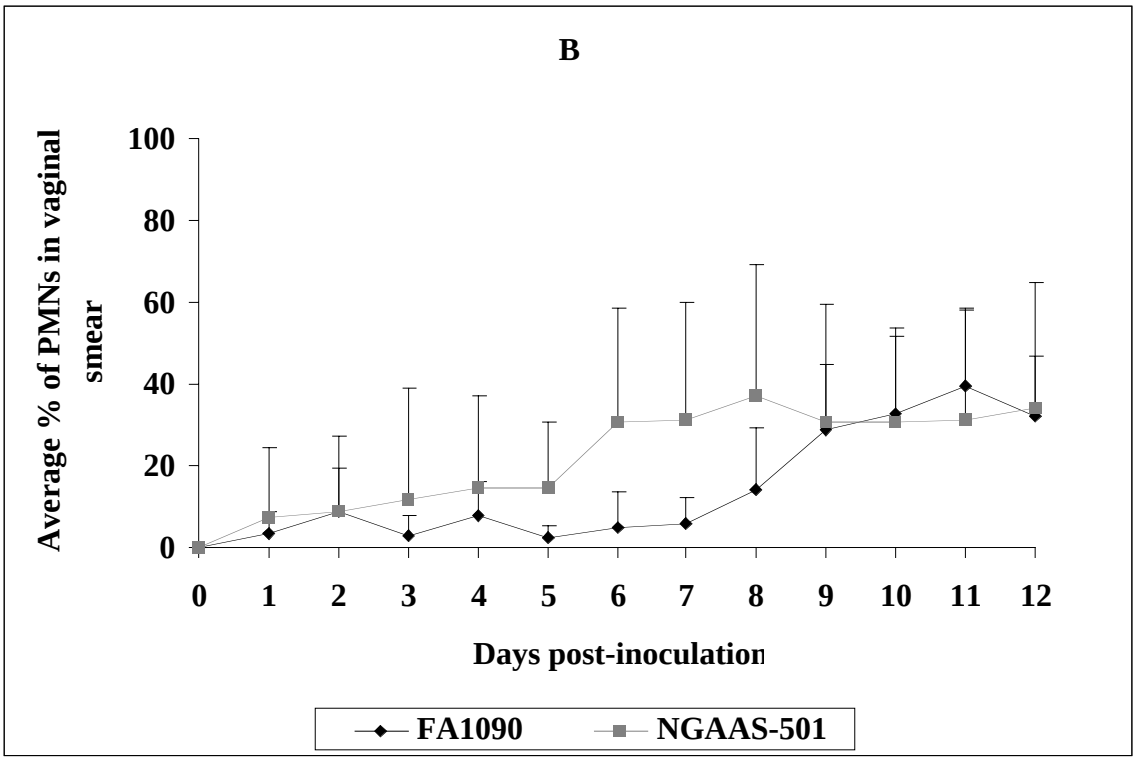
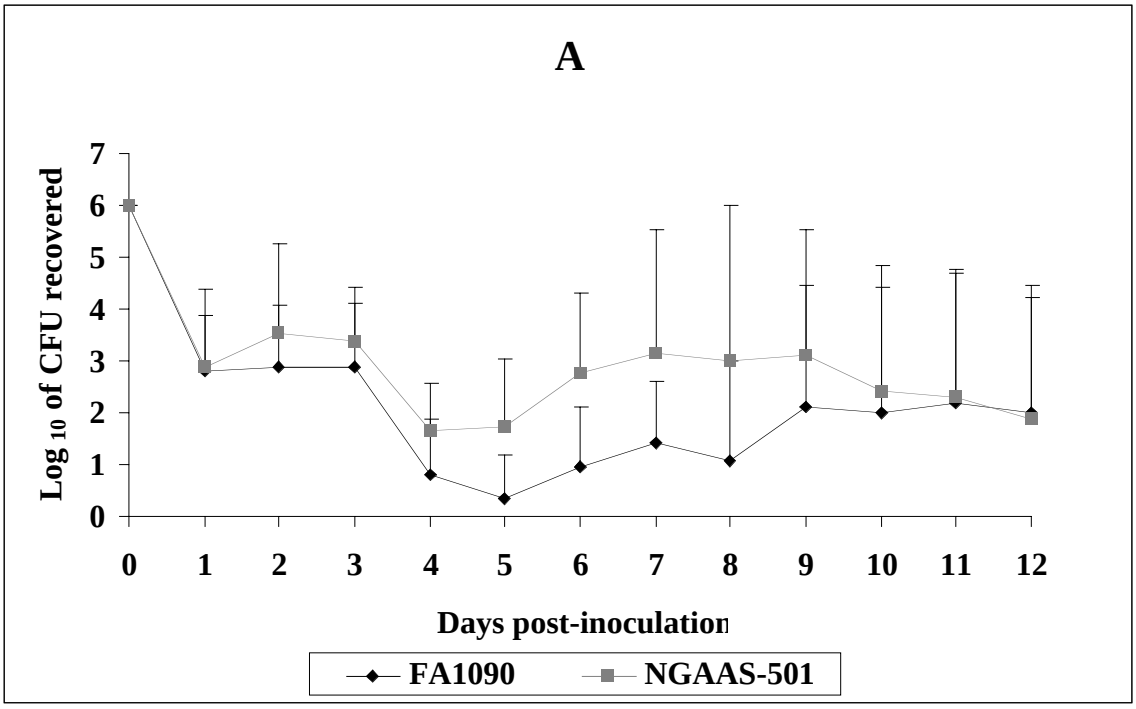

B
Average % of PMNs in vaginal smear
0
20
40
60
80
100
0 1 2 3 4 5 6 7 8 9 10 11 12
Days post-inoculation
FA1090
NGAAS-501

Figure 53. Average log $_{10}$ of CFU recovered (**A**) and percent of PMNs (**B**) in vaginal smears from mice infected either with FA1090 or NGAAS-501 (individually infected mice with either wild-type or the mutant, experiment III).


A
Log 10 of CFU recovered
0
1
2
3
4
5
6
7
0 1 2 3 4 5 6 7 8 9 10 11 12
Days post-inoculation
FA1090
NGAAS-501

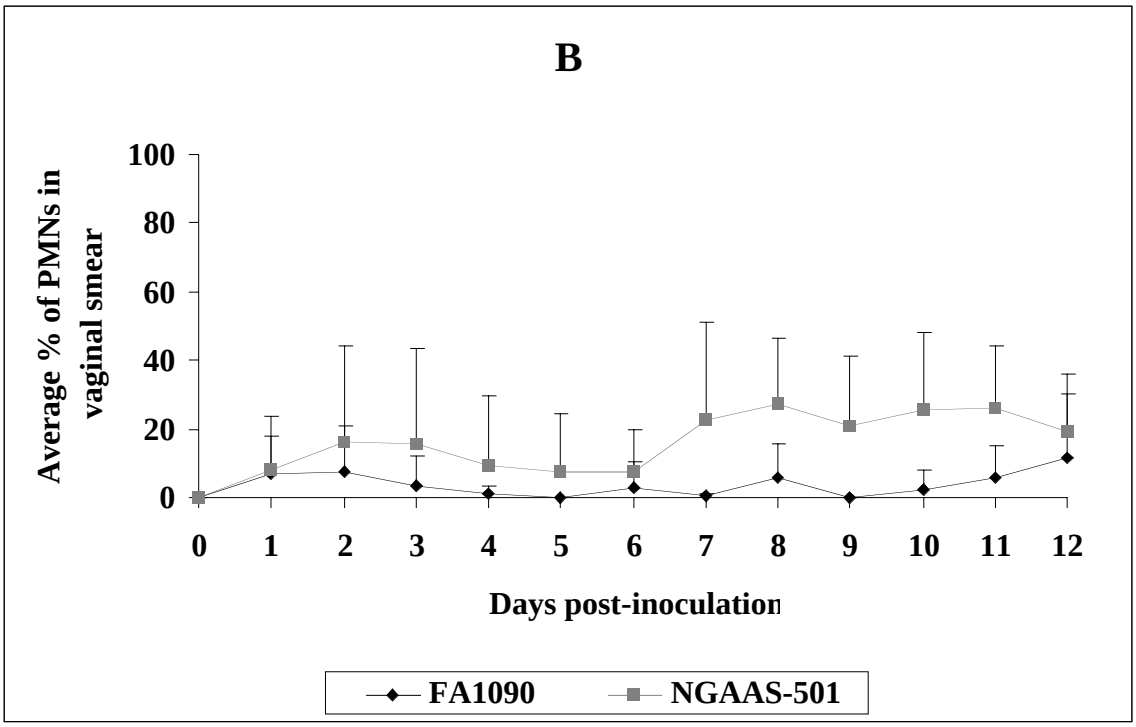
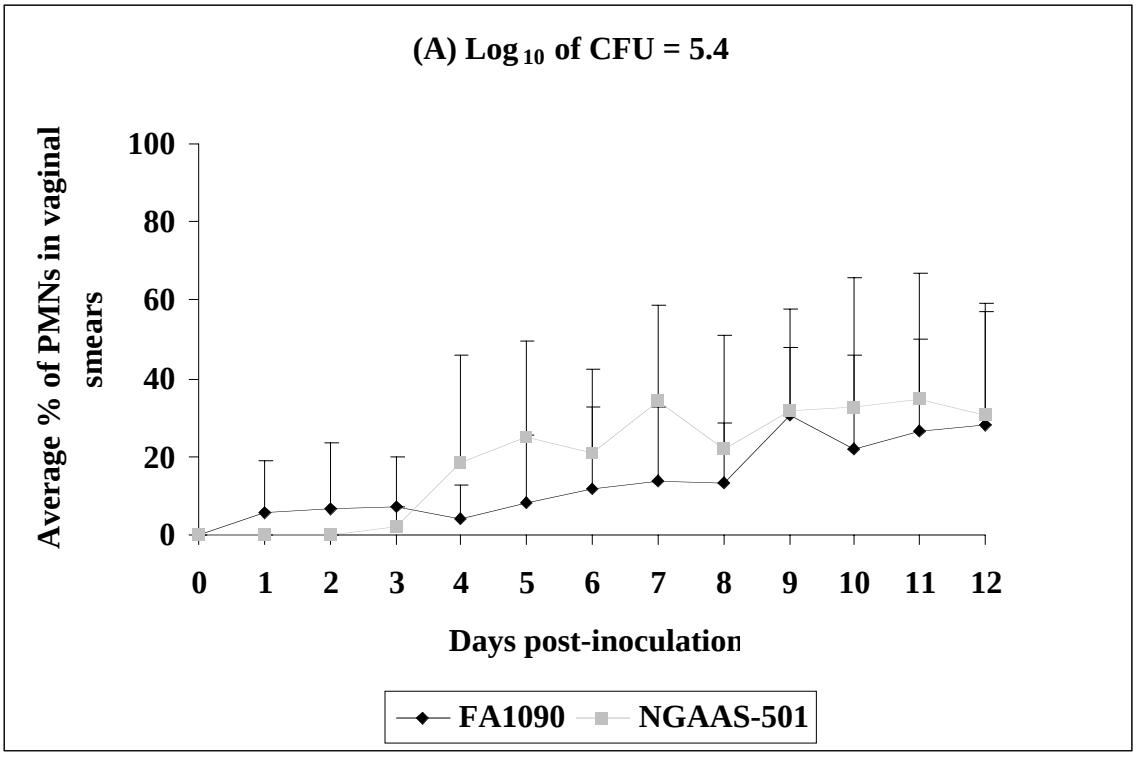
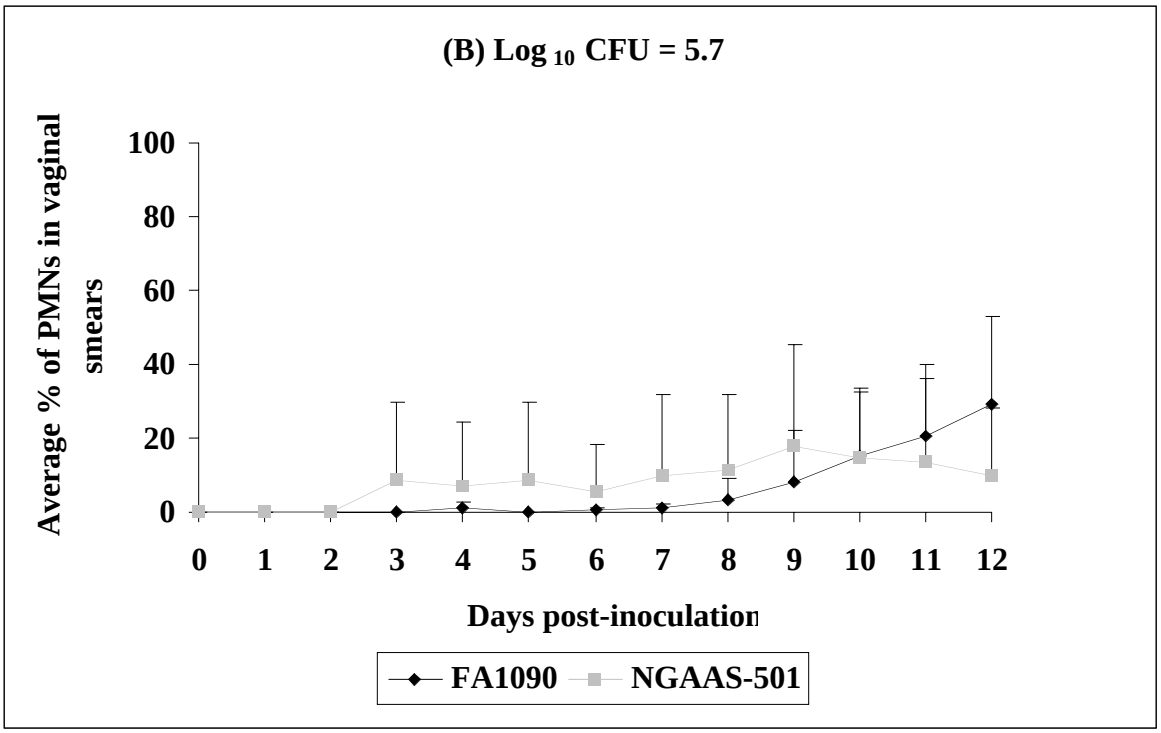
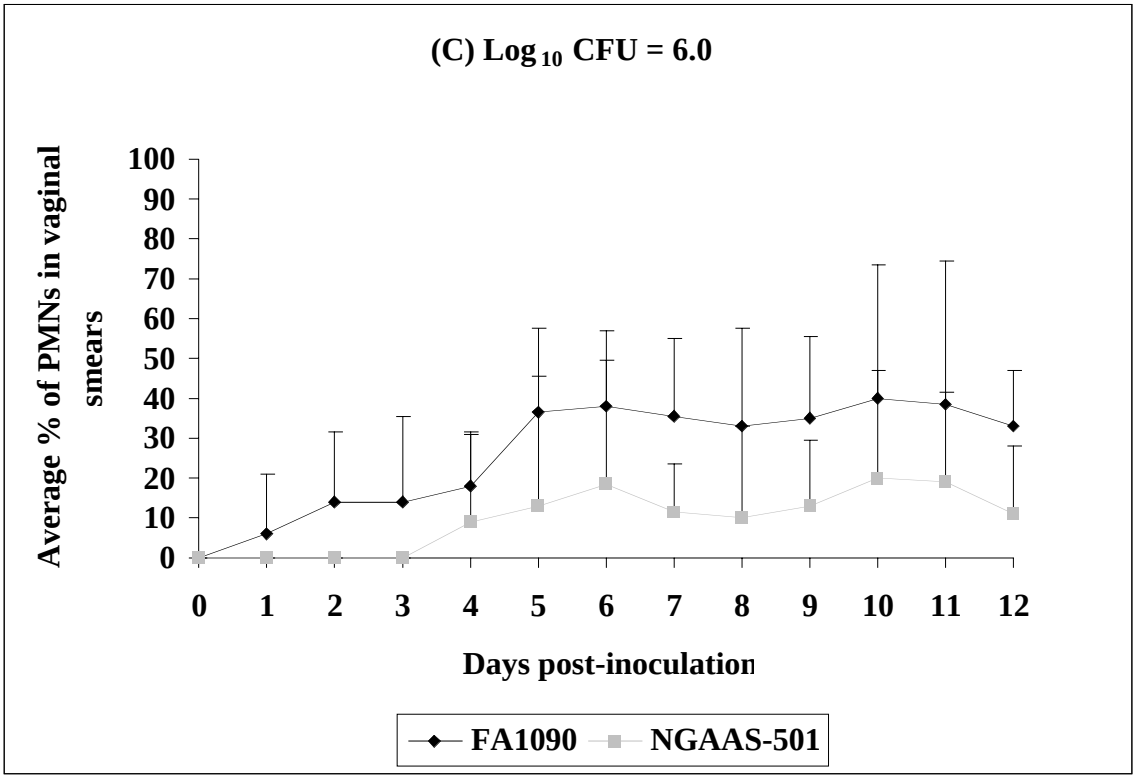

B
Average % of PMNs in vaginal smear
0
20
40
60
80
100
0 1 2 3 4 5 6 7 8 9 10 11 12
Days post-inoculation
FA1090
NGAAS-501

Figure 54. Percent of PMNs in vaginal smears from mice infected either with FA1090 or NGAAS-501 in dose response experiments shown in **Fig. 51-53**. Log $_{10}$ of CFU in inoculum (**A**) 5.4, (**B**) 5.7 and (**C**) 6.0.


(A) Log 10 of CFU = 5.4
Average % of PMNs in vaginal smears
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Days post-inoculation
FA1090
NGAAS-501


(B) Log 10 CFU = 5.7
Average % of PMNs in vaginal smears
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Days post-inoculation
FA1090
NGAAS-501


(C) Log 10 CFU = 6.0
Average % of PMNs in vaginal smears
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Days post-inoculation
FA1090
NGAAS-501

statistically significant, the tendency of the mutant to elicit a higher influx of PMNs should be noted. The reason for this observation remains unknown.

B. Mixed infection experiments

The use of mixed inocula to compare the survival of a wild-type bacterium versus that of an isogenic mutant in an experimental host is a sensitive way to detect differences due to the mutation in question. The advantage of this type of experiment is that both strains are evaluated within the same mouse, thereby minimizing any effects of animal variability. Again, estradiol-treated mice were inoculated intravaginally with a 1:1 mixture of FA1090 and NGAAS-501. The percentage of each strain among vaginal isolates was determined by bubbling assay. In two mixed infection experiments, the mutant NGAAS-501 was no longer recovered after 4 to 6 days post-infection (**Tables 12 and 13**). In contrast, FA1090 was recovered for an average of 11 days post-inoculation. This result suggests that the *kat* mutant is attenuated *in vivo* in that wild-type GC has a competitive advantage when within the same mouse.

The combined data from infection experiments using estradiol-treated mice lead to several hypotheses. The persistence of the catalase mutant strain during single infection experiments, even in the presence of a PMN response, suggests that GC may possess other mechanisms besides catalase that are more relevant as a defense against PMNs, or that can compensate for the its lack during infection. It is also possible that the persistence of the *kat* mutant in estradiol-treated mice is due to a potential limitation of the model, namely suppression of PMN functions by estradiol. The latter possibility is supported by reports that hormones can exert a regulatory role in the functions of several

Table 12. Recovery of wild-type versus *kat* mutant GC from estradiol-treated mice inoculated with mixtures of the two bacteria.

Experiment I - Dose: 6.5 x 10^5 cfu / mouse (= 50% wild-type, 50% *kat* mutant)

Mouse #	**Duration of recovery (days)**	**Percentage of wildtype FA1090 recovered on each day of culture**											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	12	95	99	100	-*	-	100	100	100	100	100	100	100
2	9	-	-	-	100	-	99	100	-	-*	-	-	-
3	12	93	100	-*	100	-*	-*	-*	-	100	100	100	100
4	11	83	100	98	99	-*	-	-	-	-*	-	100	-
5	10	80	96	98	-*	-	100	100	100	100	100	-	-
Mean	10.8 days												

-*, fewer than 42 colonies isolated; -, culture negative. The percentage of wild-type in the inocula and among vaginal isolates was determined by testing ~ 150 colonies (range 42 - 622) by assay

Table 13. Recovery of wild-type versus *kat* mutant GC from estradiol-treated mice inoculated with mixtures of the two bacteria.

Experiment II - Dose: 1.2 x 10^6 cfu / mouse (=59% wild-type, 41% *kat* mutant)

Mouse #	**Duration of recovery (days)**	**Percentage of wildtype FA1090 recovered on each day of culture**											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	12	-*	100	100	97	100	100	100	100	100	100	100	100
2	12	84	95	99	-*	100	100	100	100	100	100	100	100
3	12	91	100	100	100	100	100	-*	100	100	100	-	100
4	12	96	94	99	97	99	100	100	100	100	100	-*	100
5	12	96	94	98	99	100	100	100	100	100	100	100	100
Mean	12.0 days												

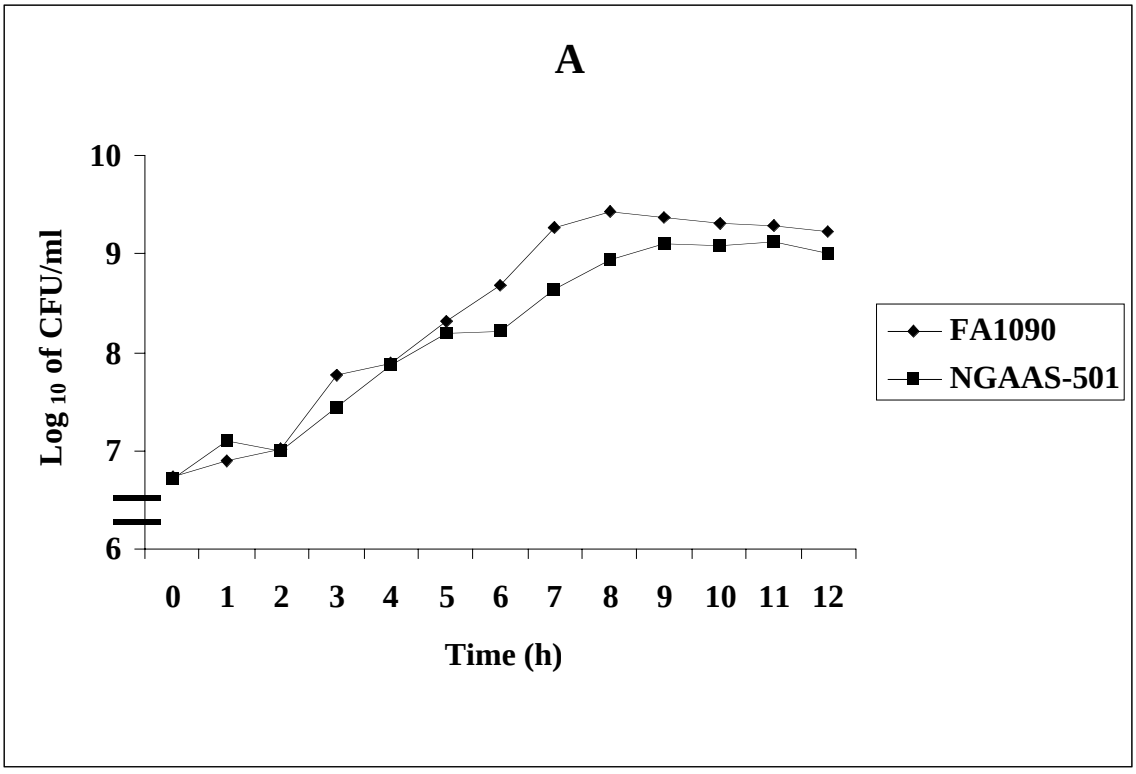
-*, fewer than 42 colonies isolated; -, culture negative. The percentage of wild-type in the inocula and among vaginal isolates was determined by testing ~ 150 colonies (range 42 - 622) by assay

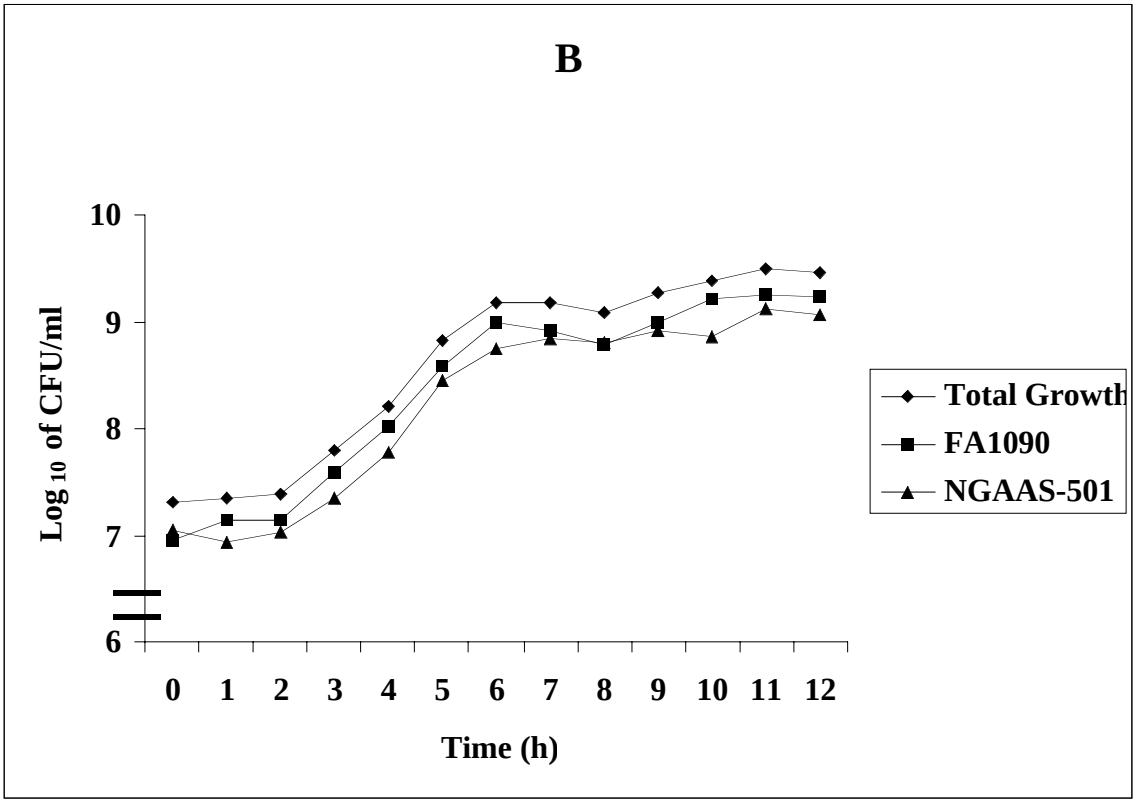
cell types including neutrophils (Bodel et al., 1972; Hulka et al., 1965; Mitchell et al., 1970). It is also known that estradiol treatment increases the susceptibility of mice to systemic gonococcal infection (Kita et al., 1985), and that this susceptibility may be due to impairment of some components of the respiratory burst required for clearance of the infection.

The observed attenuation of the catalase mutant in the mixed infection experiments leads to the following hypotheses. First, the attenuation observed could be due to factors not related to the catalase deficiency such as differences in growth rate due to an unknown factor. Second, it is possible that the wild-type strain may inhibit the growth of the *kat* mutant. Third, expression of different phase-variable phenotypes such as pili, Opas or LOS by the stock culture of wild-type and *kat* mutant GC that were used in these experiments might result in functional differences. Last, the wild-type strain may generate an environment *in vivo* in which the catalase mutant is unable to survive.

To test the possibility that differences in growth rate between the wild-type strain and the *kat* mutant are responsible for the enhanced survival of the wild-type bacteria *in vivo,* or that the wild-type strain inhibits the *kat* mutant, single and mixed growth curves were performed by inoculating GC broth with equal numbers of FA1090 or NGAAS-501, or a 1:1 ratio of both. No significant differences were observed in the growth patterns of either strain when cultured individually (**Fig. 55 A**) or in mixed cultures (**Fig. 55 B**). These results suggest that the observed attenuation of strain NGAAS-501 during mixed infections was not due to differences in growth rate or to inhibition of growth by the wild-type strain.

Figures 55. Growth rate of FA1090 and NGAAS-501. (**A**) Growth curves of wild-type and *kat* mutant cultured independently and (**B**) of a 1:1 mixture of both strains.


A
10
9
8
7
6
Log 10 of CFU/ml
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (h)
FA1090
NGAAS-501


B
10
9
8
7
6
Log 10 of CFU/ml
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (h)
Total Growth
FA1090
NGAAS-501

Gonococci require certain surface molecules to establish a productive infection in humans, namely pili, opacity (Opa) proteins and LOS. One characteristic that is common to each of these molecules is the capacity to undergo phase variation (Meyer et al., 1990). To rule out the potential role that these phase variable molecules may play in murine infection, the predominant piliation, opacity and LOS phenotypes of the frozen stocks of FA1090 and NGAAS-501 that were used to infect mice were determined. The degree of piliation was evaluated as a reflection of transformation frequency since piliation is required for natural competence in GC. Strains FA1090 and NGAAS-501 were exposed to different concentrations of genomic DNA carrying the antibiotic marker rifampicin. Transformation was performed as described in Materials and Methods, and the frequency was determined as the number of rifampicin resistant colonies per number of recipients. Both bacteria showed similar transformation frequencies, suggesting they were equally piliated (**Table 14**). The Opa phenotype of wild-type and *kat* mutant stocks was determined by colony immunoblot of 20 colonies from each stock, using antisera specific for the different known Opa proteins in FA1090 (Jerse et al., 1994). Both strains showed a predominant Opa B/D phenotype (**Table 15**).

The LOS species expressed by the wild-type and *kat* mutant was detected by electrophoretic analysis of membrane extracts in an 8-16% gradient PAGE gel, followed by silver staining. No difference in LOS species was observed (**Fig. 56**). In conclusion, it is unlikely that the attenuation observed during the mixed infection experiments was due to differences in the phase variable phenotypes that were tested. The hypothesis that the wild-type strain may generate an environment *in vivo*, in which the catalase mutant is

Table 14. Transformation frequency as a reflection of degree of piliation of FA1090 and NGAAS-501

Transfer Frequency (rif ^{r} colonies/Num. recipients)			
DNA concentration (ng)	**FA1090**	**NGAAS-501**	**MS11 B2[1]**
100	5.4×10^{-6}	8.6×10^{-6}	0
500	9.34×10^{-6}	2.5×10^{-5}	0
1000	3.0×10^{-5}	4.4×10^{-5}	0

[1] *N. gonorrhoeae* MS11 B2 was included as a nonpiliated control

Table 15. Predominant Opa phenotype of stock cultures of FA1090 and NGAAS-501

Opa protein (antibody)	**FA1090 (# of positive colonies/total tested)**	**NGAAS-501 (# of positive colonies/total tested)**
A (H138)	0/20	0/20
B/D (H4)	11/20 (± 4/20)	20/20
E/K (H164)	0/20	0/20
C (Rbc)	0/20	0/20
F (Rbf)	0/20	0/20

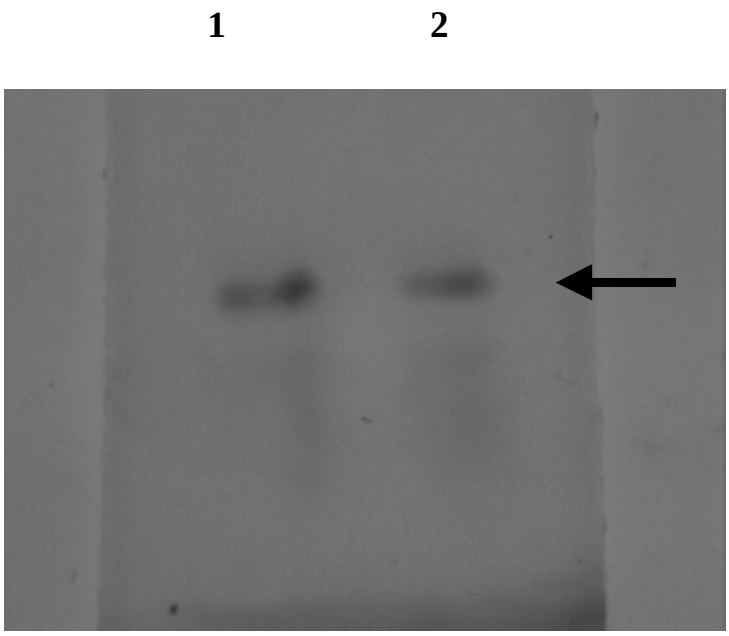


Figure 56. LOS phenotype of FA1090 and NGAAS-501. LOS from GC FA1090 and NGAAS-501 was obtained by micro-extraction using proteinase K digestion. Five ∝l of each sample were loaded in a 8-16% pre-cast gradient PAGE gel. Lane **1**, FA1090 and **2**, NGAAS-501. No differences in LOS species were observed among the strains.

unable to survive, will be addressed based on data described in the Results and analyzed in the Discussion.

IV-Role of catalase as a survival mechanism against PMN killing

The demonstration that mutation of the gonococcal catalase gene rendered the bacteria more sensitive to H_2O_2 and H_2O_2-producing lactobacilli *in vitro*, and reduced survival in mixed mouse infection experiments supports the hypothesis that catalase is an important defense mechanism for GC survival in the host. Based on these data it can be predicted that catalase may play a role against the H_2O_2 produced by phagocytes during the inflammatory response. To further study the relevance of catalase as a mechanism of defense against phagocytes, a diverse array of assays that allow one to study the interactions between gonococci and mouse PMNs were performed.

A. Optimization of the acridine orange-trypan blue staining assay

The acridine orange-trypan blue staining method allows one to measure the total number of bacteria associated with a eukaryotic cell based on staining with the fluorescent stain, acridine orange. This assay also allows one to distinguish extra- versus intracellular bacteria based on counter-staining with trypan blue, which quenches extracellular fluorescent bacteria. Before using the assay to compare the association of the wild-type strain and *kat* mutant with PMNs, the assay was optimized for the following parameters: effect of phorbol myristate acetate (PMA), percentage of serum, multiplicity of infection and time of incubation.

The sensitivity of GC strain FA1090 to murine serum was first determined because it is known that normal human serum possesses bactericidal properties against some GC strains. GC FA1090 was exposed to different concentrations of sera from estradiol-treated and untreated mice for 30 min. As a control for possible bactericidal activity, heat inactivated mouse serum was also tested. The degree of bactericidal activity was measured as the ratio of the $\log_{10}$ of bacteria recovered after exposure to normal mouse serum (NMS) as compared to the $\log_{10}$ of bacteria recovered after exposure to heat-inactivated normal mouse serum (HI-NMS). Normal human serum (NHS) was used as a positive bactericidal control. Neither serum from estradiol-treated or untreated mice killed strain FA1090. As expected, NHS decreased the population of FA1090 in a dose dependent manner, and heat inactivation of the NHS abrogated the bactericidal activity (**Fig. 57**).

Second, the effects of PMA and serum concentration on the association of GC with PMNs were tested simultaneously. PMA was used due to its property of increasing receptor expression on the surface of neutrophils (Rest and Farrell, 1990). Mouse PMNs were exposed to bacteria for 90 min, and the acridine orange staining was performed as described in the Materials and Methods. A greater percent of PMNs was associated with GC in the presence of PMA, compared to those without the stimuli. Serum enhanced the association of bacteria to PMNs in concentrations from 5 to 20% (**Fig. 58**). To further characterize the effect of serum on the association of gonococci to PMA-treated PMNs, the number of GC-associated with each PMN was categorized as 0, 1-2, 3-5 or >5 GC per PMN (**Fig. 59**). Again, the total number of bacteria associated with PMNs increased with

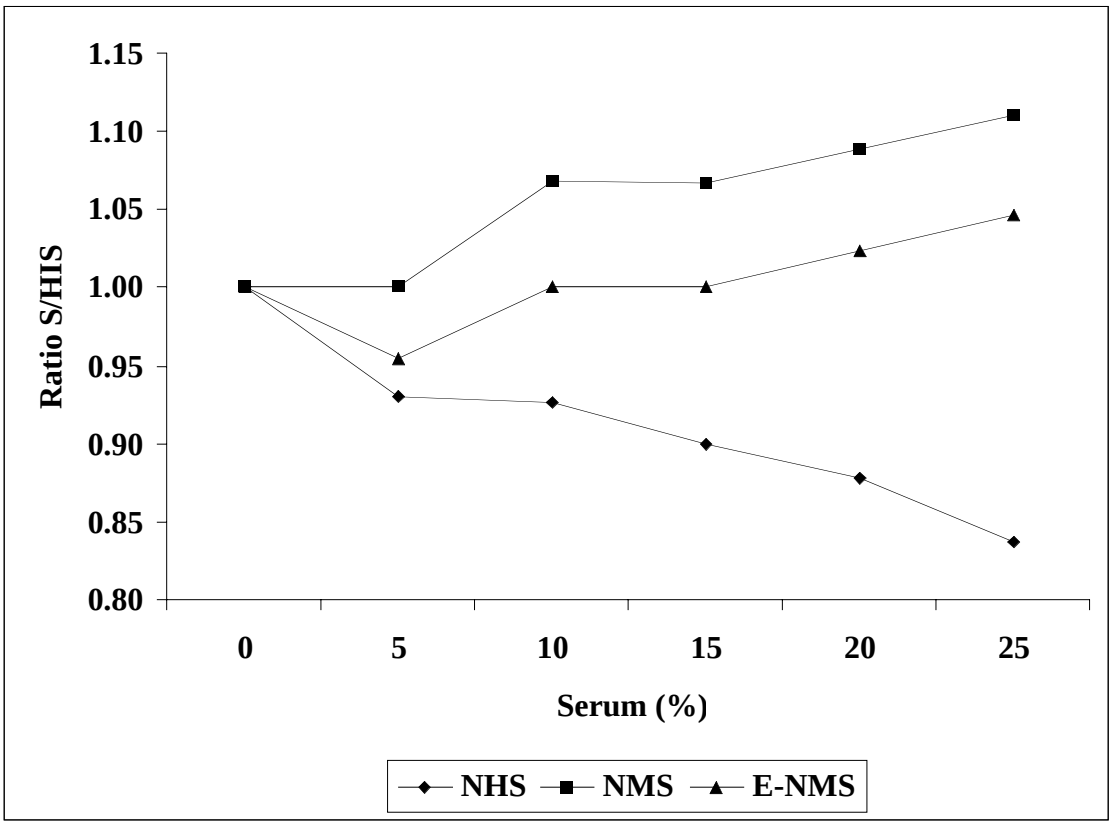


Figure 57. FA1090 serum sensitivity. The serum sensitivity of FA1090 was tested against mouse serum from untreated (NMS) and estradiol-treated mice (ENMS). Normal human serum (NHS) was used as a positive control of bactericidal activity. The Y axis corresponds to the ratio of GC recovered after exposure to murine or human serum (S) as compared to its corresponding heat-inactivated serum (HIS). The experiment was performed twice in triplicate

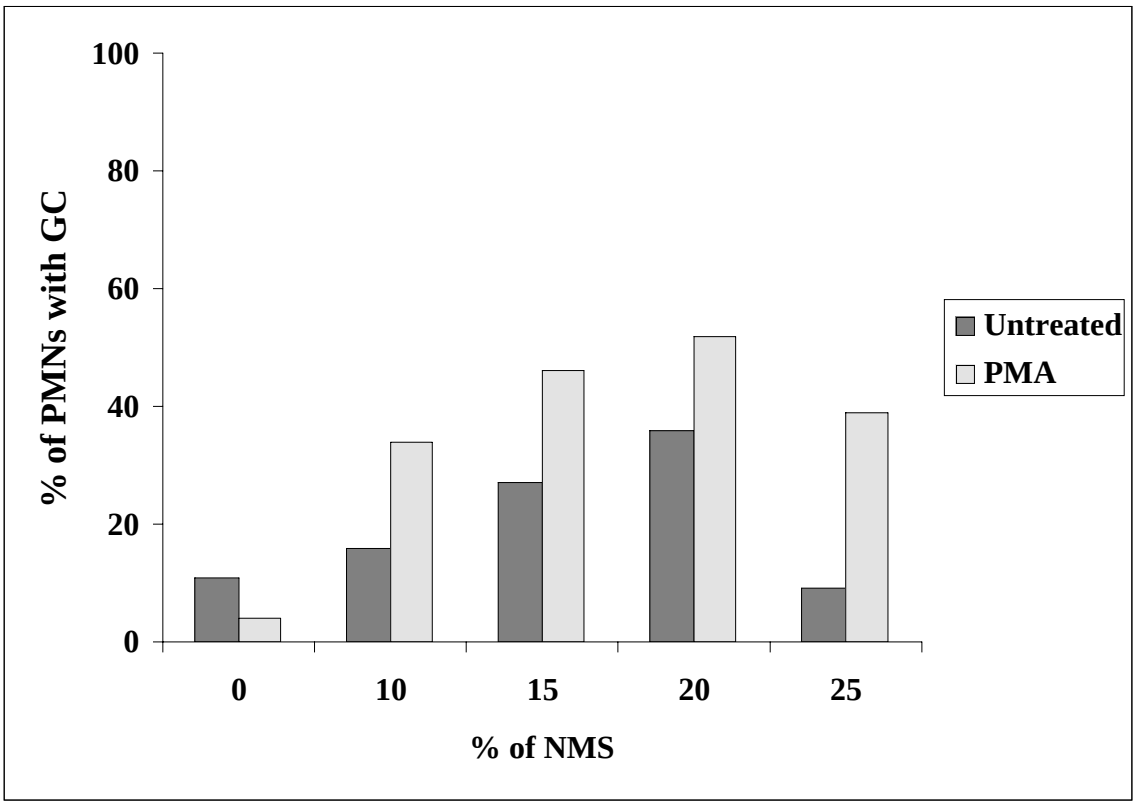


Figure 58. Effect of PMA and serum concentration on the association of gonococci to murine PMNs as measured by the acridine orange assay.

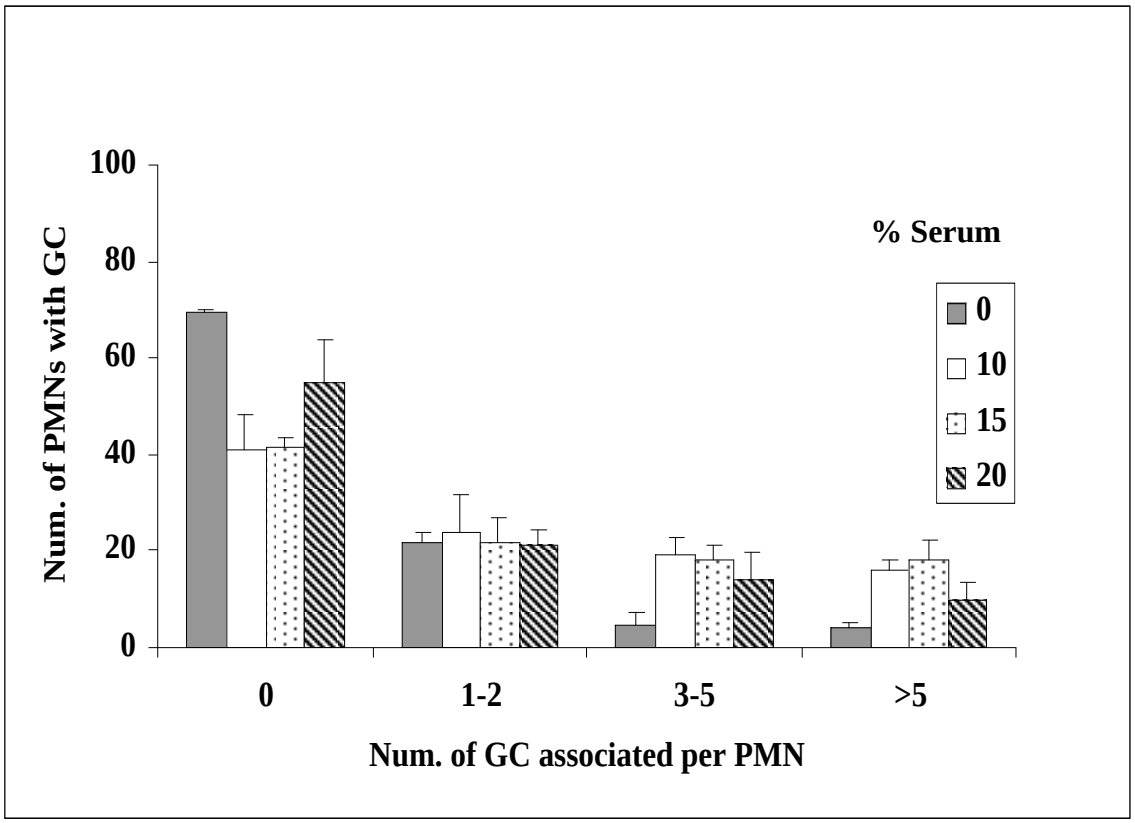


Figure 59. Effect of serum concentration on the association of gonococci to PMA-treated PMNS. The concentrations of serum tested are shown in the key.

the presence of serum, however the average number of GC per PMN did not increase with increasing concentrations of serum. The amount of serum selected for future tests was 10%, a concentration that has been used in previously reported assays using human PMNs and one that represents a reasonable amount of mouse serum to obtain for each assay.

The multiplicity of infection (MOI) is also an important factor to consider when optimizing a bacterial adherence assay, given that extremely high numbers of bacteria may give a high background. The use of low numbers of bacteria minimizes background levels, however, bacterial association may also be reduced. As described before, several MOIs were tested in the acridine orange assay in the presence of 10% NMS. As predicted, bacterial association increased with increasing MOI (**Fig. 60**). There was no significant difference in the number of bacteria associated with PMNs for MOIs of 10 versus 20. However, a higher background level of non-associated bacteria was observed at an MOI of 20 compared to an MOI of 10 (data not shown). Therefore, an MOI of 10 was chosen as the optimum MOI for further studies.

Optimization of the acridine orange assay was tested at a single time point, 90 min through out the assay. To determine if the association of GC with PMNs can be measured kinetically, murine PMNs were exposed to GC at a MOI of 10 in the presence of 10% serum. Coverslips were stained at 30, 45, 60 and 90 min post-inoculation. The percent of PMNs with GC and the number of bacteria associated per PMN increased between 30 and 60 min; no appreciable increase in the association occurred at late time points (**Fig. 61**). Based on these data, it was decided that 30, 60 and 90 minutes would be used to measure adherence in future studies.

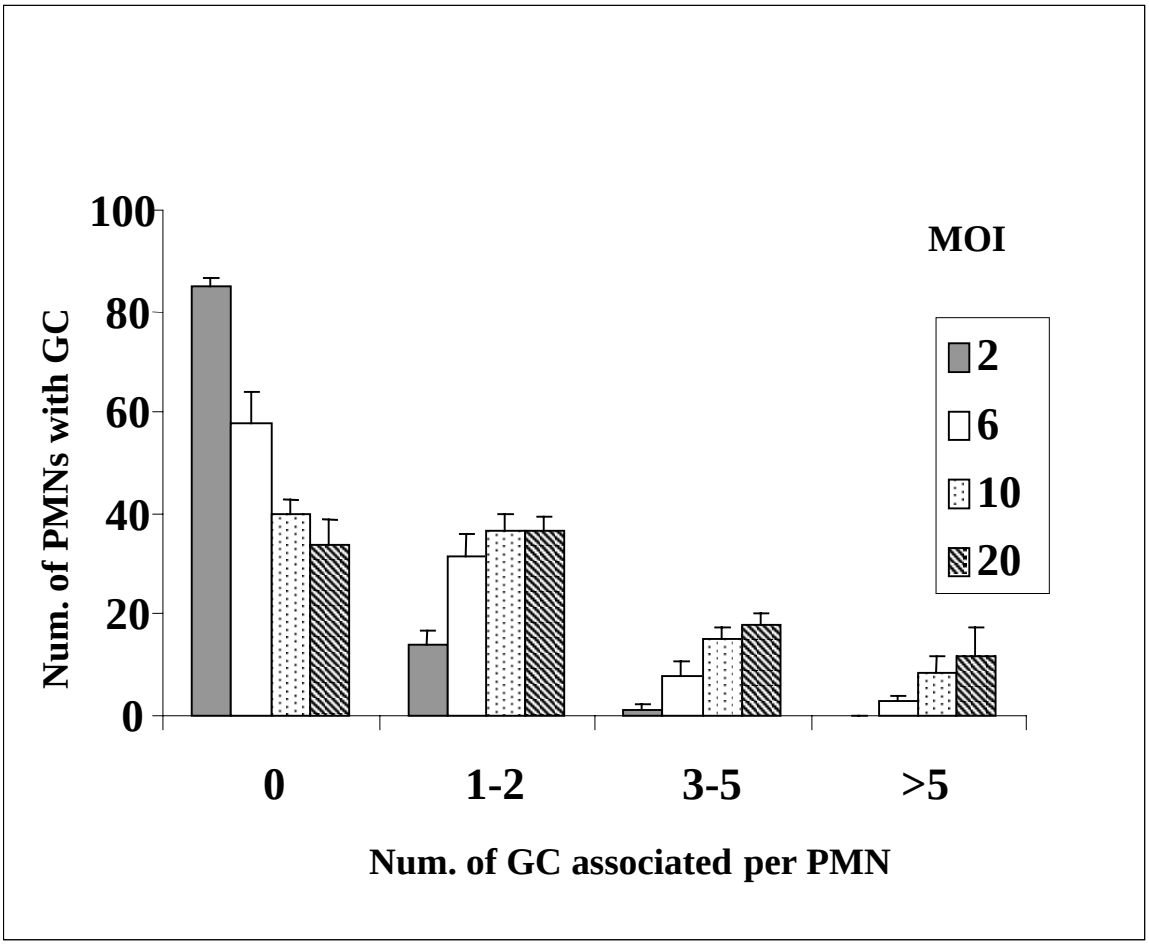


Figure 60. Effect of multiplicity of infection on association of gonococci to PMA-treated PMNs. The MOIs tested are shown in the key.

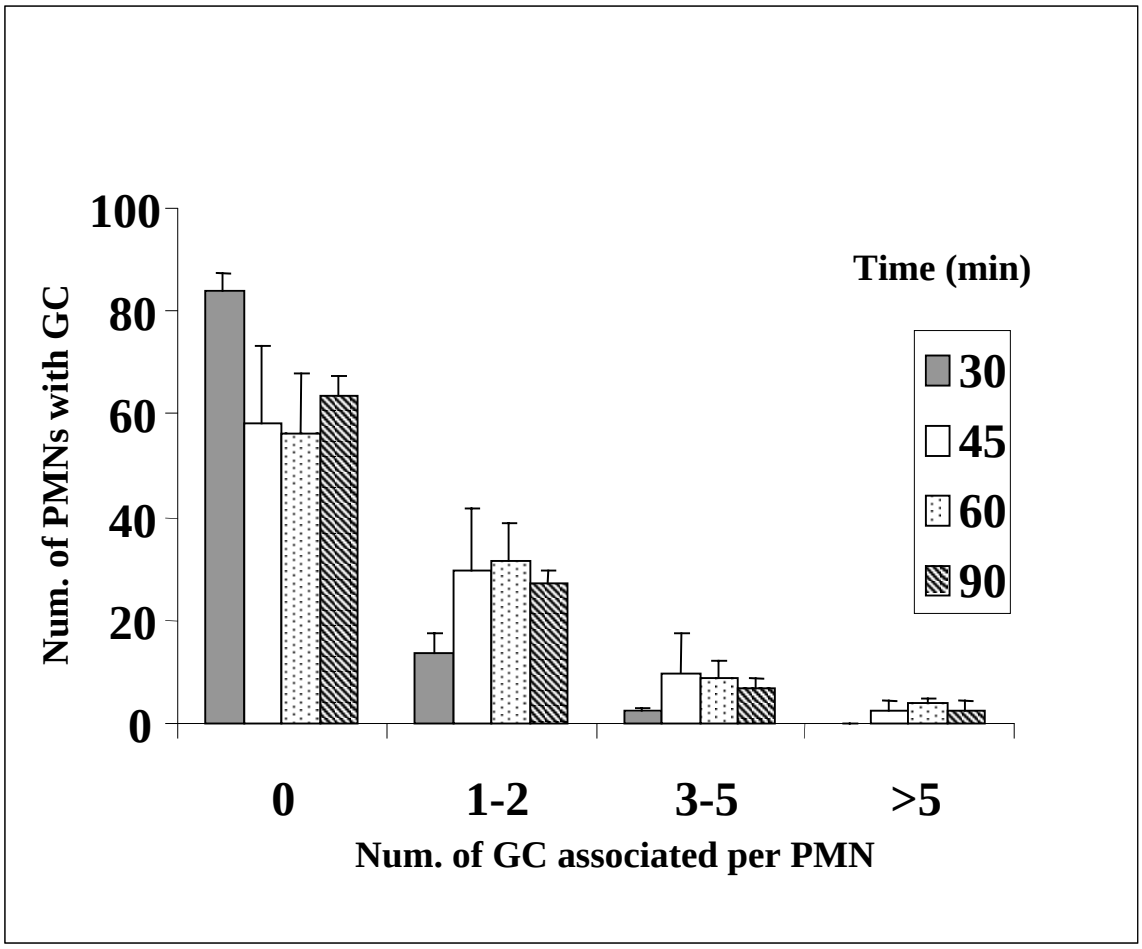


Figure 61. Effect of time on association of gonococci with PMA-treated PMNs. The incubation times tested (in minutes) are stated in the key.

The original acridine orange assay described the use of 0.04% crystal violet as a counterstain for quenching the fluorescence of extracellular bacteria. Unfortunately after several attempts, crystal violet was not successful in my hands as a counterstain. Hed (1986), reported that crystal violet is lysosomotropic meaning that it can enter phagolysosomes and quench the fluorescence of intracellular bacteria as well as extracellular bacteria. Therefore, trypan blue was used instead of crystal violet as a counter-stain to quench the signal emitted from extracellular bacteria.

B. Optimization of the PMN killing assay

Several protocols have been described for measuring the killing capacity of neutrophils against GC. These protocols vary in the composition of the assay buffer, atmosphere of incubation, MOI, presence or absence of serum and other factors. Most reports concerning the killing of GC by PMNs are based on data obtained from experiments using human PMNs. Human PMNs can take up GC by three different receptors, namely the C3b, Fc and CGM1 receptors. The first two are serum dependent with the difference that immune serum is required for the Fc receptor. The CGM1 receptor interactions are mediated by specific opacity proteins in the absence of sera. This receptor is not present in murine PMNs. In this study, a PMN killing assay was optimized to measure the killing capacity of estradiol-treated murine PMNs isolated by peritoneal lavage. **Table 16** describes all the conditions tested that led to the final protocol used during the present research. The final protocol is described in detail in the Materials and Methods.

Table 16. Optimization of PMN killing assay. Conditions in bold font represent the conditions that were used in the final assay.

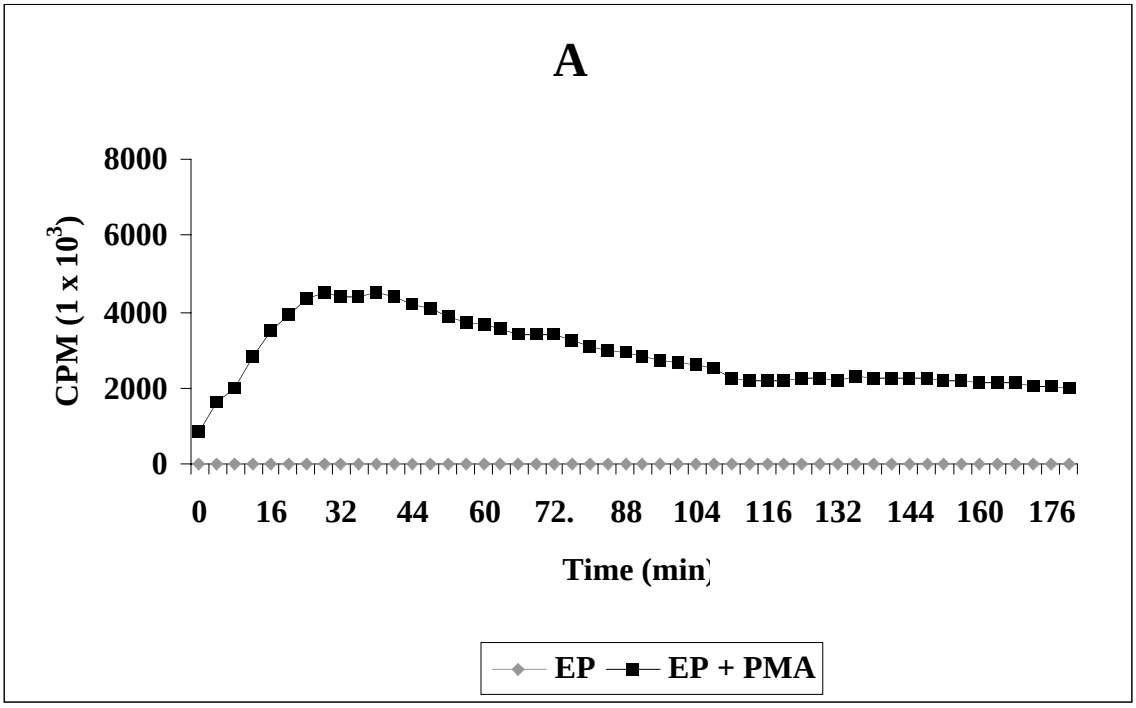
Parameter	Conditions	Results
PMN elicitation	Intraperitoneal injection of thioglycollate into the mouse 1) **2.5 ml for 5 hours** 2) 1 ml for 19 hours	No significant difference was observed in the number of PMNs obtained with either extraction method. Extraction of PMNs after 5 h incubation was selected to limit the time of exposure to thioglycollate.
Buffer	1) HBSS + 1% gelatin 2) HBSS + 1% gelatin, 0.1% $CaCl_2$, 0.1% $MgCl_2$ 3) **HBSS + 1% gelatin, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM glucose**	Different recipes of buffer were tested initially for bacterial stability. HBSS complete II (HBSS + 1% gelatin, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM glucose) reproducibly supported bacterial viability compared to the other buffers or D-MEM.
Tubes	1) 5 ml snap cap tube 2) **2 ml microcentrifuge tube**	Reactions tubes should not be larger than five times the size of the reaction volume (1 ml) (Clark et al., 1997). The 2 ml microcentrifuge tubes were selected for the assay.
Ratio (PMN:bacteria)	1) 10:1 2) **1:1**	Phagocytes possess a limited phagocytic capacity. High numbers of bacteria can inhibit phagocytosis by saturation. Published data indicate that human PMNs can take up 1-2 gonococci. A ratio of 1:1 was therefore selected for the assay.
Killing kinetics	1) 30, 60 and 90 min 2) **45, 90 and 135 min**	45, 90 and 135 min were selected as time points to determine the kinetics of killing. Using *S. aureus* as a control, 50-60% decrease in viable bacteria occurred at 90 min. No significant change was observed at 30 or 60 min.
Serum	1) nonopsonic 2) opsonic 3) **opsonization of bacteria prior to PMN exposure**	It was determined that serum is required for PMN killing of GC given that in its absence, no decrease in bacterial viability was observed. Opsonic-dependent killing was only observed when bacteria were opsonized for 15 min prior to exposure to PMNs.
Atmosphere	1) CO_2 incubation 2) **in ambient air**	Although killing was observed during incubation in CO_2, a higher degree of killing was observed during incubation at ambient air.

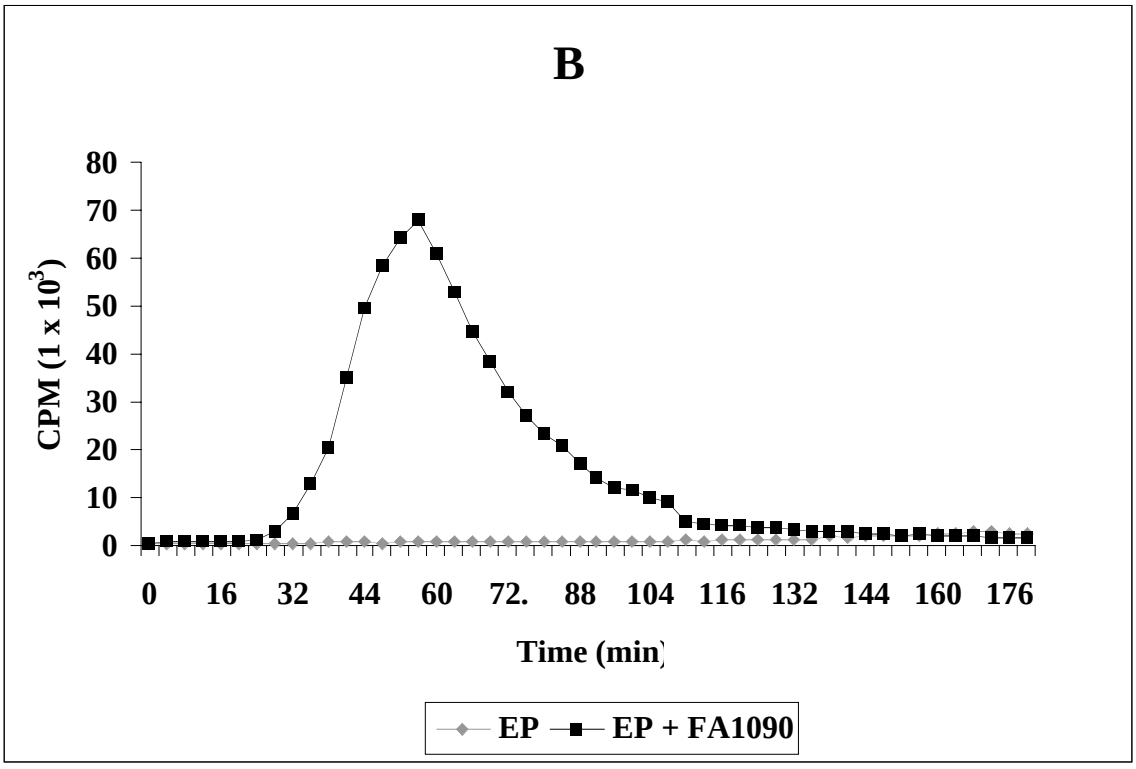
The use of thioglycollate to induce PMN infiltration has been a concern given that this agent will activate PMNs before harvesting them, and the degree of activation might affect the results of the assays in which the cells will be used. Differences in the degree of activation of PMNs elicited with thioglycollate and glycogen have been observed (Baron and Proctor, 1982; Cunningham et al., 1979), and it is known that continuous stimuli might lead to fatigue of PMNs (Matheisz and Allen, 1979). To test thioglycollate-induced PMNs extracted by peritoneal lavage from estradiol-treated mice for responsiveness to stimuli, the chemiluminescence assay was performed as described in Materials and Methods. The chemiluminescence signal emitted by estradiol-treated PMNs was measured with and without PMA and GC. As shown in **Figure 62 A** and **B**, negligible background levels were detected in unstimulated thioglycollate-induced estradiol-treated PMNs and a higher chemiluminescence signal was observed in the presence of PMA or gonococci. This result suggests that exposure of mouse PMNs to thioglycollate broth does not render them unresponsive to stimuli.

C. Characterization of estradiol-treated and untreated murine PMNs

As shown previously (**Fig. 48-49** and **Tables 10-11**), no significant difference was observed in the recovery of FA1090 or its isogenic catalase mutant NGAAS-501 from the lower genital tract of estradiol-treated mice. It can be hypothesized that the estradiol treatment that the mice received might impair some functions in the murine phagocytes, an event that might explain the inability of estradiol-treated mice to clear the catalase mutant. This hypothesis is based on a series of observations. Kita et al. (1985), measured several parameters of phagocytic functions and susceptibility to GC in

Figure 62. Chemiluminescence induced in thioglycollate-elicited estradiol-treated PMNs (EP) obtained by peritoneal lavage following stimulation with PMA (**A**) or GC (**B**). Experiment was performed three times.

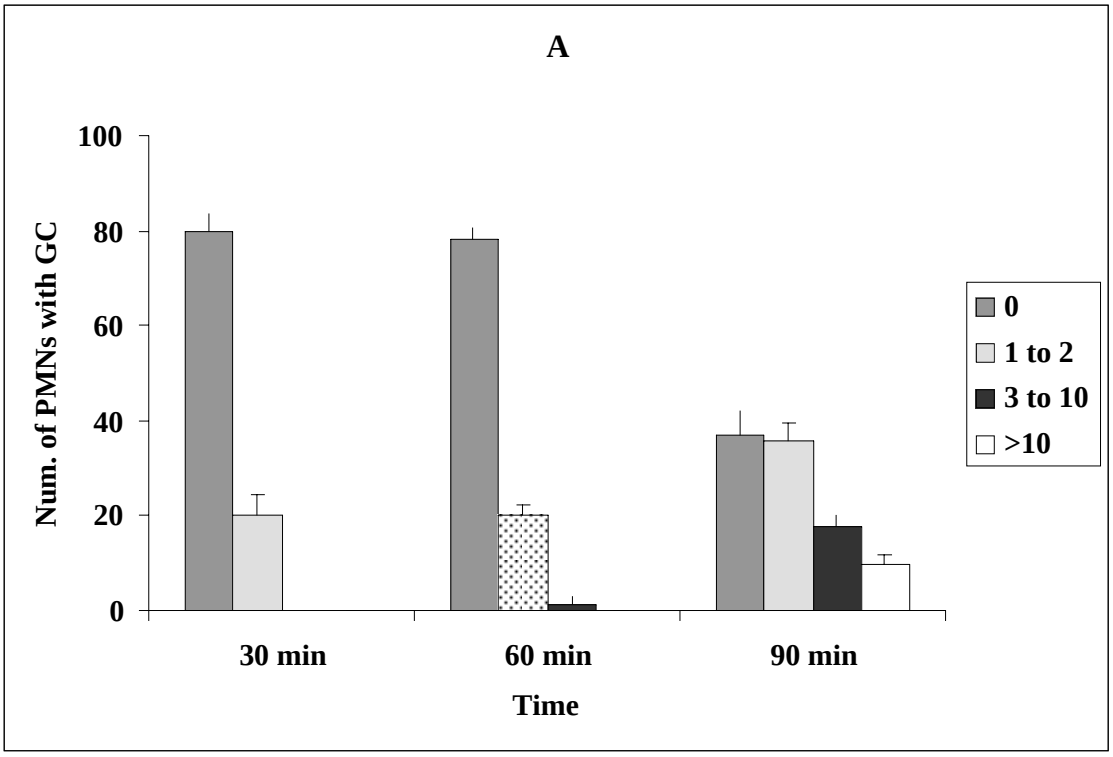

A
8000
6000
4000
2000
0
CPM (1 x 10^3)
0 16 32 44 60 72. 88 104 116 132 144 160 176
Time (min)
EP
EP + PMA


B
80
70
60
50
40
30
20
10
0
CPM (1 x 10^3)
0 16 32 44 60 72. 88 104 116 132 144 160 176
Time (min)
EP
EP + FA1090

untreated mice and in mice treated with estradiol or progesterone. Estradiol rendered the mice more susceptible to *N. gonorrhoeae* infection as evidenced by the development of bacteremia after intraperitoneal inoculation with GC. PMNs obtained from mice treated with estradiol exhibited a four-fold decrease in MPO activity and a diminished killing capacity compared to PMNs from untreated mice. On the other hand, no difference was observed in superoxide generation and bacterial uptake by PMNs obtained from estradiol-treated mice. Bodel et al. (1972), Hulka et al. (1965), Mitchell et al. (1970), and Roth et al., 1982; showed that both progesterone and estrogen depressed leukocyte metabolism resulting in no increase in hexose-monophosphate shunt activity during phagocytosis. These workers also demonstrated that progesterone depressed antibody formation while estradiol increased it.

To investigate the effect of estrogen on PMN function, a series of assays were performed. Although large numbers of gonococci associated with PMNs were observed in vaginal smears from estradiol-treated mice, it was not possible to differentiate between intra- and extracellular bacteria in these stained smears (**Fig. 50**). Therefore, to investigate possible differences in GC adherence to, and uptake by estradiol-treated and untreated PMNs, the acridine orange-trypan blue assay was used as described previously. PMNs from estradiol-treated and untreated mice were exposed to gonococci as described before. As a negative control for GC association, wells with heat-inactivated murine serum were tested in parallel. Estradiol-treatment did not affect the patterns of association of GC with PMNs as compared to untreated PMNs (**Fig. 63 A and 64 A**). Bacterial association increased as a function of time, and heat inactivation of mouse serum reduced

Figure 63. Pattern of association of GC to untreated PMNs as viewed by acridine orange staining. (**A**) NMS and (**B**) heat-inactivated NMS. The experiment was performed three times in triplicate. The number of GC per PMN with associated GC was categorized as 0, 1-2, 3-10 and >10 as shown in the key. Error bars represent the standard deviation of the mean.


A
100
80
60
40
20
0
Num. of PMNs with GC
0
1 to 2
3 to 10
>10
30 min
60 min
90 min
Time

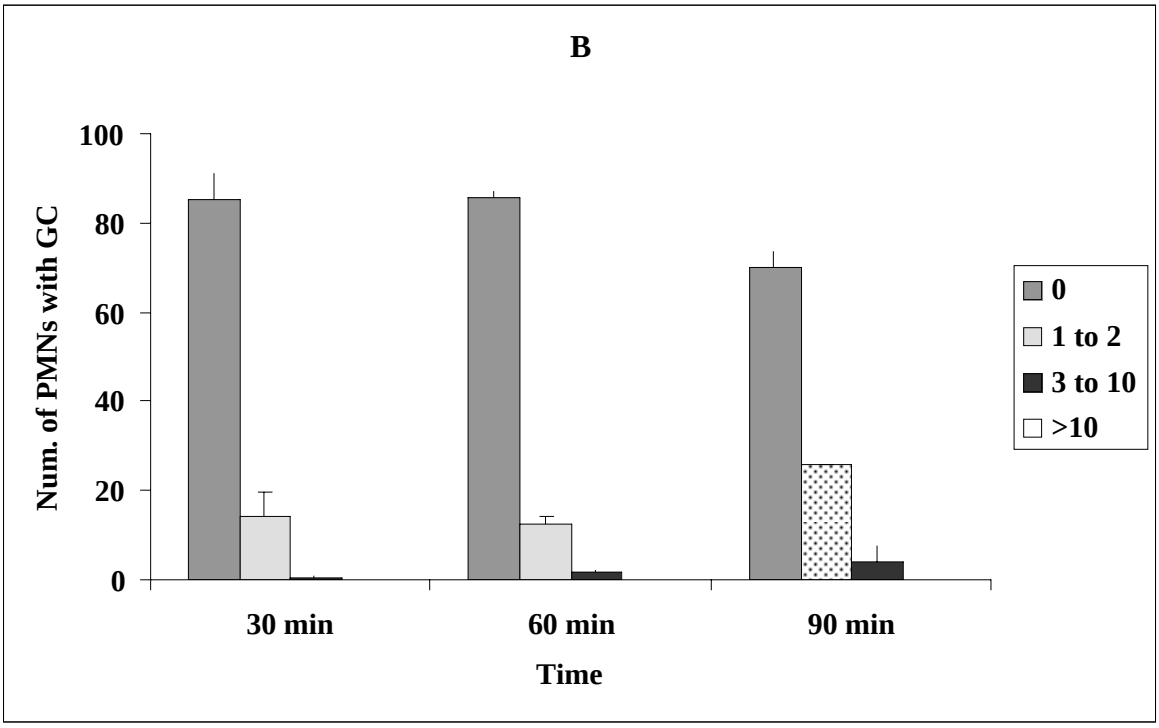
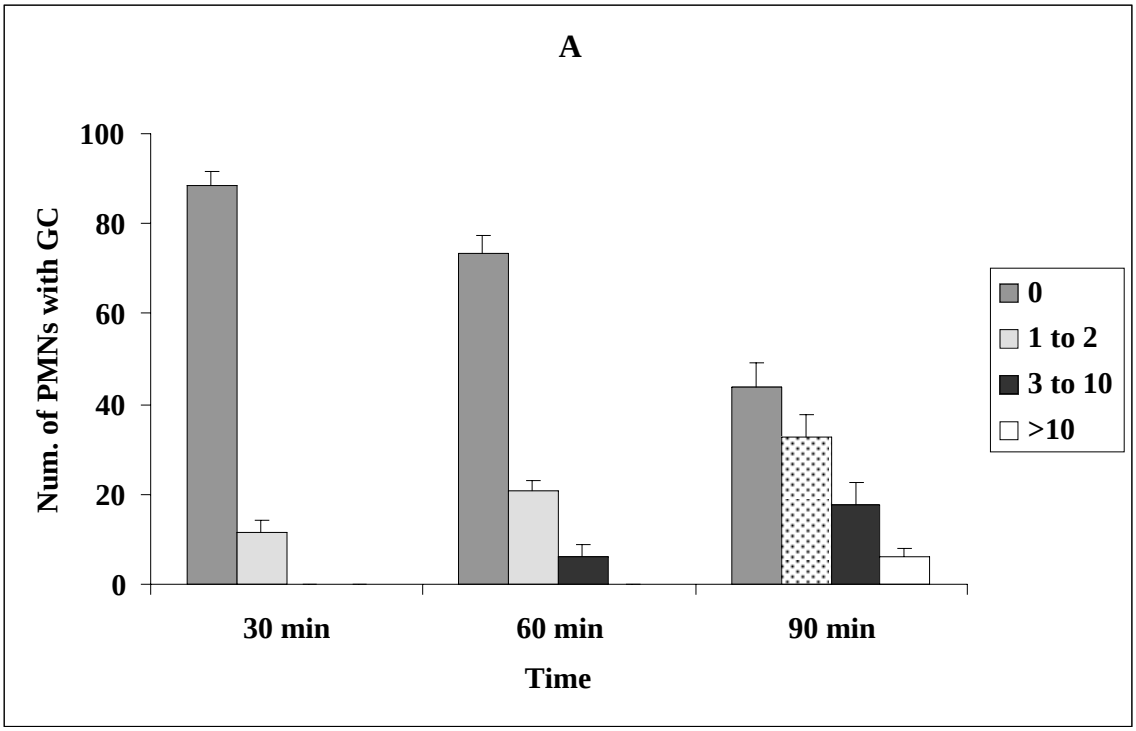
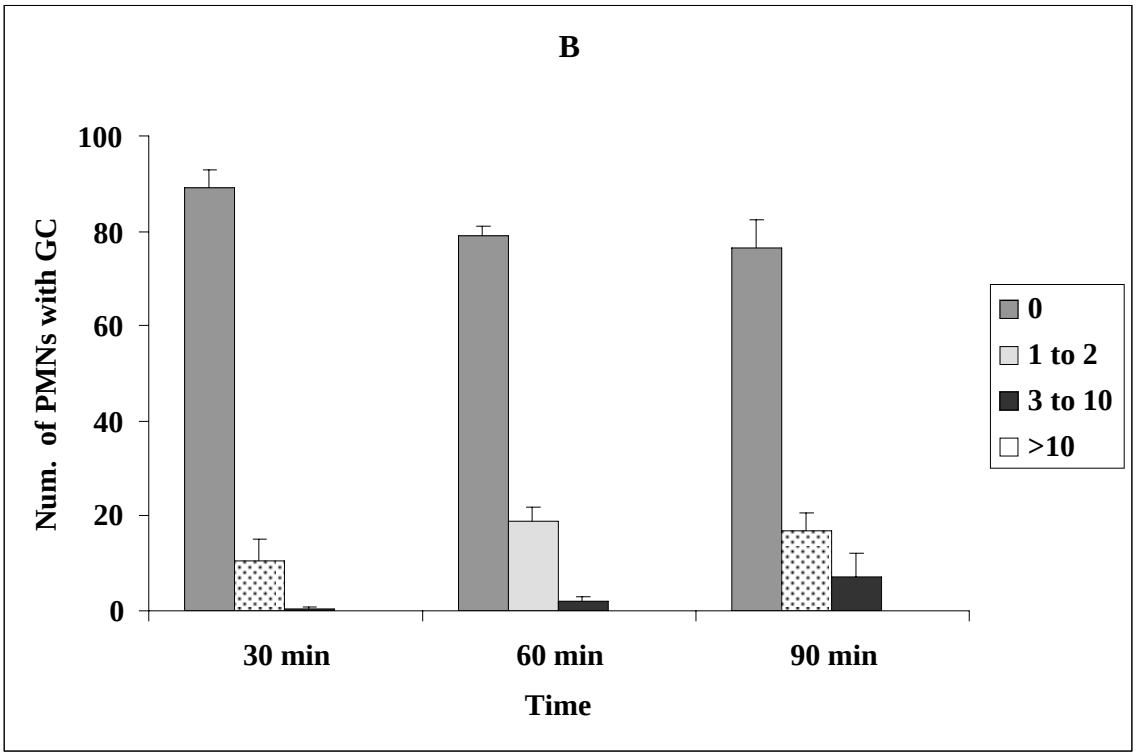

B
100
80
60
40
20
0
Num. of PMNs with GC
0
1 to 2
3 to 10
>10
30 min
60 min
90 min
Time

Figure 64. Pattern of association of GC to estradiol-treated PMNs as viewed by acridine orange staining. (**A**) NMS and (**B**) heat-inactivated NMS. The experiment was performed 3 times in triplicate. The number of GC per PMN with associated GC was categorized as 0, 1-2, 3-10 and >10 as shown in the key. Error bars represent the standard deviation of the mean.


A
100
80
60
40
20
0
Num. of PMNs with GC
0
1 to 2
3 to 10
>10
30 min
60 min
90 min
Time

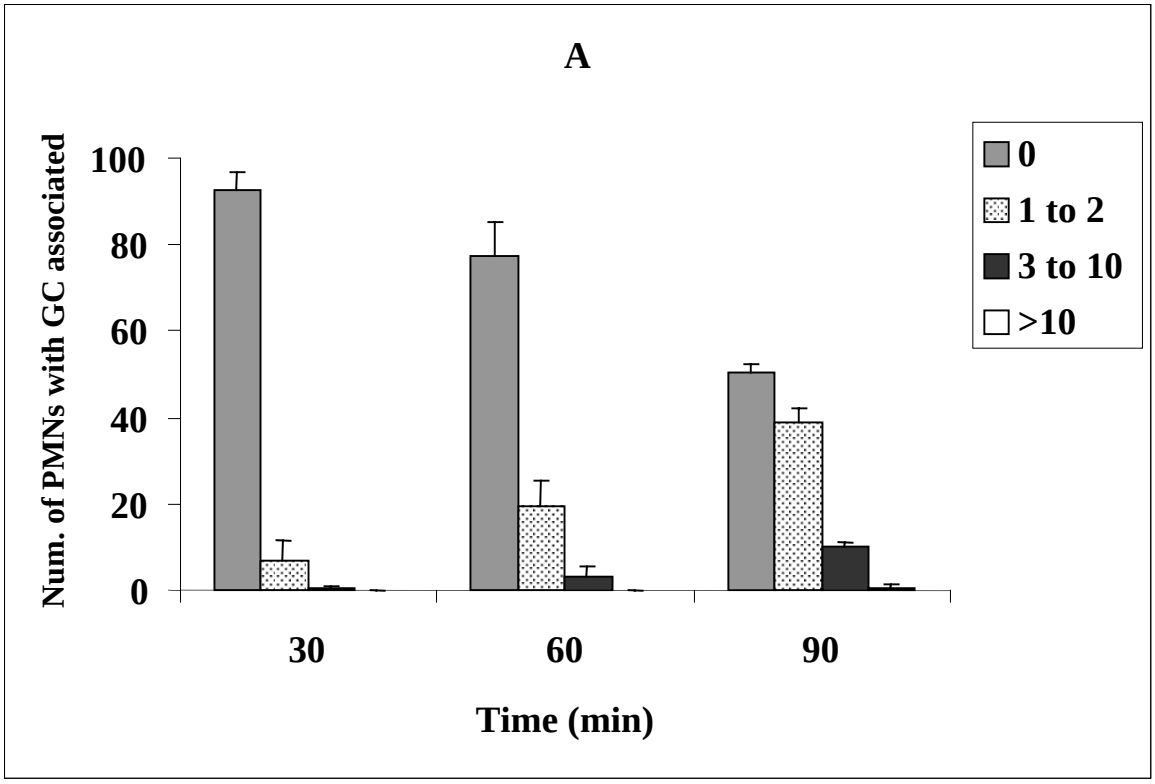

B
100
80
60
40
20
0
Num. of PMNs with GC
0
1 to 2
3 to 10
>10
30 min
60 min
90 min
Time

gonococci association with both types of PMNs (**Fig. 63 B and 64 B**). Estradiol-treated and untreated PMNs exhibited the same pattern of association (**Fig. 65 A and 66 A**) and subsequent internalization (**Fig. 65 B and 66 B**) as determined by trypan blue counterstaining. **Figure 67** shows examples of the categories used to quantitate number of GC associated per PMN and counter-staining with trypan blue.

Kita et al. (1985) reported a decrease in the activity of MPO in PMNs due to estradiol treatment. To explore the effect of estradiol on the capacity of murine PMNs to generate toxic oxygen radicals, a chemiluminescence assay was used initially to measure overall oxidative burst in PMNs from estradiol-treated and untreated mice. This assay utilizes the substrate luminol as a bystander substrate for the oxidative species generated during activation of PMNs. Light emission is dependent initially on superoxide ion and MPO, and later the light emission is totally dependent on the MPO-H_2O_2 system. To measure oxidative burst induced by GC, estradiol-treated and untreated PMNs were exposed to GC at different MOI as described in Materials and Methods. Both types of PMNs showed the same increase in chemiluminescence signal, which increased with increasing MOI (**Fig. 68 A and B**). The chemiluminescence signal in estradiol-treated PMNs were slightly higher compared to that of untreated PMNs. This result suggests that there is no repression in the activity of MPO in estradiol-treated PMNs.

The killing capacity of estradiol-treated PMNs was assessed utilizing *S. aureus* as the target in the PMN killing assay that was standardized as described previously. PMNs and bacteria were mixed together at a ratio of 1:1. The number of viable *S. aureus* was determined after 90 min of incubation. The assay was performed in the presence and

Figure 65. Pattern of association and internalization of GC to untreated PMNs as determined by acridine orange staining and trypan blue counterstaining, respectively. (**A**) Association and (**B**) internalization. Experiment was performed twice in triplicate. The number of GC per PMN with associated GC was categorized as 0, 1-2, 3-10 and >10 as shown in the key. Error bars represent the standard deviation of the mean.


A
Num. of PMNs with GC associated
100
80
60
40
20
0
30
60
90
Time (min)
0
1 to 2
3 to 10
>10

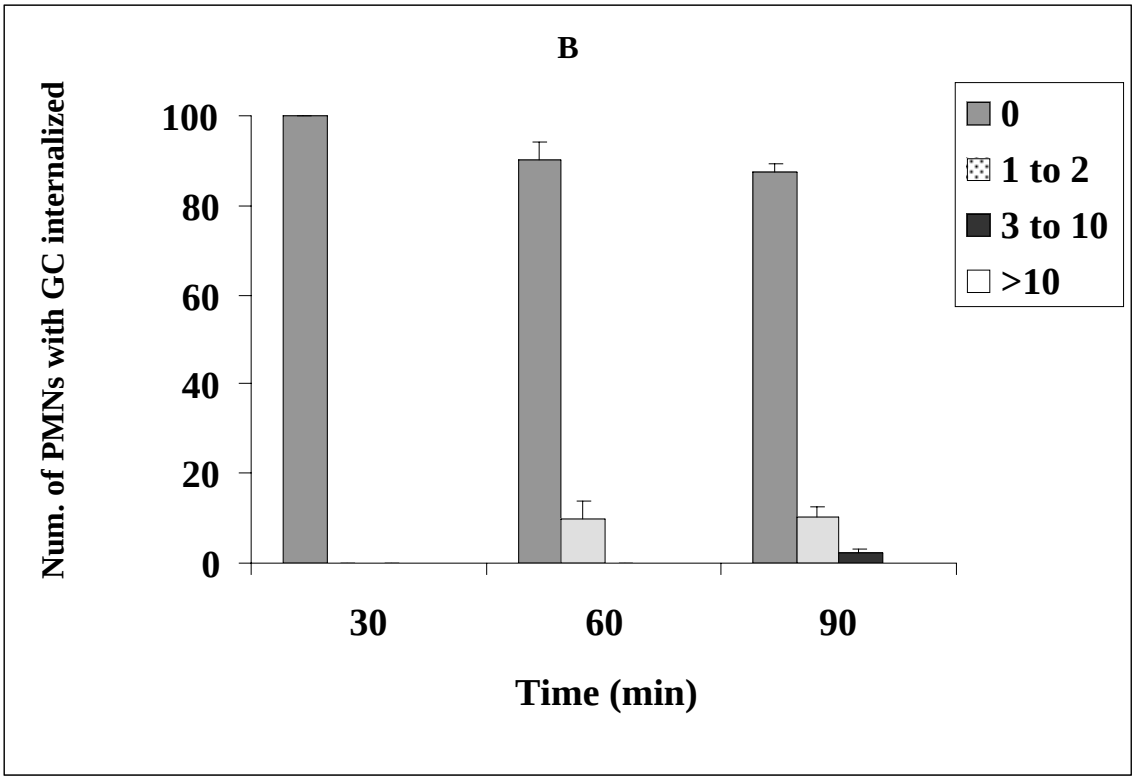
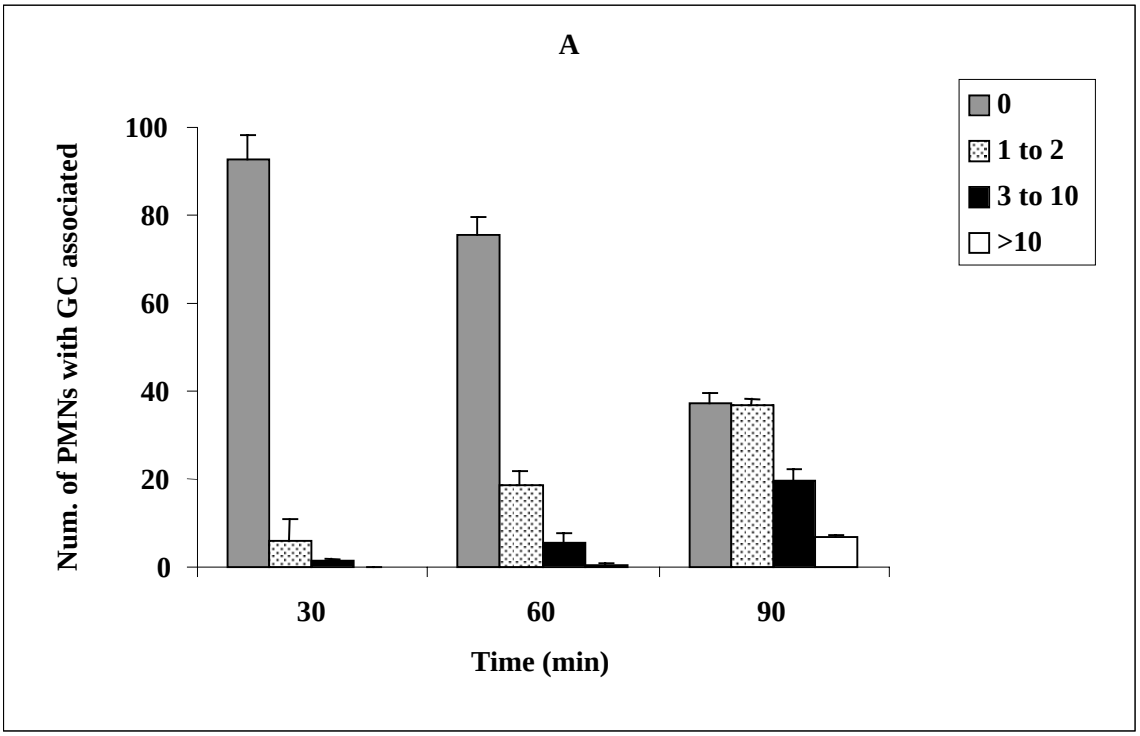
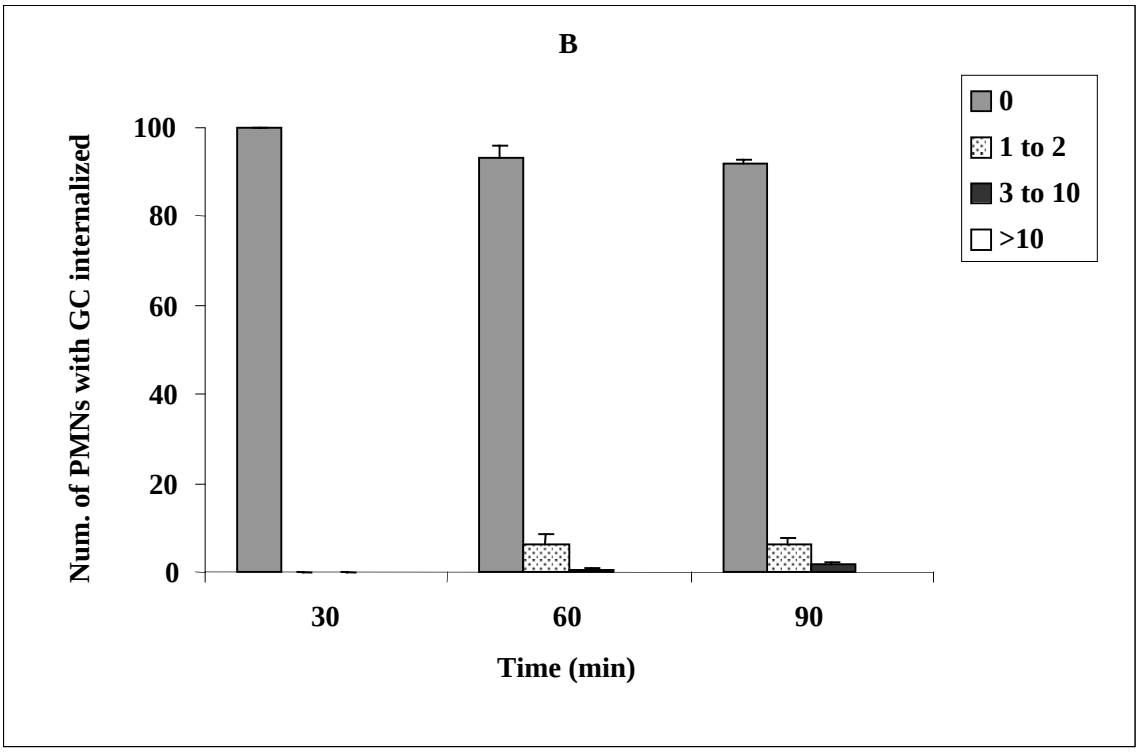

B
Num. of PMNs with GC internalized
100
80
60
40
20
0
30
60
90
Time (min)
0
1 to 2
3 to 10
>10

Figure 66. Pattern of association and internalization of GC to estradiol-treated PMNs as determined by acridine orange staining and trypan blue counterstaining, respectively. (**A**) Association and (**B**) internalization. The experiment was performed twice in triplicate. The number of GC per PMN with associated GC was categorized as 0, 1-2, 3-10 and >10 as shown in the key. Error bars represent the standard deviation of the mean.


A
Num. of PMNs with GC associated
100
80
60
40
20
0
30
60
90
Time (min)
0
1 to 2
3 to 10
>10


B
Num. of PMNs with GC internalized
100
80
60
40
20
0
30
60
90
Time (min)
0
1 to 2
3 to 10
>10

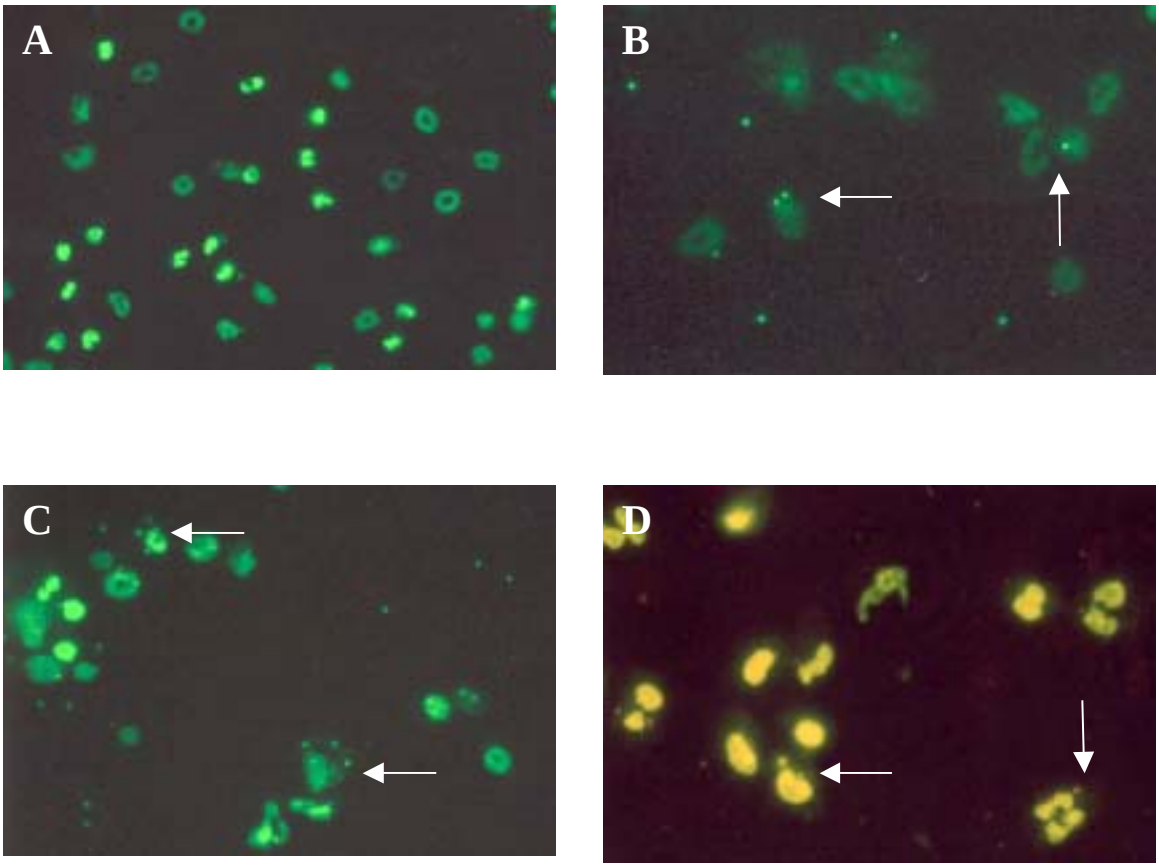
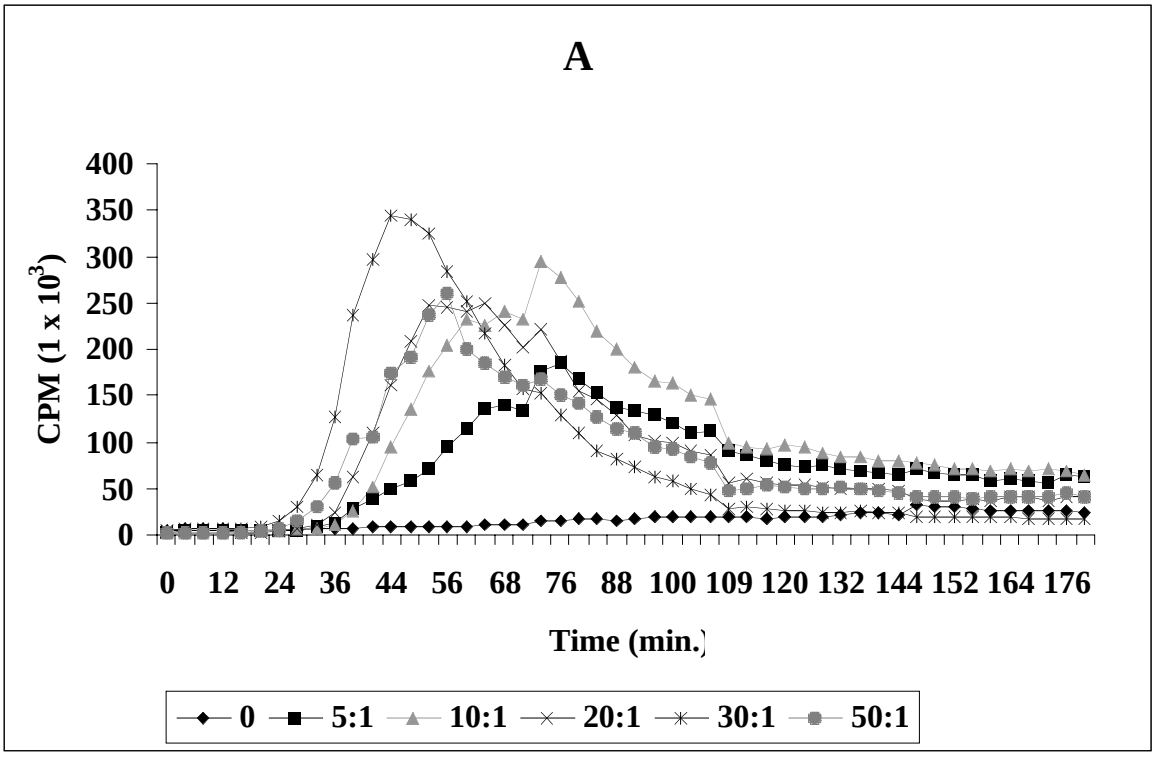
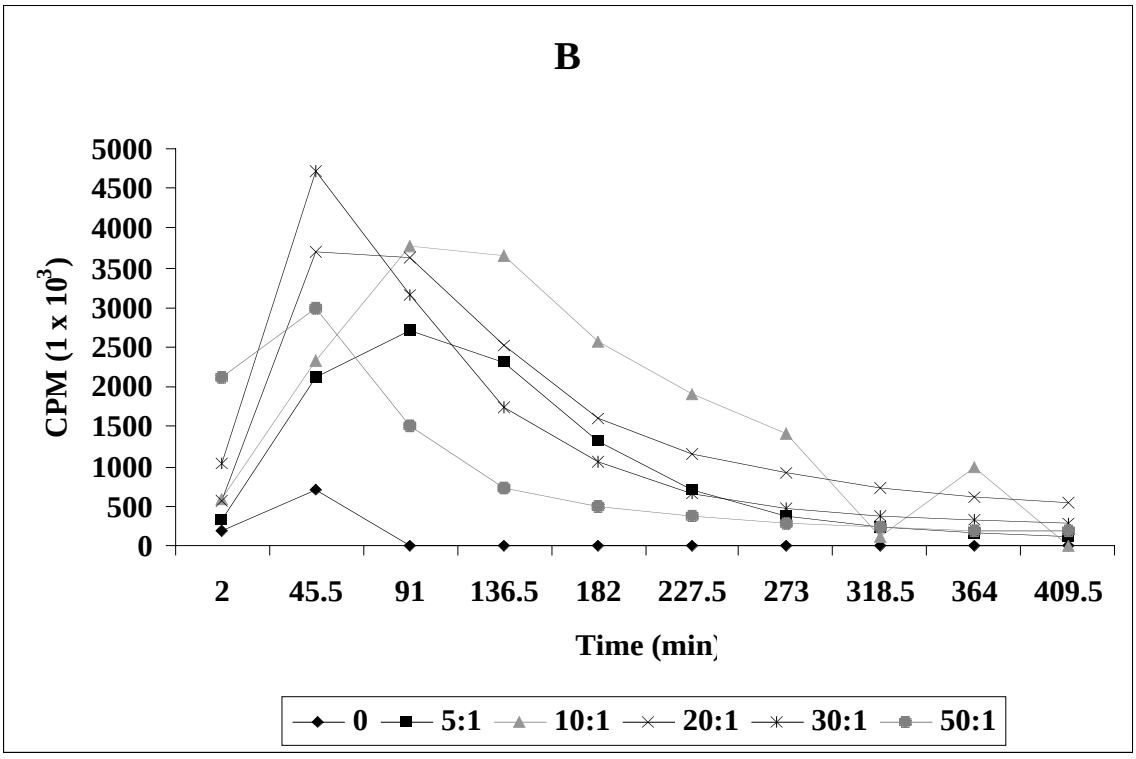


Figure 67. Association of GC to murine PMNs as measured by acridine orange-trypan blue staining. Categories of GC association to murine PMNs. (**A**) PMNs with no GC associated, (**B**) PMNs with 1–2 or (**C**) 3-10 GC associated, and (**D**) trypan blue counterstaining showing intracellular GC. White arrows indicate PMNs with gonococci associated.

Figure 68. GC-induced chemiluminescence signal in untreated (**A**) and estradiol-treated (**B**) PMNs with respect to MOI. Experiment is a representative of 6 replicas.


A
400
350
300
250
200
150
100
50
0
CPM (1 x 10^3)
0 12 24 36 44 56 68 76 88 100 109 120 132 144 152 164 176
Time (min.)
0 5:1 10:1 20:1 30:1 50:1


B
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
CPM (1 x 10^3)
2 45.5 91 136.5 182 227.5 273 318.5 364 409.5
Time (min)
0 5:1 10:1 20:1 30:1 50:1

absence of mouse serum. *S. aureus* was selected as a control due to the accepted use of this bacterium as a standard control in similar assays (Kalmar, 1997). No significant difference was observed in the killing capacity of the estradiol-treated and untreated PMNs (**Table 17**). Glucose consumption and MPO activity in untreated and estradiol-treated PMNs were measured as described in Materials and Methods. No significant difference was observed in glucose consumption induced by PMA or by GC exposure. The same amount of MPO units were produced by untreated and estradiol-treated PMNs upon exposure to FA1090 (**Table 17**).

In summary, measurements of GC association and internalization, chemiluminescence, killing, MPO and glucose consumption suggest no functional difference between untreated and estradiol-treated PMNs. In conclusion, we do not have evidence to support the possibility that the use of estradiol in the mouse model impairs the phagocytic functions of murine PMNs.

D. Interactions of FA1090 and NGAAS-501 with estradiol-treated murine PMNs

The catalase mutant (NGAAS-501) was attenuated compared to the wild-type strain in mixed infection experiments. The *in vitro* growth rate of both strains were the same in single and mixed cultures, suggesting that the observed level of attenuation of the *kat* mutant during mixed infections was due to catalase deficiency. Based on the H_2O_2 susceptibility exhibited by NGAAS-501 to increasing concentrations of H_2O_2, it can be hypothesized that the *kat* mutant is more susceptible to oxygen-dependent killing by phagocytes *in vivo*. To further explore the reason behind the observed attenuation of the

Table 17. Killing capacity, glucose consumption and MPO activity of untreated and estradiol-treated PMNs

Assay[1]	**Conditions**	**PMNs**	
		Untreated	**Estradiol-treated**
Killing (n = 3) [2]	Nonopsonic[3] Opsonic[4]	0 53	0 57
Glucose Consumption (∝M/ml)	PMNs + FA1090 PMNs + PMA	7.9 ± 0.3 18 ± 1.1	9.8 ± 0.8 19.1 ± 0.8
MPO (U/ml)	PMNs + FA1090 PMNs	2.7 ± 0.06 0.4 ± 0	2.4 ± 0 0.2 ± 0.06

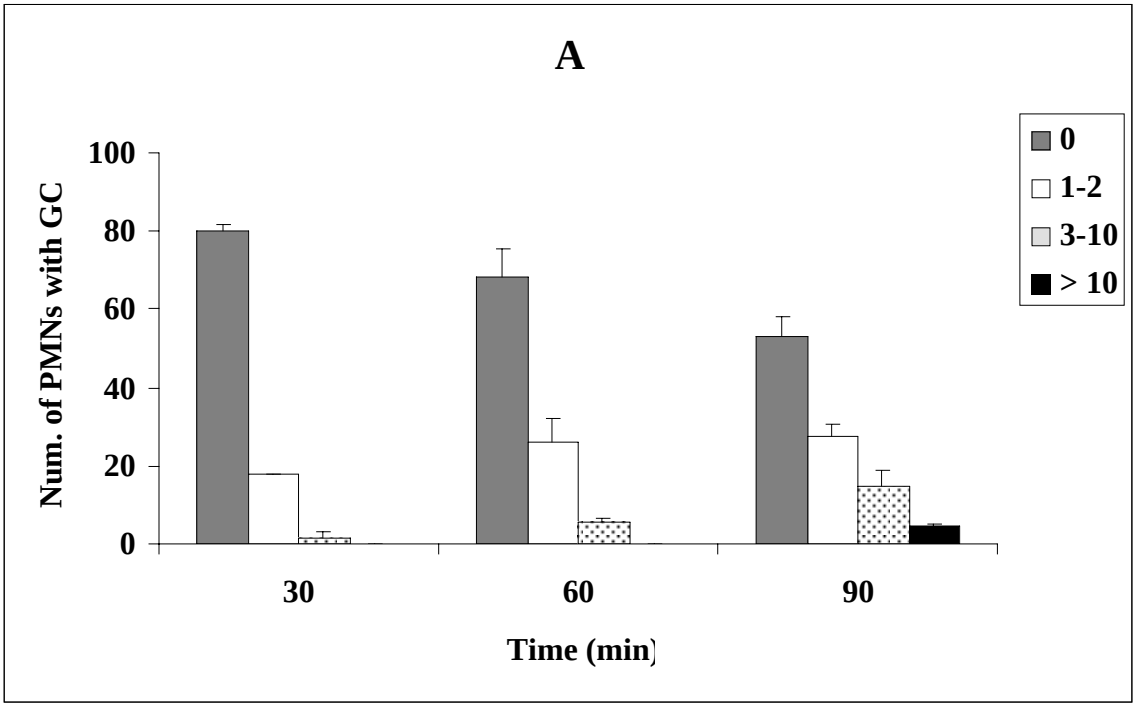
1 All the measurements were taken 90 minutes post-exposure to the stimuli.
2 *S. aureus* was used as the microorganism control in the PMN killing assay.
3 The assay was performed in the absence of serum. Killing was assessed as the percentage of bacteria killed in the presence of PMNs versus bacteria recovered from tubes in the absence of PMNs.
4 The assay was performed in the presence of 10% serum. Bacteria were opsonized for 15 minutes prior to exposure to PMNs. Percentage of killing was determined as the number of bacteria recovered in the PMNs versus that number of bacteria recovered in tubes containing PMNs plus heat-inactivated mouse sera.

kat mutant in mixed infections, the interaction among wild-type and *kat* mutant GC with estradiol-treated PMNs was analyzed. First, the pattern of association and internalization of NGAAS-501 was compared to that of FA1090 utilizing the standardized acridine orange-trypan blue assay. Both strains exhibited the same degree of association (**Fig. 69 A** and **70 A**). Again, as a negative control for association, heat-inactivated ENMS was used (**Fig. 69 B** and **70 B**). Internalization was also measured using longer time points (135 min) with the assumption that internalization of the bacteria would require a longer period of time. As expected, internalization increased as a function of time, but no difference in the internalization of wild-type and *kat* mutant GC was observed at 90 and 135 min (**Fig. 71** and **72**). These data suggest that wild-type and *kat* mutant GC associate with, and are taken up by, mouse PMNs at similar levels.

Killing of the wild-type strain versus the *kat* mutant by estradiol-treated PMNs was measured in the presence of ENMS versus HI-ENMS. Both bacteria were killed by estradiol-treated PMNs. A significant killing of the wild-type FA1090 was observed at 135 min ($p= 0.039$) as compared to the GC recovered at 45 min after exposure to mouse PMNs (**Fig. 73 A**). The *kat* deficient mutant was killed more quickly than wild-type GC in that a larger drop in CFU recovered was first observed at 90 minutes ($p = 0.049$) and continued to 135 min ($p = 0.0008$) subsequently (**Fig. 73 B**).

Additional PMN killing experiments were performed in which cytochalasin B was included. Cytochalasin B is an agent that inhibits phagocytosis and glucose consumption and as a consequence, abrogates the oxidative burst induced by bacteria. It has also been shown that cytochalasin B triggers an increase in degranulation in the PMNs, and therefore its use should allow one to study the effect of neutrophils on wild-type and *kat*

Figure 69. Pattern of association of FA1090 with estradiol-treated PMNs in the presence of (**A**) estradiol-treated normal murine serum (ENMS) and (**B**) heat-inactivated estradiol-treated normal murine serum (HI-ENMS). Legend describes the number of GC associated per PMN. The experiment was performed three times in triplicate. Error bars represent the standard deviation of the mean.


A
Num. of PMNs with GC
100
80
60
40
20
0
30
60
90
Time (min)
0
1-2
3-10
> 10

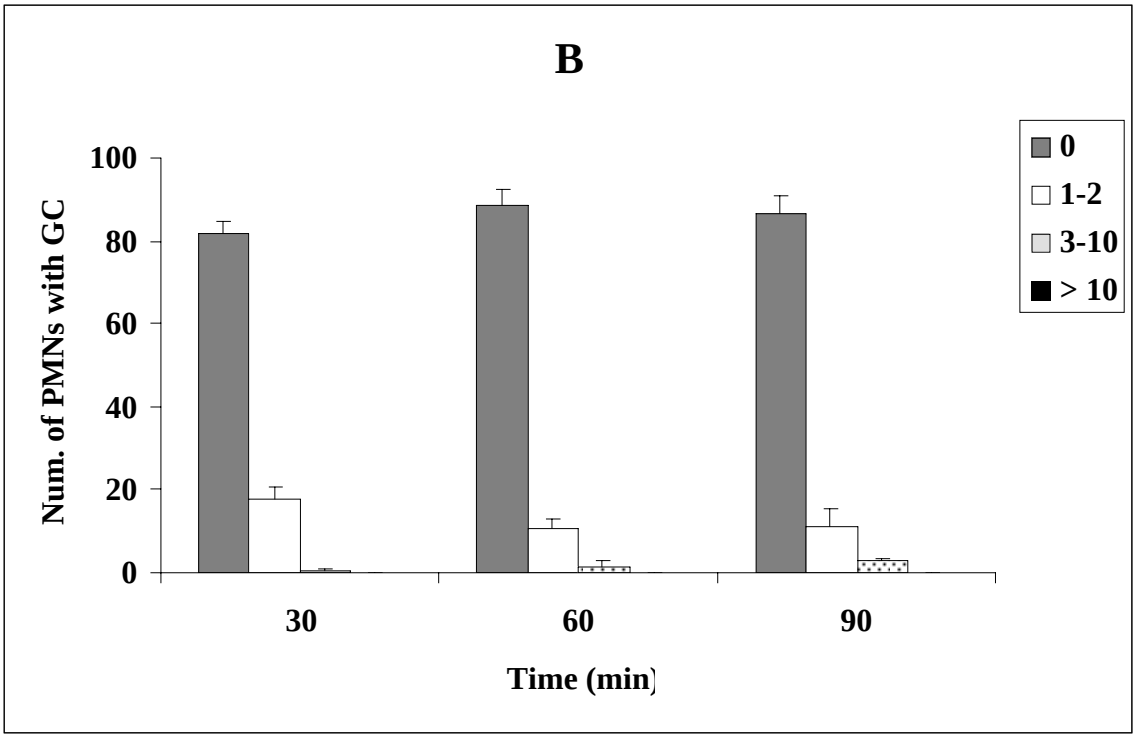
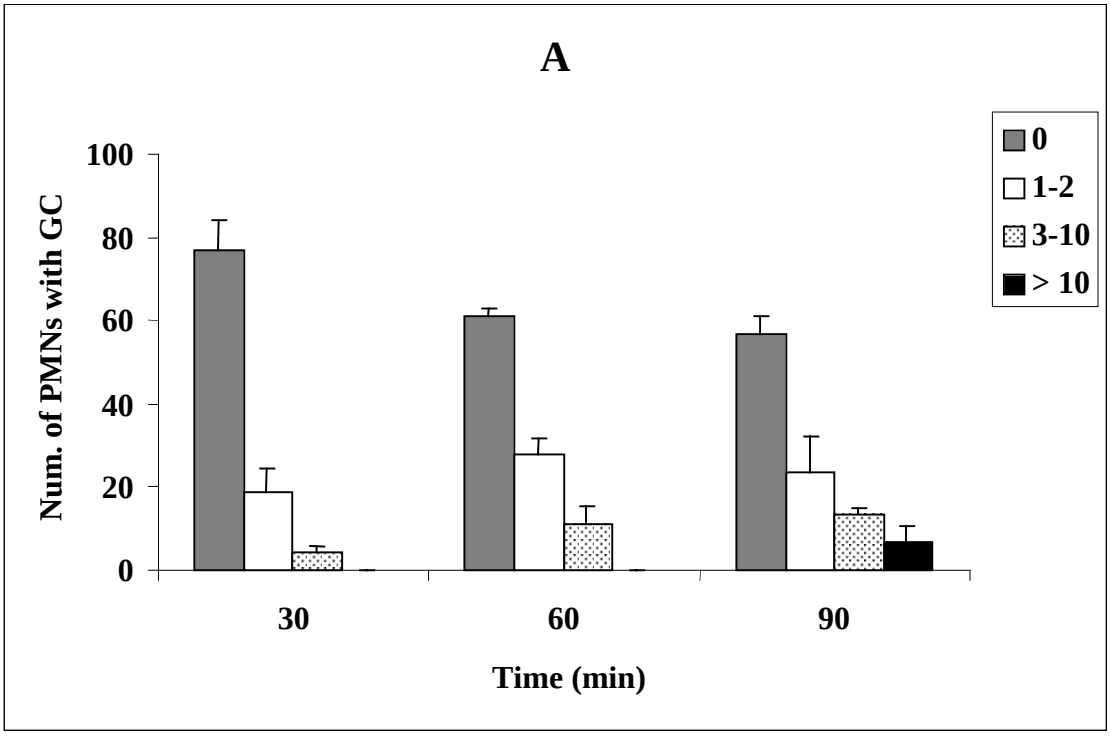

B
Num. of PMNs with GC
100
80
60
40
20
0
30
60
90
Time (min)
0
1-2
3-10
> 10

Figure 70. Pattern of association of NGAAS-501 with estradiol-treated PMNs in the presence of (**A**) estradiol-treated normal murine serum (ENMS) and (**B**) heat-inactivated estradiol-treated normal murine serum (HI-ENMS). Legend describes the number of GC associated per PMN. The experiment was performed three times in triplicate. Error bars represent standard deviation of the mean.


A
100
80
60
40
20
0
Num. of PMNs with GC
0
1-2
3-10
> 10
30
60
90
Time (min)

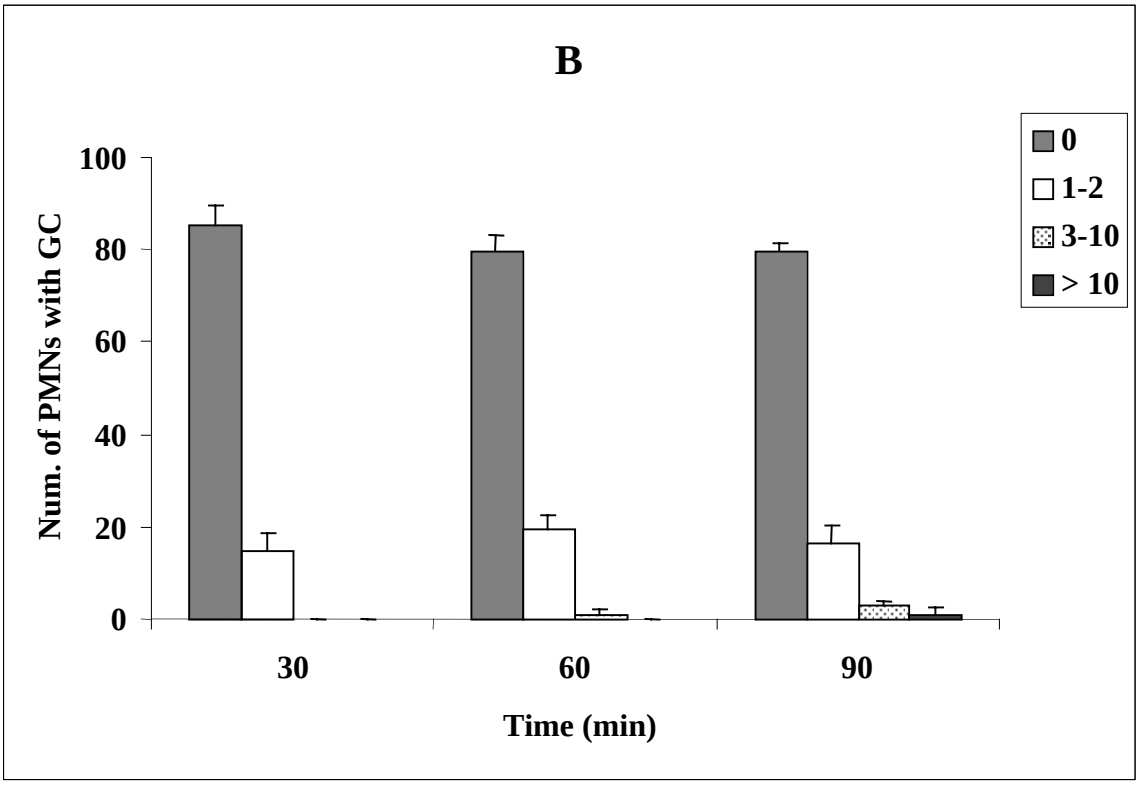
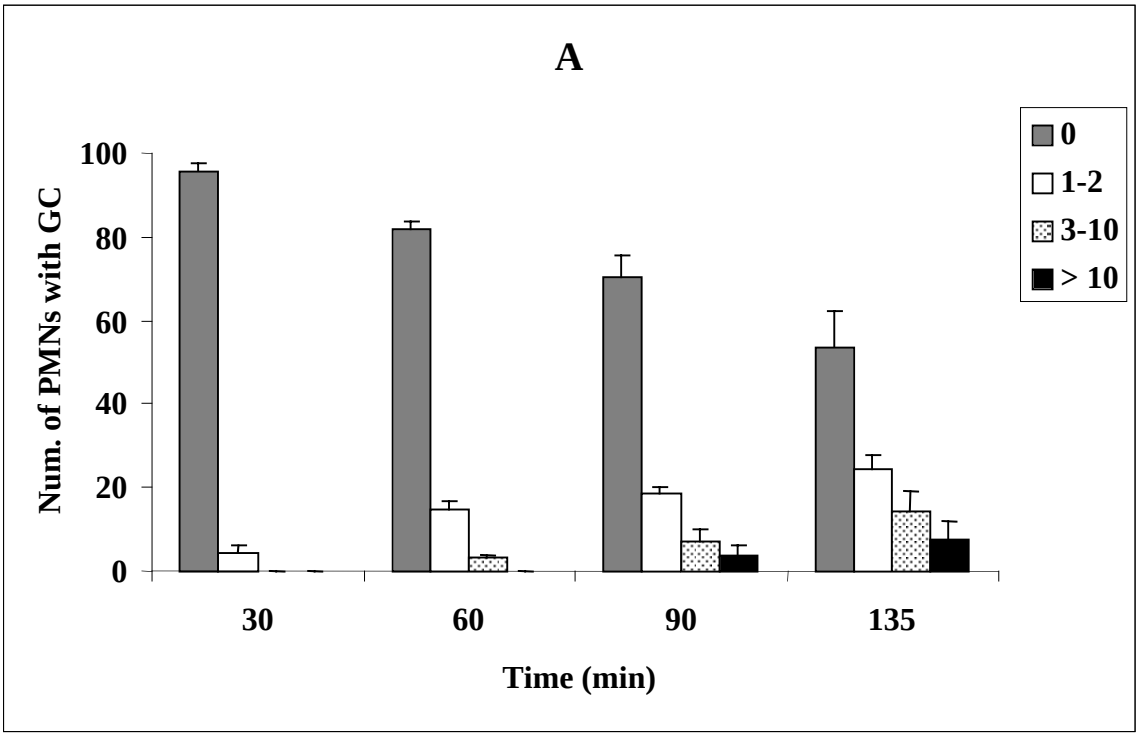

B
100
80
60
40
20
0
Num. of PMNs with GC
0
1-2
3-10
> 10
30
60
90
Time (min)

Figure 71. Pattern of association and internalization of FA1090 to estradiol-treated PMNs. (**A**) Association and (**B**) internalization. Legend describes the number of GC associated per PMN. The experiment was performed three times in triplicate. Error bars represent standard deviation of the mean.


A
Num. of PMNs with GC
100
80
60
40
20
0
30
60
90
135
Time (min)
0
1-2
3-10
> 10

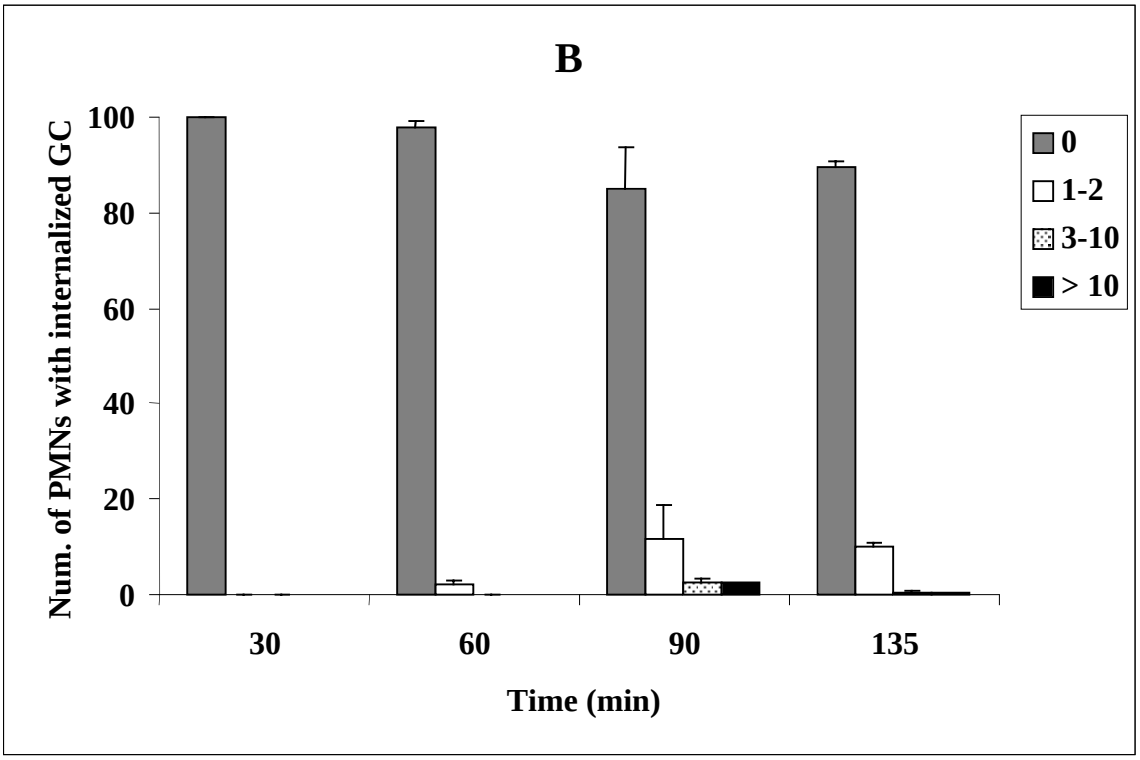
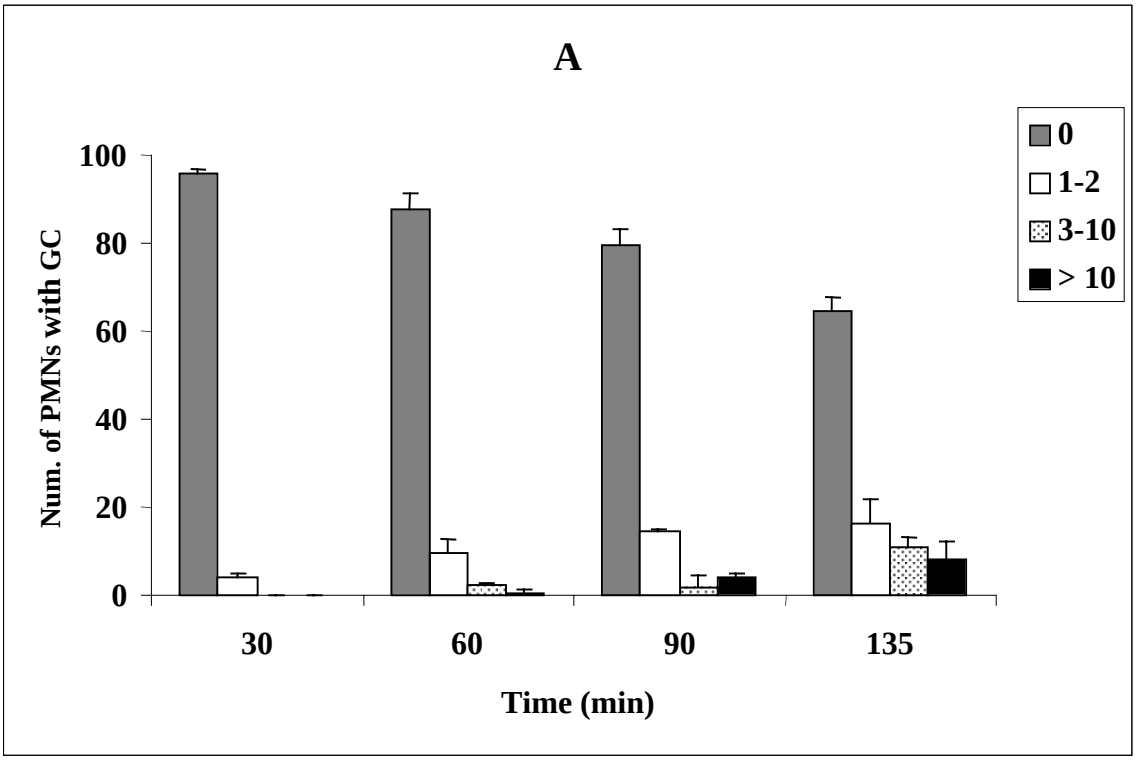

B
Num. of PMNs with internalized GC
100
80
60
40
20
0
30
60
90
135
Time (min)
0
1-2
3-10
> 10

Figure 72. Pattern of association and internalization of NGAAS-501 to estradiol-treated PMNs. (**A**) Association and (**B**) internalization. Legend describes the number of GC associated per PMN. The experiment was performed three times in triplicate. Error bars represent standard deviation of the mean.


A
Num. of PMNs with GC
100
80
60
40
20
0
30
60
90
135
Time (min)
0
1-2
3-10
> 10

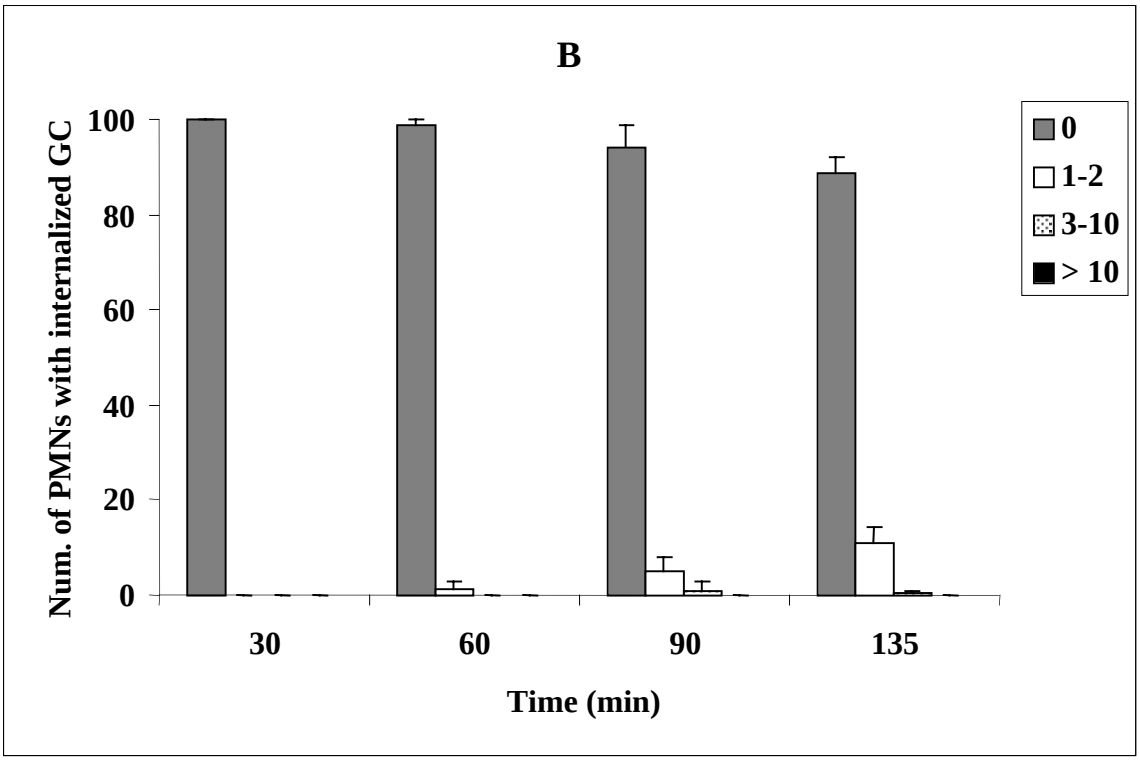
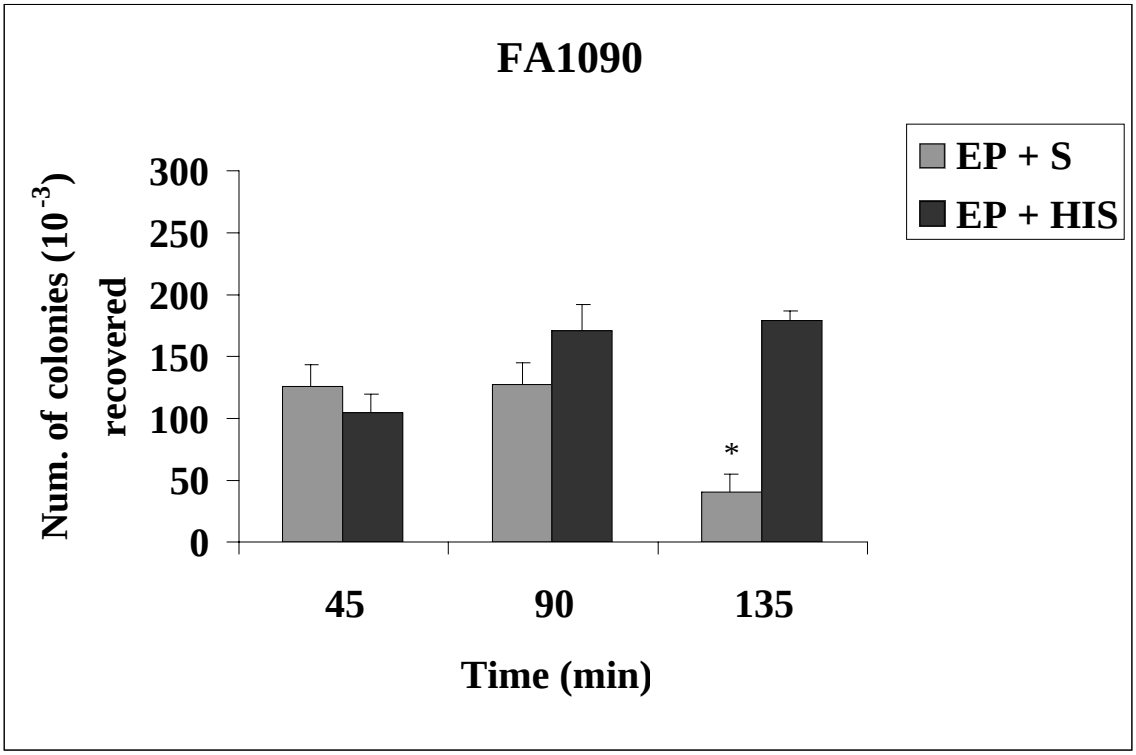
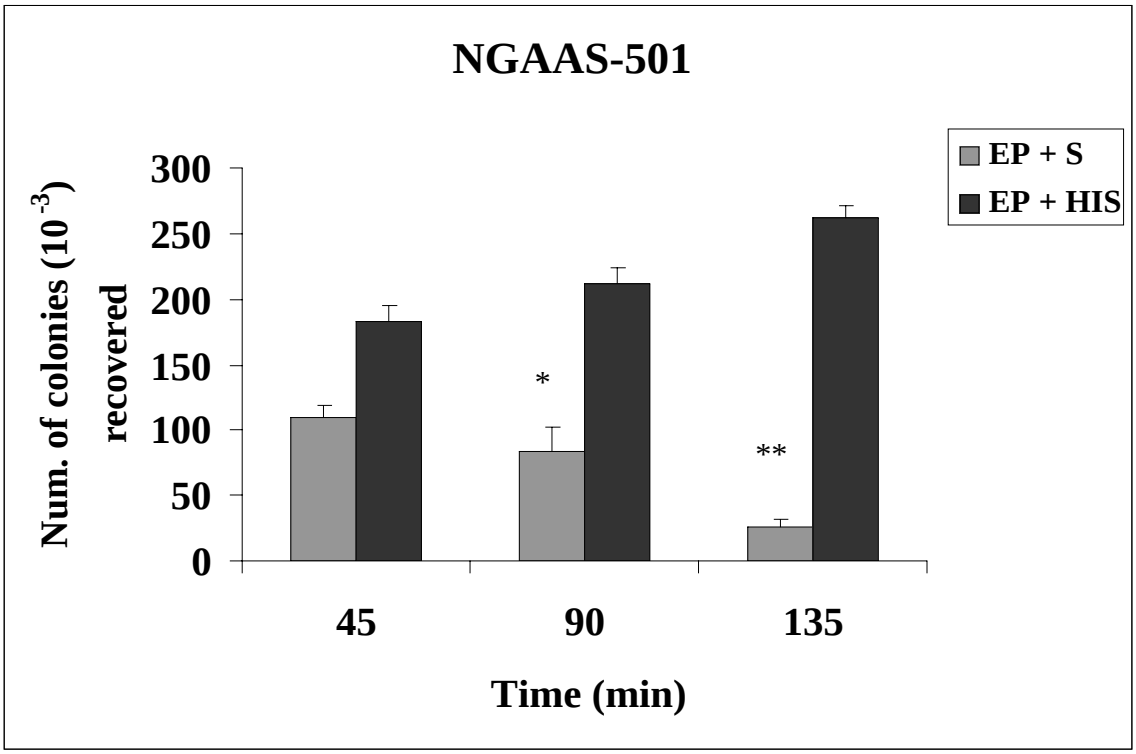

B
Num. of PMNs with internalized GC
100
80
60
40
20
0
30
60
90
135
Time (min)
0
1-2
3-10
> 10

Figure 73. Number of viable FA1090 (**A**) or NGAAS-501 (**B**) after exposure to estradiol-treated PMNs. **EP + S**, Estradiol-treated PMNs (EP) in the presence of 10% serum, and **EP + HIS** corresponds to estradiol-treated PMNs in the presence of 10% heat-inactivated mouse serum. The experiment was performed at least three times in triplicate. Error bars correspond to standard deviation of the mean. (*) $p < 0.05$ and (**) $p < 0.005$.


FA1090
EP + S
EP + HIS
Num. of colonies (10^{-3}) recovered
300
250
200
150
100
50
0
*
45
90
135
Time (min)

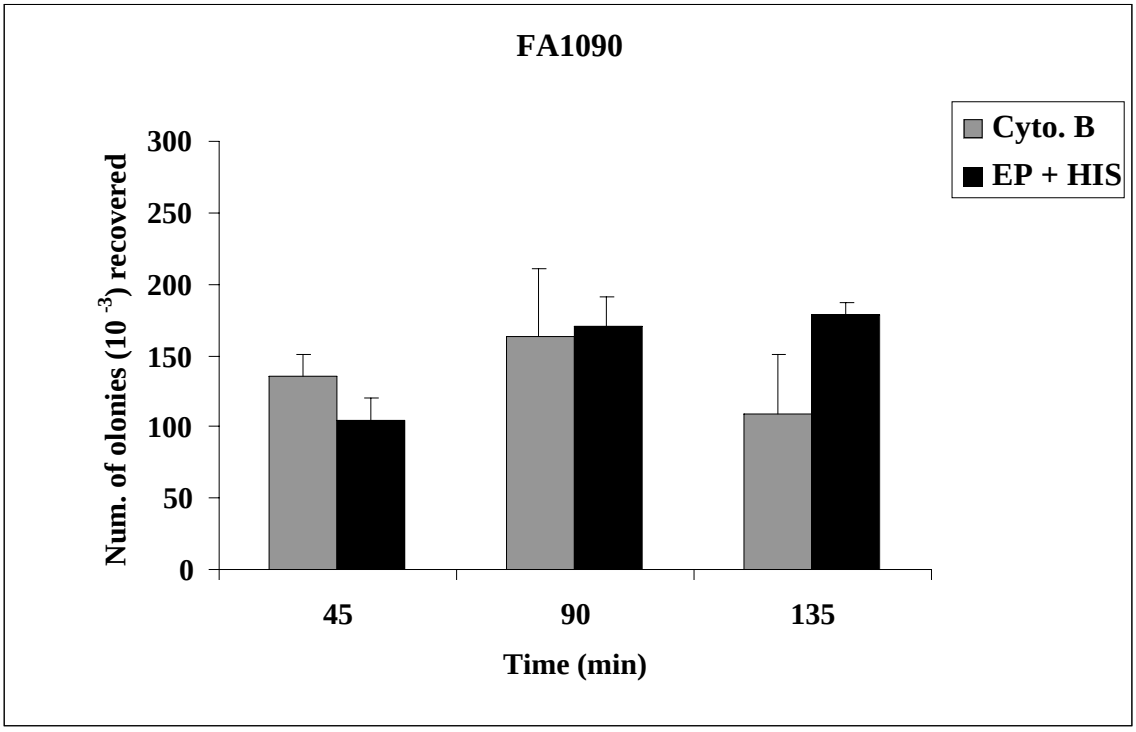

NGAAS-501
EP + S
EP + HIS
Num. of colonies (10^{-3}) recovered
300
250
200
150
100
50
0
*
**
45
90
135
Time (min)

mutant GC. No significant difference was observed in the viable FA1090 population recovered at any time point tested in the presence of cytochalasin B. In contrast the kat mutant exhibited a constant decline at each time point as compared to wild-type GC when kat mutant bacteria recovered was compared to the HI-ENMS (**Fig 74 A** and **B**). This suggests that *kat* deficiency renders gonococci more sensitive to O_2-independent killing. of cytochalasin B, the *kat* mutant exhibited a sharper decline as compared to the wild-type strain (**Fig. 74 A** and **B**).

PMN killing assays using a 1:1 mixture of FA1090 and NGAAS-501 were consistent with the results using single strains (**Fig. 75 A**). At 135 min a significant decline in the total GC population was observed ($p = 0.0054$). The distribution of the wild-type and *kat* mutant in the recovered total populations was determined by bubbling assay. Both strains were recovered at similar percentages at 45 and 90 min (**Fig 75 B** and **C**). At 135 min, a greater decline in the number of NGAAS-501 CFUs ($p = 0.00406$) occurred compared to that of FA1090 ($p = 0.034$).

In summary, the decline of the *kat* mutant within the total population of GC during single and mixed PMN killing experiments, suggests that the lack of catalase renders GC more susceptible to the antimicrobial mechanisms utilized by PMNs in inflammatory foci. These data supports the hypothesis that catalase may be one of the mechanisms used by gonococci to survive in the presence of PMNs as has been postulated before (Zheng et al., 1992). Alternatively the presence of FA1090 together with NGAAS-501 may generate an environment in which the catalase deficient strain is unable to survive.

Figure 74. Number of viable FA1090 (**A**) or NGAAS-501 (**B**) in the presence of cytochalasin B after exposure to estradiol-treated PMNs. **Cyto. B,** estradiol-treated PMNs in the presence of 10% serum and cytochalasin B; and **EP + HIS** correspond to estradiol-treated PMNs in the presence of 10% heat-inactivated mouse serum. Experiment was performed three times in triplicate. Error bars represent the standard deviation of the mean. (*) $p < 0.05$ and (**) $p = 0.005$ as compared to EP + HIS.


FA1090
Cyto. B
EP + HIS
Num. of olonies (10 -3) recovered
300
250
200
150
100
50
0
45
90
135
Time (min)

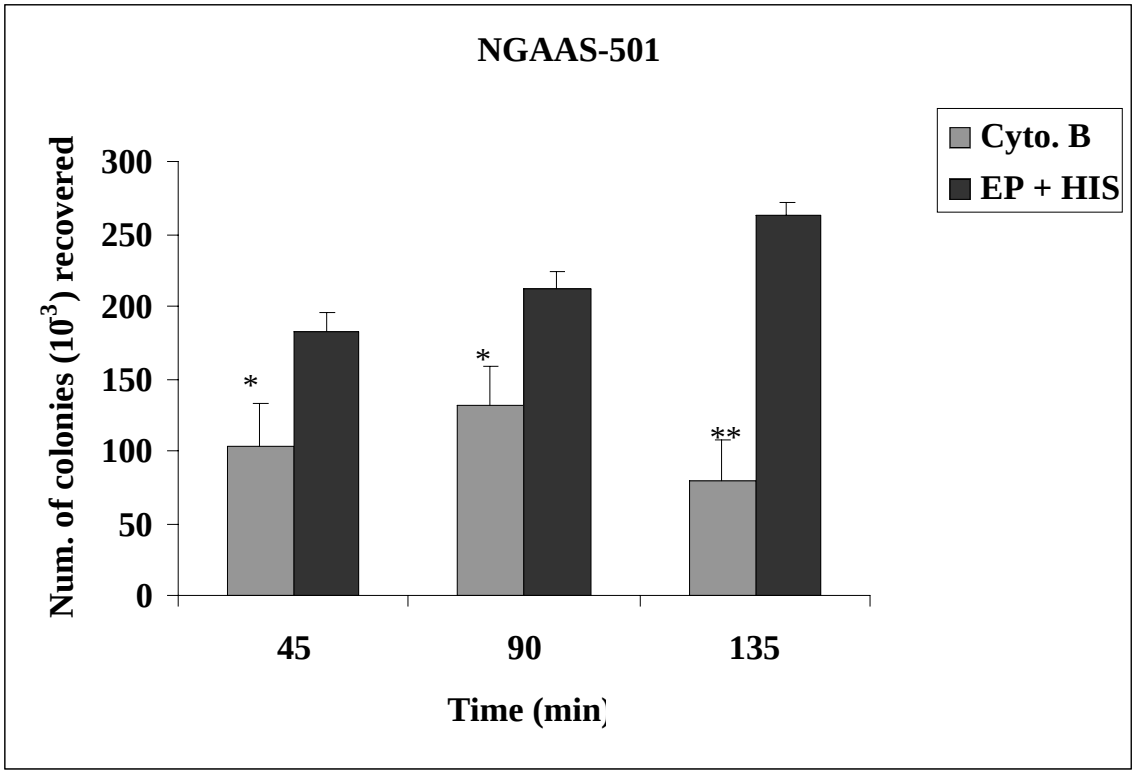
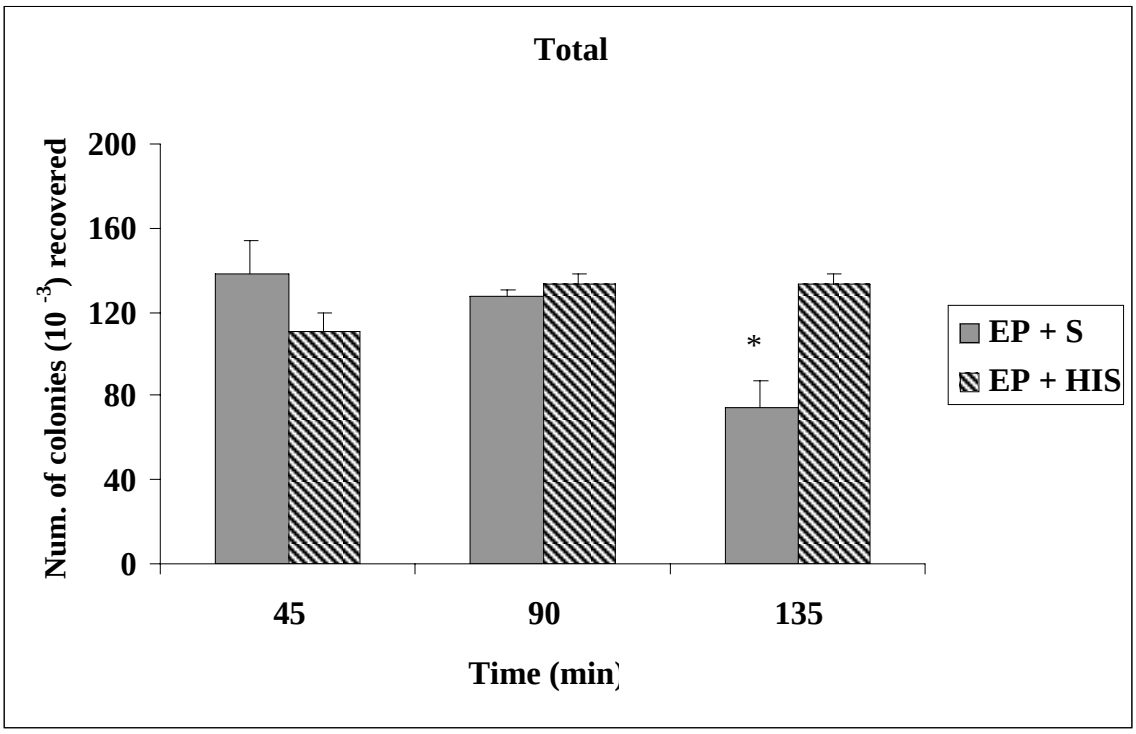
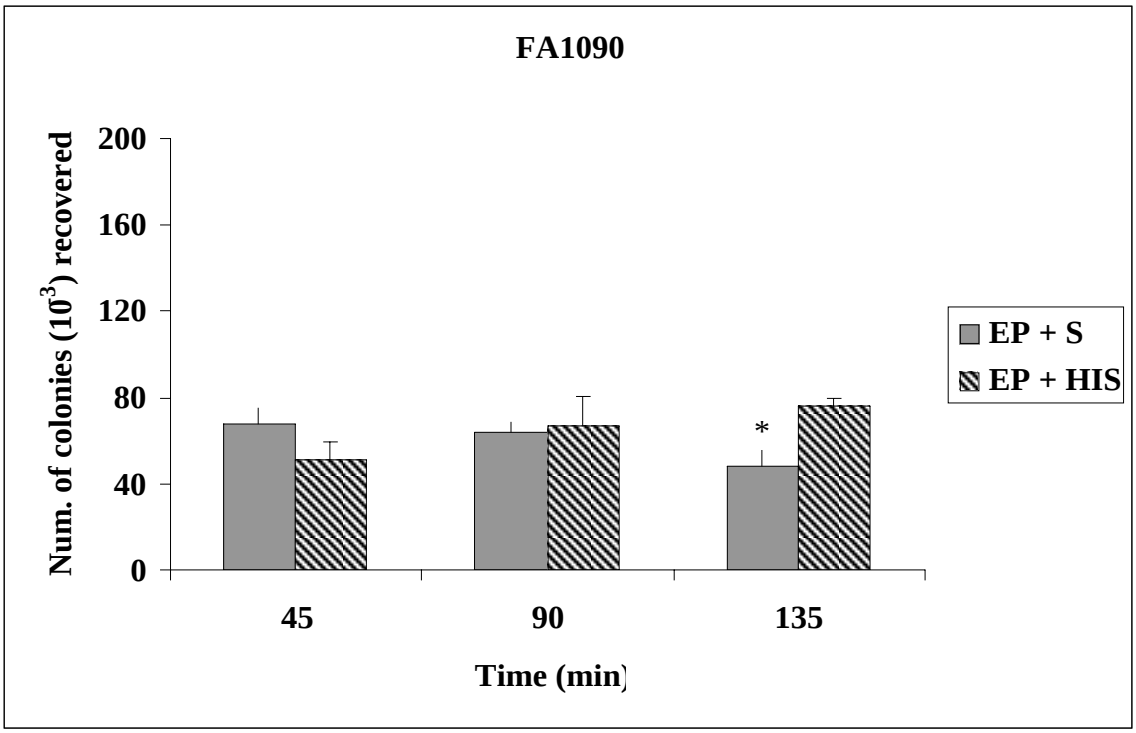
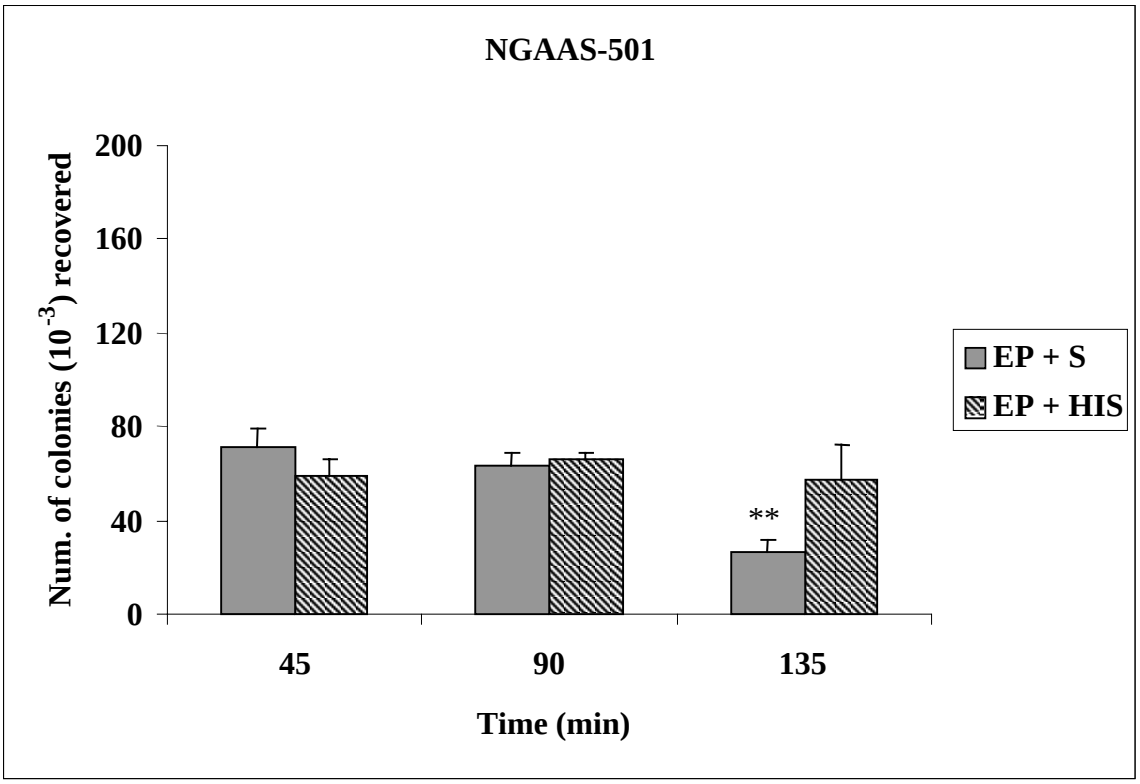

NGAAS-501
Cyto. B
EP + HIS
Num. of colonies (10^3) recovered
300
250
200
150
100
50
0
*
*
**
45
90
135
Time (min)

Figure 75. Number of viable bacteria recovered after exposure of 1:1 mixture of FA1090 and NGAAS-501 to estradiol-treated mouse PMNs. (**A**) Total killing, (**B**) FA1090 and (**C**) NGASS-501. **EP + S**, estradiol-treated PMNs in the presence of 10% serum, and **EP + HIS** corresponds to estradiol-treated PMNs in the presence of 10% heat-inactivated mouse serum. The experiment was performed three times in triplicate. Error bars represent standard deviation of the mean. (*) $p < 0.05$ and (**) $p < 0.005$.


Total
Num. of colonies (10 -3) recovered
200
160
120
80
40
0
*
45
90
135
Time (min)
EP + S
EP + HIS

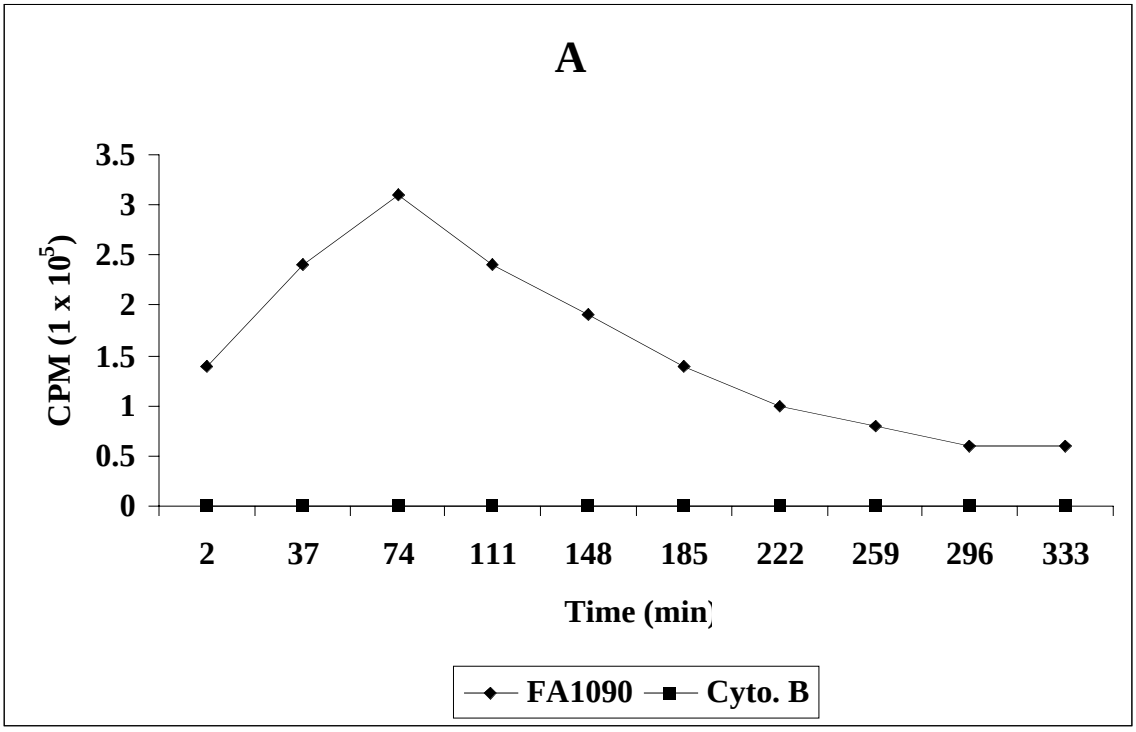

FA1090
Num. of colonies (10^3) recovered
200
160
120
80
40
0
*
45
90
135
Time (min)
EP + S
EP + HIS

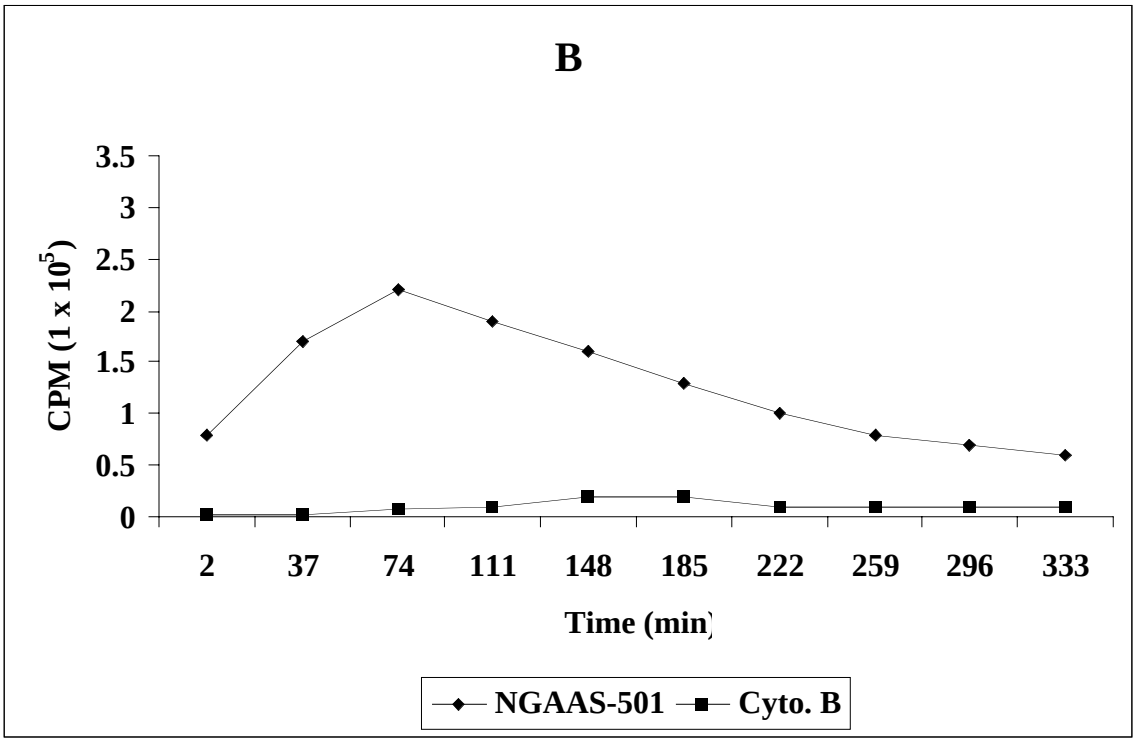

NGAAS-501
Num. of colonies (10^{-3}) recovered
200
160
120
80
40
0
45
90
135
Time (min)
**
EP + S
EP + HIS

To elucidate the response of estradiol-treated PMNs upon exposure to FA1090 and NGAAS-501, the chemiluminescence assay was used. Both GC strains induced a chemiluminescence signal (**Fig. 76 A** and **B**). To confirm that the chemiluminescence obtained was due to exposure to the bacteria, cytochalasin B or D was added to abrogate bacterial induced oxidative burst. As expected chemiluminescence was totally abrogated in the presence of cytochalasin B (**Fig. 76 A** and **B**; and **77**). Cytochalasin D inhibits phagocytosis without inhibiting glucose consumption. The chemiluminescence signal in the presence of cytochalasin D was inhibited 52 and 25% in PMNs exposed to FA1090 and NGAAS-501, respectively. This finding suggests that internalization of gonococci is not an essential requirement for the induction of PMN oxidative burst (**Fig. 77**). This observation combined with the patterns of adherence (**Fig. 71 A** and **72 A**) and internalization (**Fig. 71 B** and **72 B**) observed indicate that GC association is sufficient for the induction of oxidative burst in murine PMNs. Comparison of the chemiluminescence signal induced by FA1090 and NGAAS501 in estradiol-treated PMNs was performed as before. As shown in **Fig.78**, FA1090 induces a relatively higher signal compared to NGAAS-501, suggesting a stronger oxidative burst environment is induced in the presence of FA1090.

Induction of oxidative burst can be predominantly intra- or extracellular. To distinguish between intra- or extracellular oxidative burst induced by FA1090 and NGAAS-501, the chemiluminescence assay was utilized. To measure extracellular chemiluminescence**,** 4 U of HRP and 1mM sodium azide were added to estradiol-treated PMNs in the presence of either wild-type or *kat* mutant. In contrast, intracellular

Figure 76. Effect of FA1090 (**A**) and NGAAS-501 (**B**) on the induction of chemiluminescence in the presence and absence of cytochalasin B by estradiol-treated PMNs.


A
3.5
3
2.5
2
1.5
1
0.5
0
CPM (1 x 10^5)
2
37
74
111
148
185
222
259
296
333
Time (min)
FA1090
Cyto. B


B
3.5
3
2.5
2
1.5
1
0.5
0
CPM (1 x 10^5)
2
37
74
111
148
185
222
259
296
333
Time (min)
NGAAS-501
Cyto. B

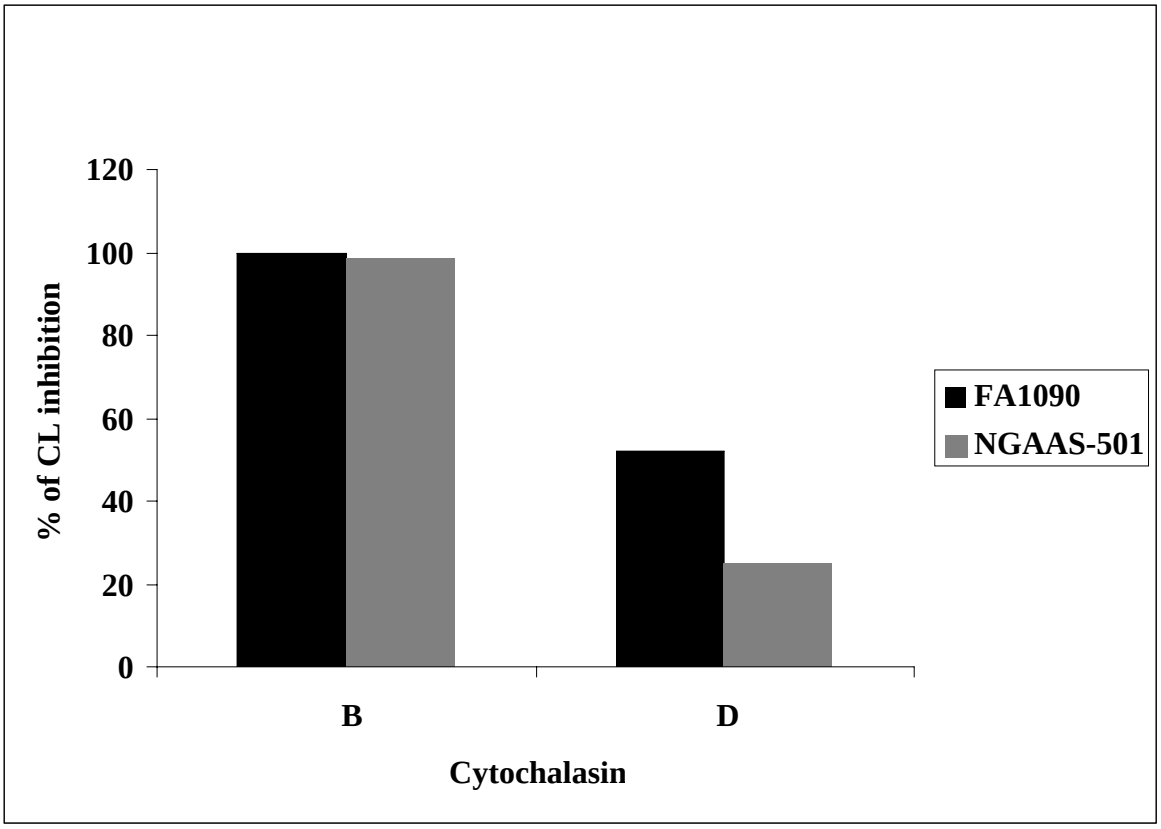


Figure 77. Effect of cytochalasin B and D in the chemiluminescence induced by FA1090 and NGAAS-501 on estradiol-treated PMNs. **CL**, chemiluminescence. Bars represent an average of six replicas.

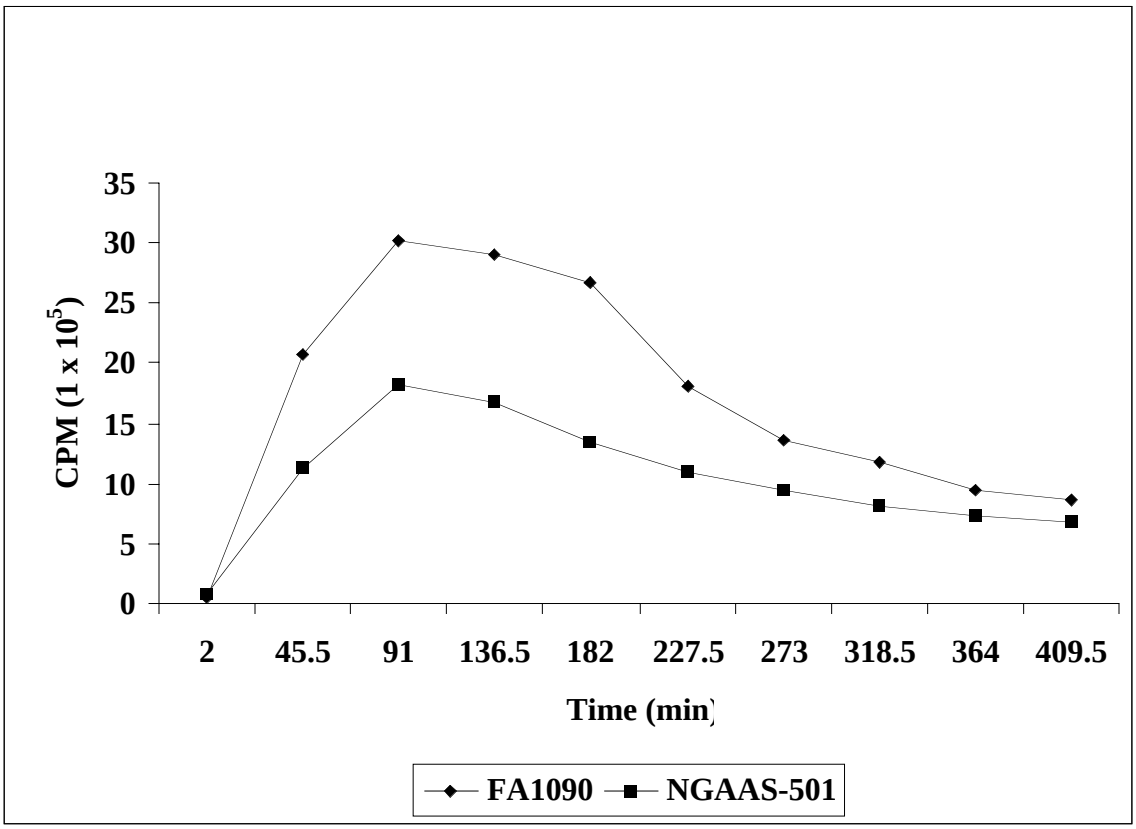


Figure 78. Comparison of chemiluminescence induced by FA1090 and NGAAS-501 in estradiol-treated PMNs. Representative of six experiments in triplicate.

oxidative burst was measured by the addition of 2,000 U of catalase to the scintillation vial.

The effect of the HRP and catalase was expressed as the percent of chemiluminescence inhibition as compared to chemiluminescence vial without any additive. As shown in **Fig. 79**, catalase inhibited 57 and 30 % of chemiluminescence signal induced by FA1090 and NGAAS-501, respectively. No inhibition was observed in the presence of HRP and sodium azide suggesting the oxidative burst induced by both GC strains is partially intracellular. To verify that the lack of inhibition in the presence of HRP and sodium azide was due to induction of oxidative burst, cytochalasin B was added to the assay. As expected, the oxidative burst was abrogated by 99 and 95 % for FA1090 and NGAAS-501, respectively.

To elucidate possible differences in the induction of toxic oxygen radicals and/or components of the oxidative burst by FA1090 and NGAAS-501, superoxide ion, H_2O_2 and MPO activity were measured. Superoxide ion production by estradiol-treated PMNs exposed to FA1090 or NGAAS-501 was measured by two assays, cytochrome C and NBT reduction. As shown in **Fig. 80** and **Table 18**, no significant difference was observed in the amount of superoxide induced by either strain. The same results were obtained for the induction of H_2O_2 and MPO activity, suggesting no major difference in the induction of toxic oxygen radicals by FA1090 and NGAAS-501. These data are in conflict with the findings in the chemiluminescence assay where an apparent higher signal was induced by FA1090.

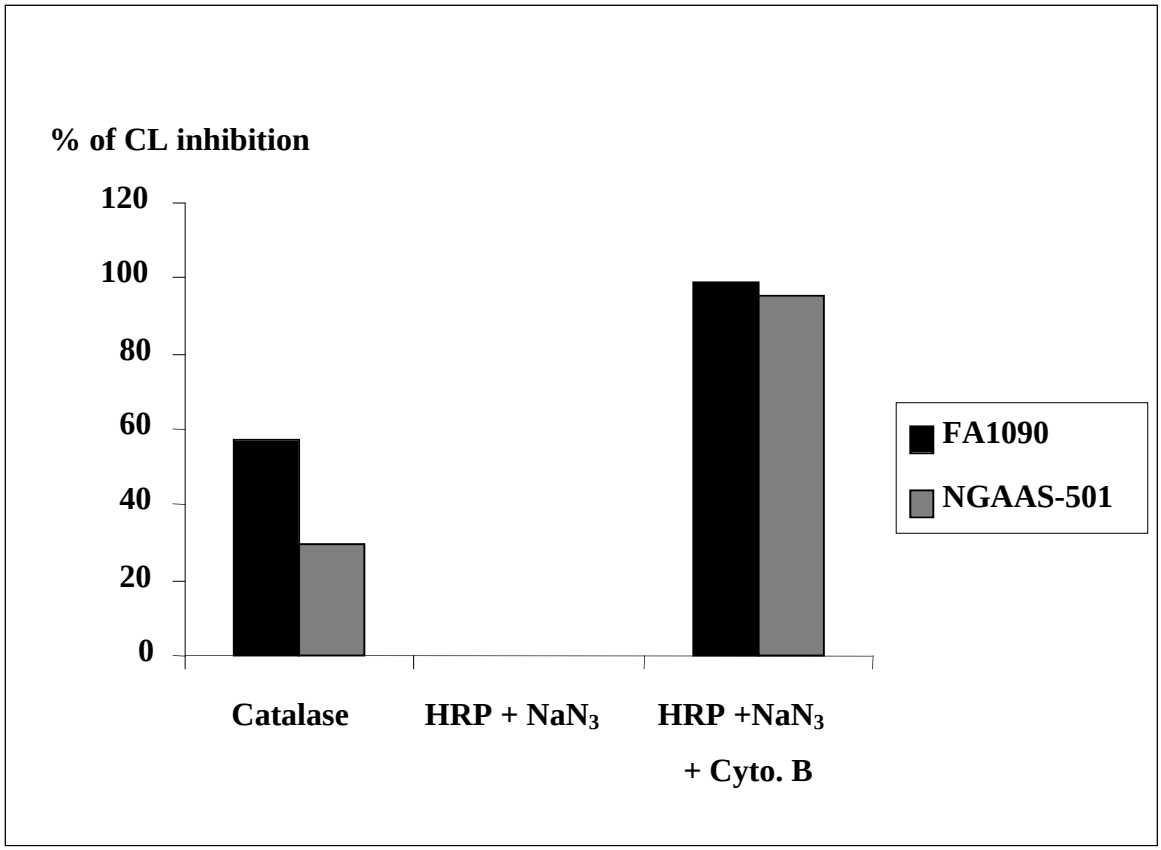


Figure 79. Measurement of intra- and extracellular oxidative burst induced by FA1090 and NGAAS-501. Bars represent an average of six replicates. CL, chemiluminescence.

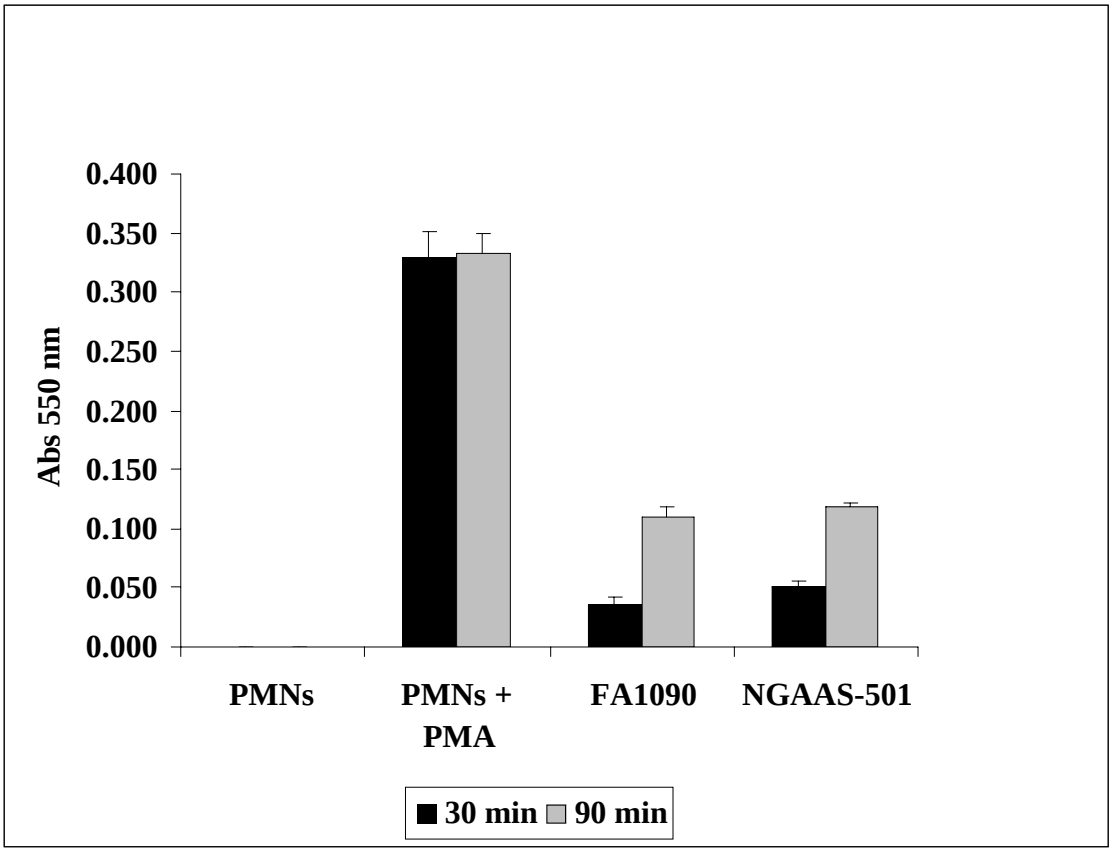


Figure 80. Determination of superoxide ion production by estradiol-treated PMNs as measured by NBT reduction.

Table 18. Superoxide, H_2O_2 and MPO production by estradiol-treated PMNs exposed to FA1090 and NGAAS-501.

Assay [1, 2]	**FA1090**	**NGAAS-501**
Cytochrome C reduction (nM/ml/10^6 cells)	7.6 ± 1.5	7.5 ± 2.3
H_2O_2 [3] **(∝M/ml/10^6 cells)**	25 ± 10	26 ± 4
MPO [4] **(U/ml/10^6 cells)**	1.7 ±0.06	1.9 ±0.1

[1] Estradiol treated PMNs were used
[2] All the samples were measured after 90 min
[3] Estradiol-treated PMNs exposed to PMA were used as positive control; 106 ∝M/ml/10^6 cells
[4] Base line levels of PMNs unexposed to any stimuli; 0.27 ± 0.06 U/ml/10^6 cells.

Discussion

I- Characterization of the detoxifying enzymes in *Neisseria gonorrhoeae* strain FA1090.

The importance of detoxifying enzymes, namely superoxide dismutase (SOD), peroxidase and catalase in the pathogenesis of several microorganisms has been explored in *in vitro* (Clements and Foster, 1999; Franzon et al., 1990; Hong et al., 1998) and *in vivo* models (Buchmeier et al., 1994; Schwartz et al., 1983). The repertoire of detoxifying enzymes in GC appears to be a bacterial strain specific phenomenon. Early studies reported that GC does not produce SOD (Archibald and Duong, 1986; Norrod and Morse, 1979), which is curious since GC is isolated from purulent exudates containing PMNs that are vigorously evolving superoxide radical ($O^{\cdot}_2$) and H_2O_2. More recently, Tseng et al. (2001) reported the presence of SOD activity (iron-dependent activity encoded by *sodB* gene) in several gonococcal strains including FA1090 by activity gel and by Southern blot. Mutation in the *sodB* gene of one of these strains, however, did not render the bacteria more sensitive to oxidative killing. Instead, a novel mechanism of manganese accumulation was found by this group to have a more important role than SOD in defense against paraquat-induced superoxide. Peroxidase is an alternative way to convert H_2O_2 to non-toxic derivatives. Although peroxidase activity has been reported for some GC strains (Archibald and Duong, 1986; Johnson et al. 1993), this activity was not detected in strain FA1090 by Moore and Sparling (1995). In contrast to the apparent strain-specific phenomena with regard to SOD and peroxidase, all GC produce catalase. It is hypothesized that gonococci use catalase as a mechanism of protection against H_2O_2

released by PMNs during phagocytosis. This hypothesis is based on the observation that GC generates high levels of catalase in the presence of sublethal concentrations of H_2O_2 and upon brief exposure to neutrophils (Zheng et al.,1992).

Among the three detoxifying enzymes that I looked by activity gel, only catalase activity was observed in strain FA1090. One copy of the catalase gene and one protein species were detected by Southern blot and catalase activity gel, respectively. The presence of a single catalase in GC is in contrast to microorganisms such as *E. coli* (Loewen, 1996) and *Bacillus subtilis* (Loewen and Switala, 1987), which possess two and multiple catalases, respectively.

II-Cloning and characterization of the gonococcal catalase gene

Catalases have been cloned from a wide variety of microorganisms (Brown et al., 1995; Hass et al., 1991; Hassett et al., 2000; Wygong et al., 1998). Johnson et al. (1996) cloned the gonococcal catalase gene from GC strain 2821. Initial attempts to clone the gene by conventional methods using *E. coli* as the background strain were unsuccessful due to instability of the plasmid vector after cloning. Therefore, these investigators prepared a random genomic library of GC DNA using a chimeric plasmid consisting of the GC cryptic plasmid and a 7.2 kb β-lactamase plasmid as a vector. The library was transformed into a spontaneous gonococcal catalase mutant (Johnson et al., 1993). Five-thousand and eight-hundred putative clones were screened by adding H_2O_2 to the colonies. Only 12 out the 5,800 screened clones were catalase positive, for an efficiency of 0.2%.

In the present study, we used information from the FA1090 genome sequence to clone the *kat* gene from this strain. The clone was isolated by complementation of the catalase-deficient *E. coli* strain UM255. *E. coli* UM255 was used previously to verify functional expression of a cloned catalase gene from *H. pylori* (Odenbreit et al., 1996). Twelve out of 433 putative clones obtained were catalase positive, for an efficacy of 5%. One interesting observation was the presence of a dark color in 100% of the *E. coli* transformants that expressed gonococcal catalase. Although the source of this color remains unknown, this second phenotype was useful for confirming catalase expression in *E. coli* UM255 in further experiments.

Plasmid pAAS184-*kat* 30 was selected for further characterization. The cloned insert was completely sequenced and exhibited 95% identity with the previously cloned gonococcal catalase gene of strain 2821 (Johnson et al. 1996). The gene encodes a protein of 504 amino acids, which exhibits similarity to catalases from *H. influenzae* (Bishai et al., 1994a), *V. fisheri* (Visick and Ruby, 1998), *B. fragilis* (Rocha and Smith, 1995), *P. aeruginosa* (Ma et al., 1999), and *B. pertussis* (DeShazer et al., 1994), among others. The catalase from FA1090 is predicted to have 4 additional amino acids compared to the catalase from strain 2821. No conclusions could be made about the difference in size, given that the 4 additional amino acids in the FA1090 catalase are scattered around the protein.

Gonococcal catalase was efficiently expressed in *E. coli* with no visible differences in terms of the relative mobility of the catalase protein in catalase activity gels. High levels of catalase activity were detected in *E. coli* carrying the cloned *kat* gene, presumably due to the dosage effect resulting from plasmid copy number. Another reason

that may explain the high levels of gonococcal catalase activity in *E. coli* UM255 is the possible differences in gene regulation on *E. coli* and GC. The differences in gene regulation will be discussed below. The functionality of the expressed catalase protein was confirmed by the increased H_2O_2 resistance of *E. coli* UM255 carrying pAAS184-*kat* 30. The identity of the recombinant protein expressed by this plasmid was confirmed to be gonococcal catalase by *N*-terminal sequence analysis and Western blot.

Most bacterial catalases are located in the cytoplasm where they prevent the entry and the detrimental effects of H_2O_2. In *E. coli*, however, HPI is present in both the periplasm and the cytoplasm, whereas HPII is strictly cytoplasmic (Heimberger and Eisenstark, 1988). The nucleotide sequence data of the gonococcal *kat* gene revealed no evidence of the presence of a typical export-directing amino acid signal in the predicted gonococcal catalase protein. This finding is consistent with the report of Zheng et al. (1992) that GC FA1090 catalase is only found in the cytoplasm. In *Pseudomonas aeruginosa*, that one of the two catalases encoded is released by cell lysis during stationary phase (Hassett et al., 2000). The released catalase was very resistance to the action of proteases indicating that the protein could persists from extended periods when is released from lysed cells. The implications of this mechanism in Pseudomonas pathogenesis could be as to prevent H_2O_2 damage in the bacterial cell and to protect cells growing in biofilms (Elkins et al., 1999; Stewart et al., 2000). Clinically, *P. aeruginosa* might release catalase as a mechanism of defense against the phagocytic cell and its O_2-dependent antimicrobial mechanisms. This mechanism can be described as being altruistic in that autolysis of individual bacteria may save part of the population during infection. The same mechanism has been proposed for the catalase of *H. pylori* (Odenbreit et al.,

1996). In contrast, however, studies of bacteriolysis in *S. aureus* revealed that H_2O_2 can inhibit the activation of autolytic enzymes, and therefore may interfere with the optimum degradation of the bacterial cell wall at the site of infection (Ginsburg, 1989).

How gonococcal catalase enzyme is exported to the extracellular environment, if that is the case, is still unknown. It can be speculated that one way by which GC may release catalase to the extracellular milieu is through autolysis (Hebeler and Young, 1976; Morse and Bartenstein, 1974). Autolysis in GC can be induced in response to acid pH (Dillard and Seifert, 1997; Elmros et al., 1976), although endolysins that act at alkaline pH have also been reported (Hebeler and Young, 1975). Although GC does not produce biofilms, the bacterial aggregation and release of GC catalase could act to protect the infective population. If this is true, we could hypothesize that GC catalase possesses an altruistic role in GC pathogenesis as proposed for *H. pylori* and *P. aeruginosa* with the same clinical implication.

Eukaryotic and prokaryotic catalases have been classified into three distinct groups: monofunctional, catalase peroxidases and non-heme or pseudo-catalases (Loewen, 1997). Based on the data collected in my studies, catalase from GC FA1090 appears to be a typical monofunctional catalase. This result is in contrast to the report that GC strain 2821 possesses a catalase-peroxidase enzyme (Johnson et al., 1993). I was unable to detect any peroxidase activity in the strain FA1090 using 4 different enzymatic assays and by peroxidase activity gel. From these data, I conclude that gonococcal catalase from the strain FA1090 is a typical monofunctional catalase.

Characterization of the catalase promoter region led to a series of interesting observations. First, the promoter in FA1090, as well as GC 2821 did not exhibit a typical

organization. While a putative Shine-Dalgano sequence was situated 8 bp in advance of the initiation codon, no regions corresponding to the consensus -10 and -35 sequences with appropriate sequence spacing were found. AT rich regions were observed in the putative promoter region and 3 FNR (fumarate-nitrate regulator) sequences were identified. Regulatory FNR sequences have been identified upstream of other gonococcal (Householder et al., 1999; Lissenden et al., 2000) and *E. coli* genes (Bates et al., 1995; Becker et al., 1996; Melville and Gunsalus, 1996; Williams et al., 1998). The lack of a typical promoter sequence and the presence of FNR sequences in the region immediately upstream of the *kat* gene leads one to postulate that the expression of the catalase gene in FA1090 is tightly regulated in the interface of an aerobic/anaerobic environment (Sawers, 1999).

As mention previously the differences in gene expression of on *E. coli* and GC can account for the high gonococcal catalase activity observed on *E. coli* UM255 expressing GC catalase. The FNR controls the expression of GC genes expressed such as *aniA*, a gene required for the anaerobic growth of GC in the presence of nitrite (Knapp and Clark, 1992; Mellies et al., 1997). In the case of the *aniA* gene, FNR induces its expression. In contrast, FNR may repress GC catalase during the transition from aerobic to anaerobic environment. Also may lead to the constitutive basal levels of catalase normally observed in GC. The GC FNR was cloned and complementation of *E. coli* FNR mutant with GC FNR did not restore *E. coli* wild-type phenotype as evidenced in the lack of transcription in tested *lacZ*-fusions consisting of *E. coli* genes possessing FNR consensus sequences. Transcription of FNR *E. coli* regulated genes was only observed when the promoter region of *E. coli* FNR was fused with the GC FNR structural gene. Amino acid similarity

analysis of *E. coli* and GC FNR demonstrated changes in protein residues with apparent critical role in FNR protein recognition and function on each bacterium. Taking that into account the high catalase activity detected in *E. coli* UM255 expressing the GC *kat* gene can be attributed to the lack of regulation of the GC *kat* gene on *E. coli* leading to a constitutive expression. Other regulators to have a role in the regulation of catalase on *E. coli* are OxyR and the sigma factor *rpoS* might be involved on GC regulation (Storz and Zheng, 2000). Johnson et al. (1996) expressed GC *kat* on *E. coli* deficient in *rpoS*. No difference in the expression of GC *kat* was observed suggesting that *rpoS* is not required for GC expression. On the other hand this observation suggest that possible differences in the RpoS protein impaired recognition of GC *kat* consensus sequences and as a consequence no effect in the GC *kat* expression on *E. coli* is observed. This suggest that although different bacteria posses similar pathways of regulation the proteins involved posses specificity for their respective host.

III-Construction, characterization and complementation of a GC catalase mutant

For several pathogenic bacteria, catalase has been postulated as a defense against killing by PMNs. A correlation between catalase activity and virulence was established long ago, based on the relationship between the lack of catalase activity, sensitivity to oxidative stress, and diminished virulence in certain strains of *M. tuberculosis* (Cohn et al., 1954; Middlebrook, 1954). Later, a similar correlation between levels of catalase activity and virulence was demonstrated in *S. aureus* (Mandell, 1975). The importance of catalase in bacterial pathogenesis is not supported by all studies, however, in that genetically defined catalase mutants of *Listeria monocytogenes, S. typhimurium, E. coli* and *S.*

flexneri, were shown to be fully virulent in animal models (Fields et al., 1986; Franzon et al., 1990; Gaillard et al., 1986).

The construction of a catalase-deficient isogenic *N. gonorrhoeae* mutant is an important pre-requisite for the study of the putative role of this enzyme in pathogenesis. In this study, two catalase mutants (NGAAS-500 and NGAAS-501) of strain FA1090 were constructed. Both mutants carried a 1.2 kb deletion of the *kat* gene. A nonpolar *aph*A-3 cassette encoding kanamycin resistance was introduced at the site of the deletion in mutant NGAAS-500. Mutation of the catalase gene resulted in total abrogation of catalase activity by activity gel and quantitative enzymatic assays, confirming the presence of only one catalase in this strain. The catalase deficiency in NGAAS-501 rendered the bacteria more susceptible to H_2O_2 as expected. Shaking during incubation enhanced this sensitivity as observed by a faster decline of the mutant population and by an increase in DNA damage on alkaline agarose gels. This fact suggests that the localization of catalase in the cytoplasmic space might help to prevent DNA damage (Odenbreit et al., 1996). Three inducers of toxic oxygen radicals (paraquat, tetracycline and streptonigrin) inhibited NGAAS-501 slightly more than FA1090. Finally, the catalase deficient bacteria were more sensitive to H_2O_2-producing lactobacilli than the wild-type, suggesting catalase may serve as an important biological defense in the competition with microflora.

Complementation of mutations in *N. gonorrhoeae* has not been a popular task due to the lack of well-characterized genetic systems for introducing cloned genes into *Neisseria sp*. In this study, restoration of catalase activity as measured by bubbling assay was accomplished in both mutants by supplying a wild-type copy of the gonococcal catalase gene *in trans* using the shuttle vector pLEE20 (pAAS20-*kat* 30). Unfortunately

catalase activity could only be quantitated by a standard spectrophotometric assay in the complemented strain NGAAS-503 (NGAAS-501 with plasmid pAAS20-*kat* 30). The catalase activity in NGAAS-503 was 1.5-fold more than that of the wild-type strain. The 2-fold increase can be attributed to the copy number of the complementing plasmid. Strain NGAAS-503 was more resistant to paraquat compared to NGAAS-501 and slightly more resistant than wild-type GC. Expression of wild-type catalase in NGAAS-503 restored wild-type levels of resistance against H_2O_2 produced by *L. crispatus*. Complementation of NGAAS-501 did not restore wild-type levels of resistance to H_2O_2, however.

In summary, in 5 out 7 phenotypes tested, the wild-type phenotype was restored in mutant NGAAS-501 by introducing a wild-type copy of the *kat* gene *in trans*. The reason why complementation did not restore resistance to H_2O_2 is not known. On activity gels, the relative mobility of the catalase protein expressed in bacteria NGAAS-503 was faster than that of wild-type catalase, suggesting possible post-translational modification. Attempts to sequence the protein expressed in the complemented mutant were unsuccessful due to a possible modification in the *N*-terminus of the protein. No obvious deletion in the *kat* gene carried by NGAAS-502 and 503 was identified, and reintroduction of pAAS20-*kat* 30 from GC NGAAS-502 and 503 into *E. coli* UM255 restored catalase activity, dark color phenotype and wild-type relative mobility in the catalase activity gel. The basis for the altered mobility of this catalase protein when expressed from pAAS20-*kat* 30 in NGAAS-501 remains unknown.

IV- Role of catalase *in vivo*

The classic approach for defining the role of a virulence factor in pathogenesis requires the construction of a mutant strain that lacks the factor thought to be important during infection but is otherwise identical (isogenic) to the parental strain. The virulence of the mutant and parent are then compared in an appropriate setting. Using a female mouse model of gonococcal genital infection (Jerse, 1999), I demonstrated that both wild-type FA1090 and catalase mutant NGAAS-501 could colonize the genital tract of estradiol-treated mice. No significant difference was observed in the duration of recovery at doses ranging between 2.5×10^5 to 1×10^6. Although the characteristic influx of neutrophils was observed in the mice during infection and gonococci were seen associated to the PMNs, no correlation could be established between the degree of inflammation and clearance of infection. At this point, the results suggested that catalase is not required for infection and persistence in the genital tract of estradiol-treated mice, or perhaps that gonococci use a different mechanism for survival in the genital tract in the absence of catalase. A third possible explanation relates to the effect that exogenous estradiol may have over some components of the neutrophil oxidative killing machinery (Kita et al., 1985). These aspects are discussed below in the section dealing with the characterization of PMNs from estradiol-treated and untreated mice.

Mixed infection experiments are a sensitive way of detecting differences between strains in that the strains being compared are exposed to exactly the same environment, decreasing the effects of animal variability that may occur in mice individually infected with either strain. A dramatic attenuation of the mutant was observed in mice inoculated with a 1:1 mixture of FA1090 and NGAAS-501 in that no mutant was recovered after 6

days. No differences other than catalase that could explain this attenuation were identified. Both the parental strain and the catalase mutant exhibited similar *in vitro* growth rates, piliation levels, and Opa and LOS phenotypes. It is important to mention that although the *kat* deficient mutant stock was composed primarily of Opa B/D variants versus the wild-type stock, which had a larger percent of Opa negative variants, it is unlikely that there is a link between the *kat* deficiency, Opa phenotype and GC pathogenesis in the mouse for two reasons. First, there are no CEACAM receptors on mouse PMNs that would be utilized by Opa proteins. Second, once GC is inoculated in the mouse a selection for Opa proteins is observed within 48 hours in most mice, and therefore that which is introduced originally is not what is obtained after colonization (Jerse, 1999). This selection has also been observed in male volunteers inoculated intra-urethrally (Jerse et al., 1994).

Also, the growth of the parental strain did not affect the growth rate of the mutant in broth culture. Although there are many factors in the mouse that might affect the viability of the mutant bacteria, one possible explanation for the attenuation observed during the mixed infections is that when *in vivo*, the wild-type strain may generate an environment unsuitable for NGAAS-501 growth. This theory is described in figures 81-83. Given that the only difference between the wild-type and the *kat* mutant is catalase, I hypothesized that the presence of the wild-type strain might generate a higher oxidative burst environment *in vivo* due to degradation of H_2O_2 by catalase leading to the production of molecular oxygen (Fig. 81). Molecular oxygen is one of the two requirements for oxidative burst, and the replenishment of O_2 by the activity of the wild-type catalase could increase the level of oxidative burst in the local environment. In

Figure 81. Interaction of FA1090 with mouse PMNs. Glucose and O_2 are the elements required to induce an active oxidative burst and toxic oxygen radical production by the PMNs. Exposure of murine PMNs to wild-type strain FA1090 might trigger an initial oxidative burst. Once the process is activated, the degradation of H_2O_2 by gonococcal catalase would replenish the molecular oxygen needed to continue the oxidative burst. Although more H_2O_2 may be produced, the wild-type FA1090 can survive this environment due to the presence of gonococcal catalase and also due to the activation of the innate detoxifying enzymes in the PMNs that will limit the amount of H_2O_2 that might become in contact with GC.

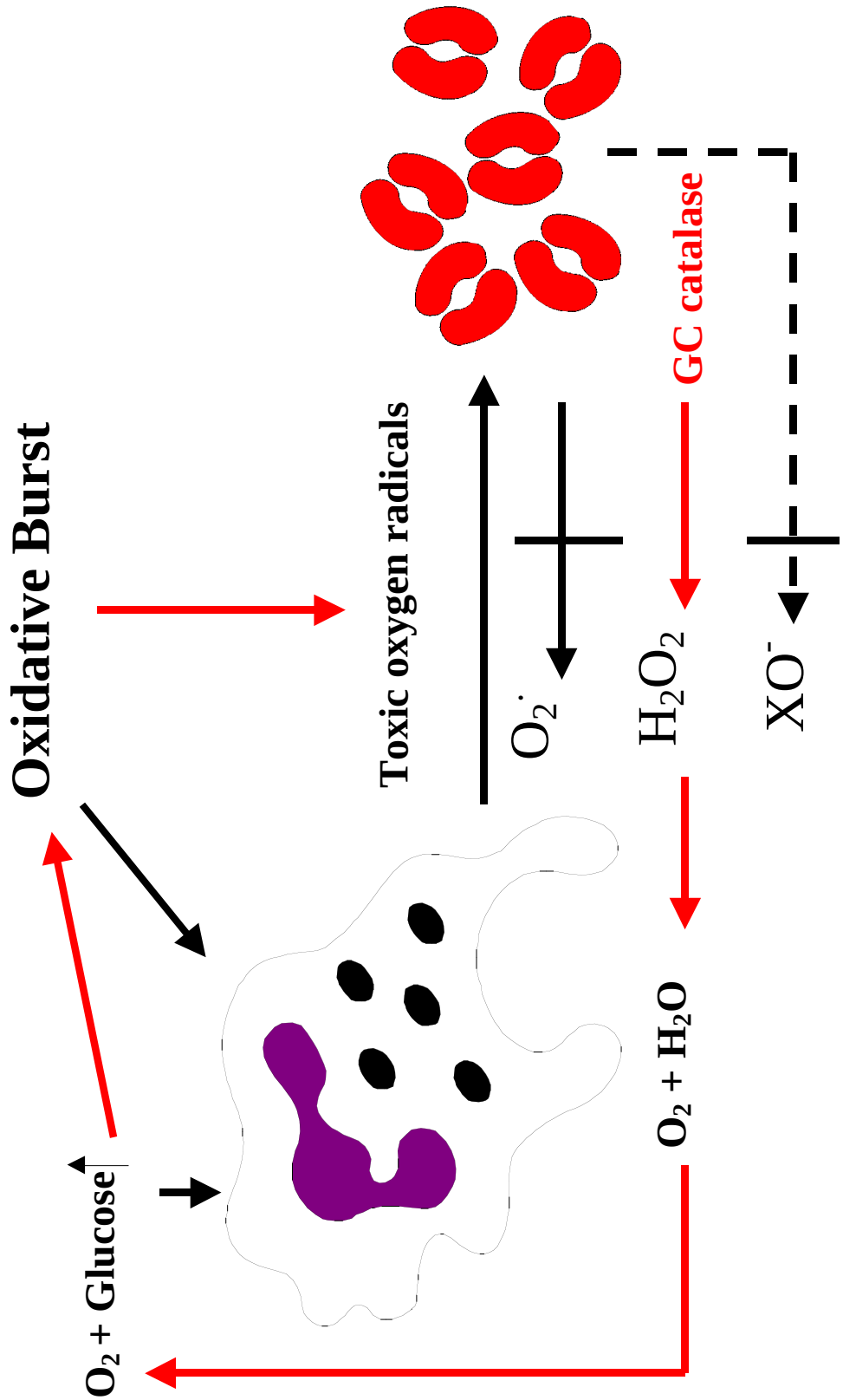

Oxidative Burst
Toxic oxygen radicals
$O_2^{\cdot}$
H_2O_2
GC catalase
XO^-
O_2 + Glucose
O_2 + H_2O

Figure 82. Interaction of NGAAS-501 with mouse PMNs. Exposure of mouse PMNs to the catalase deficient mutant NGAAS-501 as well as to the wild-type strain FA1090 might trigger an initial oxidative burst. Contrary to the wild-type strain, NGAAS-501 does not posses catalase, and as a consequence no molecular oxygen would be generated to replenish the oxidative burst environment and to perpetuate the production of toxic oxygen radicals such as H_2O_2. Gradually, oxidative burst activity will therefore decrease.

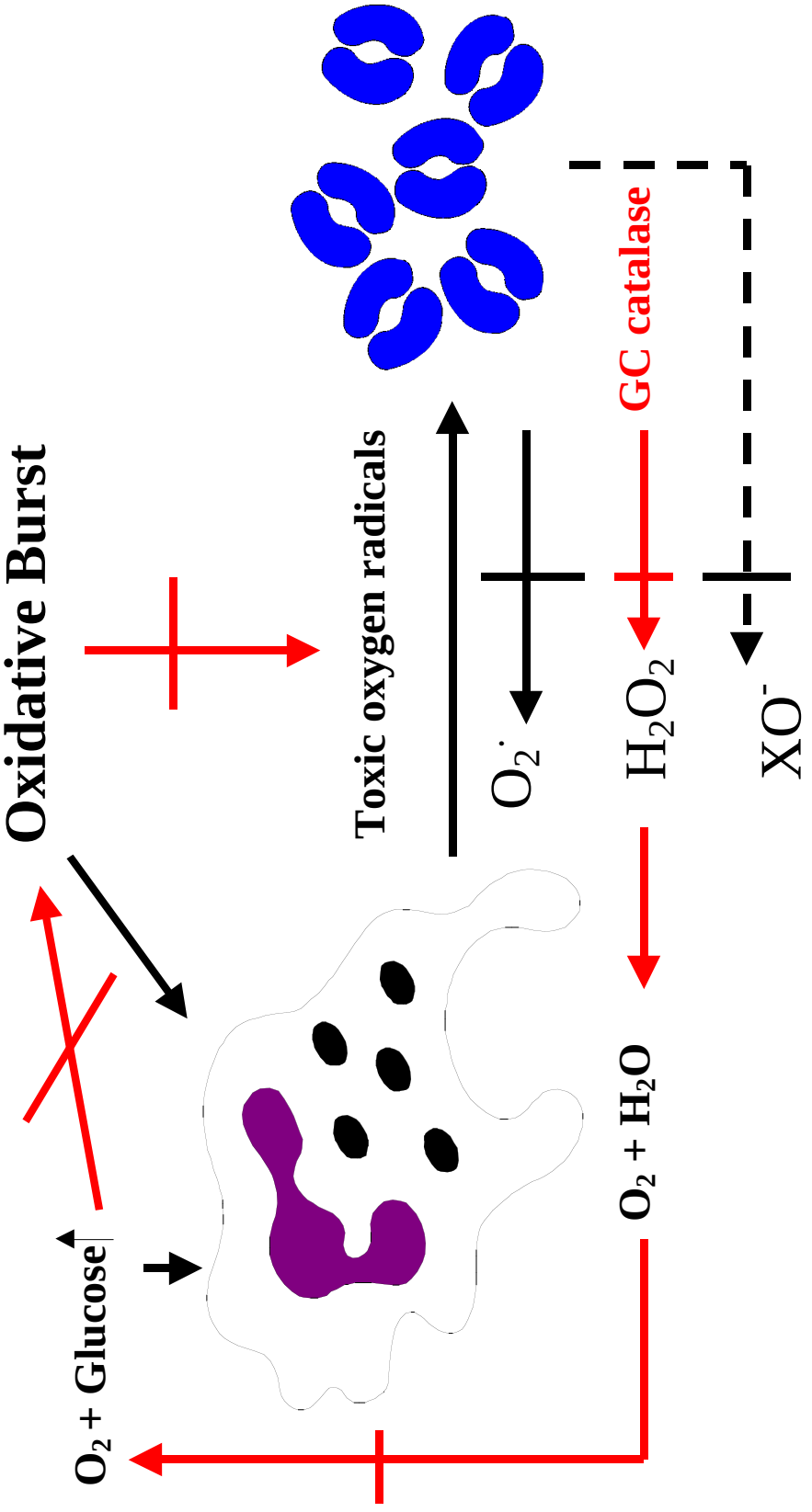
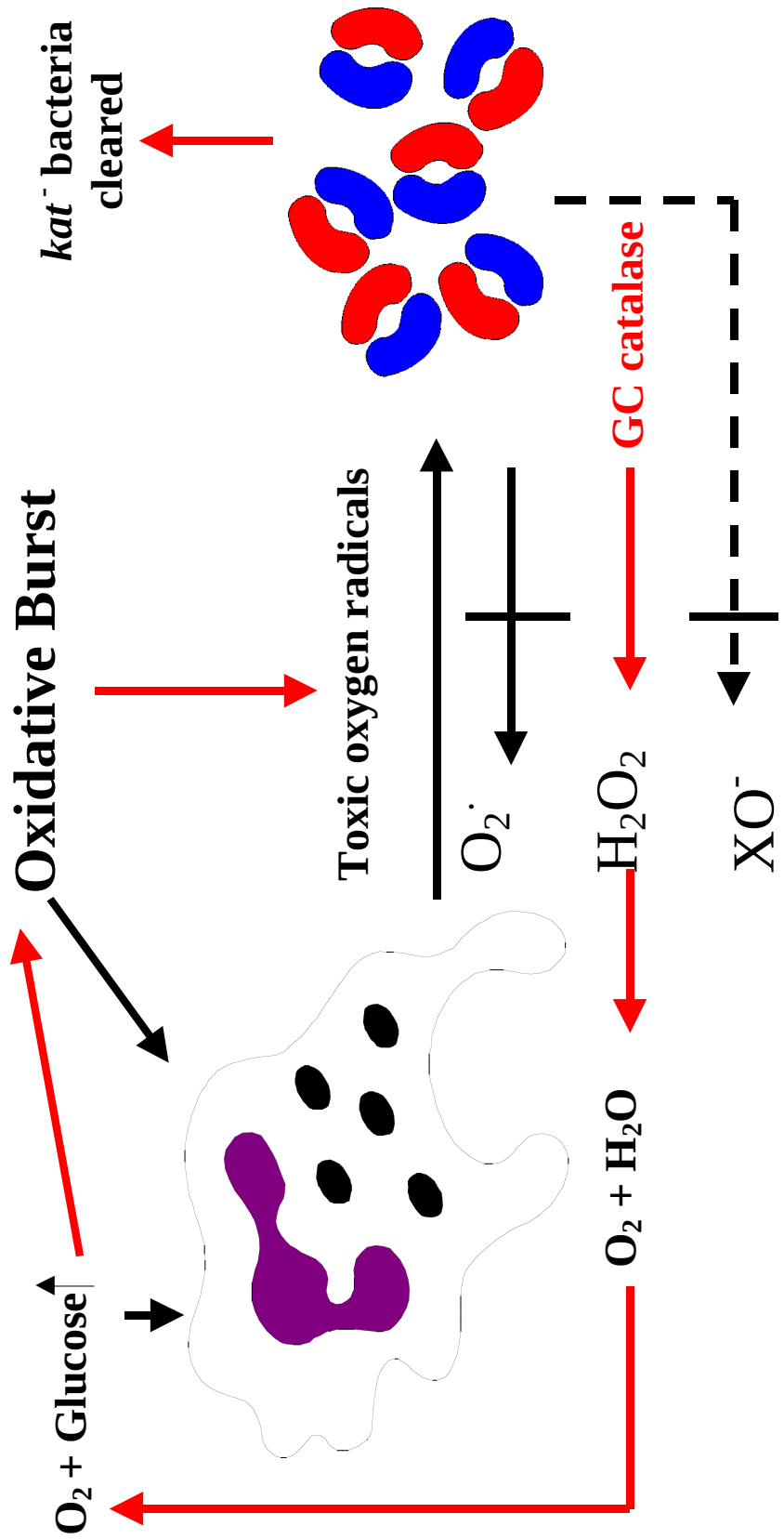

Oxidative Burst
Toxic oxygen radicals
$O_2^{\cdot}$
H_2O_2
GC catalase
XO^-
$O_2 + H_2O$
O_2 + Glucose

Figure 83. Interaction of a 1:1 mixture of FA1090 and NGAAS-501 with mouse PMNs. Exposure of mouse PMNs to FA1090 and the catalase-deficient mutant NGAAS-501 might trigger an initial oxidative burst. Once the process is activated, one of the possible sources of O_2 needed to perpetuate the production of toxic oxygen radicals such as H_2O_2 is the degradation of H_2O_2 by gonococcal catalase. Although H_2O_2 may be produced, the wild-type FA1090 can survive in this environment due to the presence of gonococcal catalase and also due to the activation of the innate detoxifying enzymes in the PMNs. In contrast, the catalase deficient NGAAS-501 would be targeted by the higher levels of toxic oxygen radicals that are produced in the presence of the FA1090.


kat- bacteria cleared
Oxidative Burst
Toxic oxygen radicals
GC catalase
O2 + Glucose
O2 + H2O
O2·
H2O2
XO-

contrast, due to the lack of catalase in the *kat* mutant, replenishment of molecular oxygen would not occur, and therefore production of toxic oxygen radicals would be lower during infection with the mutant. As a consequence, *kat* mutant GC survives (**Fig. 82**). In mixed infections an increase in toxic oxygen radicals due to the presence of the wild-type strain would affect the mutant more that the wild-type, an event that would be evidenced by a decline in the catalase deficient population (**Fig. 83**). It should be mentioned that during an active oxidative burst, the innate detoxifying enzymes of PMNs are activated in order to avoid damaging the host. This event would also decrease the amount of toxic oxygen radicals that might come in contact with gonococci, resulting in increased bacterial viability. Other data that support this model are mixed PMN killing assays in which the catalase deficient mutant was killed more than the wild-type strain, supporting the idea that in a mixed population of GC, the catalase deficient fraction is the target population. Also GC *kat* mutant was more sensitive to H_2O_2 and inducers of toxic oxygen radicals such as paraquat, streptonigrin and tetracycline suggesting that during an active oxidative burst the *kat* deficient bacteria can be affected more as compared to wild-type GC. More experiments are needed to prove this theory. Other data suggesting that the model is correct will be discussed below in relation to the interactions between wild-type FA1090 and the NGAAS-501 with estradiol-treated PMNs.

Enhanced bacterial survival by the action of degradation metabolites resulting from a bacterial enzyme has been proposed for *H. pylori*. In this study, CO_2 produced via degradation of urea by *H. pylori* urease was proven to be a scavenger of peroxynitrite, a host derived metabolite of nitric oxide. (Kuwahara et al., 2000). This was the first report describing the role as a scavenger of a degradation metabolite due to the action of a

bacterial enzyme. Although the mechanism described for *H. pylori* urease is different than the model I propose, both mechanisms describe the participation of metabolites resulting from the degradation of host substrates in the survival of bacteria.

V-Characterization of estradiol-treated PMNs

The importance of PMNs in the control of infection has been investigated in models of infection for *Chlamydia psittaci* (Buendìa et al., 1999) and Herpes simplex virus-2 (Milligan, 1990). In this study, no significant difference was observed in the recovery of FA1090 and NGAAS-501 from estradiol-treated female mice that were inoculated with wild-type or *kat* mutant GC. Although this result suggested that catalase is not important during infection, a second possible explanation is that estrogen is immunosuppressive. The effect of sex hormones on the immune system, and specifically on inflammation, has been surrounded by an extensive debate. One of the first observations of the effect of estrogen on components of the inflammation process was reported by McNaab and Jellinck (1974). Rats treated with estrogen exhibited an increase in peroxidase activity in the uterus. This increased peroxidase activity correlated with the estrogen decomposition into water soluble components. This study suggests that the increased peroxidase activity is responsible for the decomposition of estrogen. The interconversion of estrogen to other derivatives in the rat uterus was confirmed by Mabin et al. (1974), in female reproductive tract tissues.

Estrogen has also been implicated in the inhibition of neutrophil chemotaxis, although the mechanism by which this occurs is controversial. In a report by Jungi et al., 1977, gonadectomized rats were treated with estradiol for a week. The characteristic peak

of neutrophil influx normally observed at the end of the estrous cycle was detected in the gonadectomized rats only after withdrawal of estradiol. Exposure of neutrophils to estradiol and other sex hormones *in vitro* did not affect the migration of neutrophils, however, suggesting that the chemotactic factor that is responsible for the migration of PMNs is produced by other cell types in the vaginal mucosa, and that estrogen specifically interacts with these chemotactic factor-producing cells rather than with the PMNs. In contrast, Ito et al. (1995), established that estradiol directly inhibits the chemotaxis of neutrophils by binding to estrogen receptors expressed on human PMNs (Muramatsu and Inoue, 2000; Press et al., 1986). The addition of estrogen receptor-specific inhibitors abrogated the inhibition of neutrophil chemotaxis. Although it is not known why these data differ, the two groups utilized PMNs from different sources suggesting that PMNs from rats and humans may differ in their response to estradiol. Host-specific differences in the response of granulocytes to PMA have been reported previously (Young and Beswick, 1986).

The primary function of neutrophils is to phagocytize and destroy microorganisms. The release of MPO and H_2O_2 into the phagosome containing the ingested microorganism generally leads to a rapid microbicidal effect. Phagocyte response to microbial stimulation is accompanied by a burst of oxygen consumption. Most, if not all, the extra oxygen consumed during the respiratory burst is converted first to the superoxide anion and then to H_2O_2. MPO is released from the cytoplasmic granules in neutrophils and monocytes and reacts with the H_2O_2 produced by oxidative burst to form a complex that can oxidize a variety of substances such as chloride. Chloride is initially oxidized to hypochlorous acid, with the subsequent formation of chlorine and chloramines. These products of the MPO-

H_2O_2-chloride system are powerful oxidants that can have a profound biological effect which can be long-lasting (Klebanoff, 1999; Thomas, 1979a; Vigerust et al., 2000). It has been demonstrated that chlorine and chloramines can oxidize bacterial components and that the rate of oxidation correlates with bacterial killing (Thomas, 1979b). Interestingly, the catalytic products of the MPO activity are targeted directly to the bacteria by the binding of MPO to the bacterial surface (Britigan et al., 1996).

Estrogen has been shown to affect the oxidative burst in PMNs. Estrogen enhancement of MPO activity is supported by studies involving hormone replacement therapy (Békési et al., 2001) and by *in vitro* studies (Jansson, 1991). The effect of estradiol on MPO is to stimulate the peroxidase-catalyzed reactions by acting as an oxidation-reduction catalyst. The estrogen is oxidized by peroxidase and H_2O_2 to the phenoxy radical, and the latter can be reduced to the original estrogen by an electron donor whose oxidation is thus stimulated. In the absence of an appropriate electron donor or after its complete oxidation, irreversible inactivation of the estrogen occurs. Oxidation of estrogen is suggested to occur during phagocytosis, implicating MPO in PMNs as being responsible for the process. Studies with female mice by Kita et al. (1985) described a 4-fold decrease in MPO activity with estradiol treatment, and no effect on superoxide ion production. The latter finding is controversial given that other groups have reported inhibition of superoxide ion production by estradiol. Inhibition of superoxide production by estradiol would greatly affect the capacity of PMNs to kill bacteria because this reaction is the first committed step in the oxidative burst, and is therefore necessary for the subsequent H_2O_2 production required for the MPO-H_2O_2 system (Békési et al., 2000; Buyon et al., 1984). It is important to note that the reported levels of estradiol that can

inhibit oxidative metabolism and MPO system are believe to be greater than 10,000 times the physiological concentrations (Bodel et al., 1972; Klebanoff, 1977). The levels of estradiol in the female mice model are below than the reported inhibitory levels (3,000 pg/ml of serum).

In my hands no significant difference was observed between PMNs from estradiol-treated versus untreated mice in several aspects tested. Both types of PMNs showed similar patterns of gonococcal association and internalization as reflected by the acridine orange-trypan blue staining. Both PMN types exhibited the same pattern of chemiluminescence response (a measure of overall oxidative burst) to different numbers of gonococci, although estradiol-treated PMNs showed a higher signal. This result is in agreement with the enhancement of MPO activity by estradiol observed by others (Békési et al., 2001; Jansson, 1991; McNaab and Jellinck, 1974), given that chemiluminescence emission is dependent on MPO activity. The killing capacity of PMNs was not affected by estradiol as measured by killing of *S. aureus* in the standard 90 min assay. No significant difference between the two types of PMNs in glucose consumption and MPO activity was determined. I therefore conclude that the use of exogenous estradiol in the female mouse model of genital tract infection did not impair the activity of murine PMNs, and therefore, estradiol-treatment is not responsible for the ability of the *kat* mutant to persist in the genital tract of mice.

VI-Characterization of interactions of FA1090 and NGAAS-501with mouse PMNs

Both the parental strain and the isogenic *kat* mutant associated with PMNs and were internalized at the same rate. In contrast, the *kat* deficient mutant was more rapidly

killed by murine PMNs compared to the parental strain as predicted. Based on these data I conclude that catalase serves as a defense against PMNs. This conclusion is consistent with other systems. For example, the sensitivity of catalase deficient mutants to the action of PMNs has been reported in *Salmonella* (Papp-Szabo et al., 1994). Also, the association between increased resistance to toxic oxygen radicals and catalase expression in GC (Zheng et al., 1992) and *Nocardia asteroides* (Filice, 1983) is consistent with the hypothesis that catalase is a defense against PMNs.

PMNs utilize O_2-independent defense mechanisms in the absence of oxidative burst as evidenced by the killing of bacteria in the presence of cytochalasin B, an inhibitor of oxidative burst (Okamura et al., 1979). Cytochalasins inhibit cytoskeleton rearrangement in PMNs affecting phagocytosis. Cytochalasin B, but not D, affects glucose consumption, and as result inhibits oxidative burst (Densen and Mandell, 1978). In this study, PMNs killed *kat* deficient gonococci in the presence of cytochalasin B. The fact that the rate of PMN killing was faster in the catalase deficient strain compared to the parental strain in the presence of cytochalasin B suggests that catalase deficiency may increase susceptibility to killing by O_2-independent mechanisms as well. The role of O_2-independent killing has been studied for several microorganisms (Modrzakowske and Spitznagel, 1979; Wetheral et al., 1984). How catalase deficiency in GC correlates with O_2-independent killing is not known. The possibility that serum present in the PMN assay affected viability of the bacteria was ruled out by the demonstration that serum from estradiol-treated mice does not posses any bactericidal activity. Killing of GC in the presence of cytochalasin B may not be surprising given that degranulation is increased under the influence of this agent and the granules in human PMNs have been proven to exert antibacterial effects (Casey et al.,

1986; Qu et al., 1996; Qu et al., 1997; Rest, 1979; Rock and Rest, 1988). Other lines of evidence support the role of O_2-independent killing as a mechanism utilized by PMNs in the control of a GC infection (Shafer and Rest, 1989). First, it was determined that PMNs obtained form patients with chronic granulomatous disease (CGD) kill gonococci as well as normal neutrophils (Rest et al., 1982). This inheritable disorder impairs PMNs in oxidative burst and thus unable to kill bacteria by O_2-dependent mechanisms (Holmes et al., 1968). Second, no difference was observed in the killing capacity of PMNs maintained in anaerobic conditions as compared to those kept at aerobic conditions (Casey et al., 1986). Third, GC can consume the available molecular oxygen after exposure to serum or PMN-derived lactate to an extent where the oxidative burst cannot be mounted (Britigan and Cohen, 1985; Britigan and Cohen, 1986; Britigan et al., 1988). Fourth, GC is often isolated together with strictly anaerobic bacteria that suggests the presence of an extensive anaerobic environment that PMNs cannot acquire the require oxygen for oxidative killing of the bacteria (Fontaine et al., 1982; Kellogg et al., 1983). Also GC can be maintained and grown under anaerobic conditions in the presence of a surrogate electron acceptors such as sodium nitrite (Casey et al., 1986; James-Holmquest et al., 1973; Knapp and Clark, 1983).

Although both strains induced oxidative burst as confirmed by a total abrogation of the chemiluminescence signal in the presence of cytochalasin B, the catalase mutant appeared to induce lower levels of chemiluminescence as compared to the parental strain. Both strains were able to induce oxidative burst without phagocytosis as reflected by the chemiluminescence response in the presence of cytochalasin D. The induction of oxidative burst in the absence of phagocytosis has been shown before in studies with *E. coli*

(Okamura et al., 1979). The generation of oxidative burst by GC seems to be intracellular since no inhibition was observed in the presence of HRP plus sodium azide, and only between 30 - 50 % of the intracellular activity was inhibited in the presence of catalase for both strains. The latter observations contrast with other microorganisms such as *H. pylori* in which bacteria are not internalized and the oxidative burst occurs extracellularly (Ramarao et al., 2000). The manner by which GC interacts with the PMNs may determine whether toxic oxygen derivatives are primarily intracellular or extracellular. Nonopsonic interactions result in a predominantly intracellular oxidative burst, suggesting that Opa-mediated entry into the PMNs activates a different mechanism of oxidative burst (Naids and Rest, 1991). It should be noted, however, that mouse PMNs do not express Opa receptors, and the interaction between gonococci and mouse PMNs required serum, suggesting that nonopsonic interactions did not occur.

Although the catalase appears to confer a survival advantage to GC based on mixed infection data, experiments in which mice were infected with either wild-type or *kat* mutant GC demonstrate that gonococcal catalase is not essential for survival in the mouse genital tract even in the presence of a PMN influx. *In vitro* data showing that the wild type FA1090 induced a higher chemiluminescence signal than the *kat* mutant NGAAS-501 may explain the survival of the *kat* mutant in the presence of PMNs in vivo, since low oxidative burst would generate fewer oxygen radicals. This event, inconjunction with other factors *in vivo*, might allow the mutant to persist in the genital tract. Although the *kat* mutant induced lower levels of oxidative burst *in vitro*, it is important to mention that no significant differences were observed when comparing the capacity of the wild type and catalase deficient GC to induce superoxide, H_2O_2 and MPO. These findings do not

completely rule out the theory that lower levels of oxidative burst induction is the reason that catalase deficient gonococci survive in mice inoculated with the *kat* mutant alone. The lack of difference observed in the generation of superoxide, H_2O_2 and MPO may be explained by a difference in the kinetics of induction by the wild-type and *kat* mutant bacteria. Such differences would be reflected in the overall induction of oxidative burst as evidenced in the chemiluminescence measurements. It is important to emphasize that more experiments are needed in order to identify differences between the wild-type strain and its isogenic mutant, and to prove this model.

In summary, mutation of the catalase gene in *N. gonorrhoeae* rendered the bacteria susceptible to toxic oxygen radicals, H_2O_2-producing lactobacilli and PMNs, and conferred a survival disadvantage in mice. These data support the hypothesis that catalase is a defense mechanism used by GC to survive in the genital tract. This work opens the door to design better strategies for preventing gonococcal infection such as vaccines that target catalase.

Appendix summary

Construction of genetic systems for studying transcriptional regulation and complementation of mutations in *N. gonorrhoeae* has been difficult. As a consequence, an extensive survey of gonoccocal gene expression in response to environmental stimuli and the characterization of the role of GC genes are not as complete as it is for other microorganism such as *E. coli*. In part, this is due to the restriction barriers for DNA transfer that exist in *N. gonorrhoeae*, which limit conventional methods for transferring DNA in *E. coli* and related microorganisms. It is nonetheless imperative that genetic systems be developed to allow us to study how GC adapts to the host environment.

In the present appendix, several attempts to design genetics systems to study gonococcal transcriptional regulation and to perform complementation are described. The use of reporter genes such as green fluorescent protein and β-galactosidase was explored and several versions of suicide shuttle vectors were constructed to allow recombination into the chromosome and to express genes *in trans*. Although some advances were made, I was unable to construct a reliable system. Nevertheless, a detailed description of the genetic designs and results are presented in the following sections.

I- Primers and plasmid constructs

Tables 19 and **20** correspond to primers and plasmids utilized in this section.

Table 19. Primers utilized

Primer designation	Sequence	Used
3-97 F	5'-AGTTTCAGACATGTAACCGC-3'	*por IB* promoter
4-97 B	5'-AAAGTCAGGGCAATCAGG-3'	*por IB* promoter
5-97	5'-AATTCGCCGTCTGAAC-3'	GC Uptake sequence
6-97	5'-AATTCTTCAGACGGCG-3'	GC Uptake sequence
13-97 F	5'-CCGTTTACGAAATTGGAACAGG-3'	*erm* probe
14-97 B	5'-GGCGTGTTTCATTGCTTGATG-3'	*erm* probe
17-97 F	5'-GCTCGTATGTTGTGTGGAATTG-3'	pUC9 *lacZ* promoter
20-98 F	5'-AAGTTCGATACCCGGCATCTG-3'	*kat* promoter
21-98 B	5'-GCCACAAATCTTGCGTCAGC-3'	*kat* promoter
22-98 F	5'-TATGCGTTCCGCCACAAACAAC-3'	*kat* gene probe
23-98 B	5'-GAAGCTGTTGGGTTCGTAGTGC-3'	*kat* gene probe
45-99 F	5'-ACAGCCGGTATAAAGGGACCAC-3'	km probe
46-99 B	5'-ACGCAGAAGGCAATGTCATACC-3'	km probe
50-00 F	5'-CGTATAATCGCATCCATAG-3'	*kat* promoter
51-00 B	5'-GGGCATTGGAGGTAGTCAT-3'	*kat* promoter
54-00 F	5'-GGCAGACTTCGTGCGAATAAGGA-CAGTGAAG-3'	Mob-UPS

55-00 B	5’-GAAATCTGCCGGGTTCGTGTAGAC-TTTCCTTG-3’	Mob-UPS
58-01 B	5’-GAATCCGTAATCATGGTCAT-3’	*lacZ* gene
59-01 F	5’-ATACGCCTGCTTCTGACCG-3	*proBA* gene
60-01 F	5’-AGACCTGCTGATTCCGCG-3’	*proBA* gene
61-01 F	5’-CGCGGAATCAGCAGGTCT-3’	*proBA* gene
R 1	5’-CGGAAGTGGGAATCTAGGACG-3’	*opa J*
F 36	5’-AAAGGTGAACGAAAACAAGGGC-3’	*opa J*
R 9	5’-GGTGCTTCATCACCTTAGGGAAC-3’	*opa B/D*
F 54	5’-GTCAGCATCACTAGGGTAGGCG-3’	*opa B/D*
R 81	5’-CTCTAAGGTGCTGAAGCACCAAGT-3’	*opa I*
F 32	5’-GTTTTGTGCCAGCACTATGGTAGG-3’	*opa I*
F 6	5’-GTGCAGGCGGATTTAGCCTAC-3’	*opa C* and *F*
HV2C R	5’-GTTTATAATAACGGAAGTACGC-3’	*opa C*
HV2F R	5’-AACAGAGTTTCTTACCG-3’	*opa F*
F 7	5’-CCCCTAGCAAATCAGCCTATTCA-3’	*aniA* promoter
R 10	5’-AAGAAGGATTGTTGGAGAATTCGACT-3’	*aniA* promoter

Table 20. Plasmid constructs

Back bone	Relevant marker	Comments
1) pAAS201-*kat* 700	pSUP201-1 with 700 bp *kat* internal fragment in *Eco* RI site	This study
2) pAAS20-UPS	pLEE20 carrying UPS in *Eco* RI site	This study
3) pAAS20-UPS-*gfp*	pAAS20-UPS carrying *gfp* gene	This study
4) pAAS20-UPS-*lacZ*	pAAS20-UPS carrying *lacZ* gene	This study
5) pAAS51	pQF50 carrying 1.9 kb *Bam* HI mob region	This study
6) pCRAAS-Mob-UPS	pCR-Blunt carrying 145 bp Mob-UPS fragment	This study
7) pAAS-52 *kat*	pQF50 carrying kat promoter and 145 bp Mob-UPS	This study
8) pAAS-53 *ani A*	pQF50 carrying *aniA* promoter and 145 bp Mob-UPS	This study
9) pCRAAS-*proBA* 8.2	pCR-Blunt carrying 2.3 kb *proBA* gene	This study
10) pAAS184-*proBA* 8.2	pACYC184 carrying *proBA* in the *Eco* RI site	This study
11) pAAS184-*proBA* - km	pAAS184-*proBA* 8.2 carrying *aphA3* cassette in *Bss* HII site of *proBA* gene	This study

II-Optimization of conjugation mating time

Optimization of conjugation is an important parameter for a effective transfer of plasmid DNA. The time required for the DNA transfer varies from a bacterial strain to another and can be determined by the donor and the recipient used. The conjugation procedure was first optimized in terms of the mating time using the standard protocol as described in the material and methods. GC strain F62 was used for control purposes. Strain F62 showed a high frequency of transconjugants (10^{-3}) in as few as 1h of mating which increased with mating time (**Table 21**). In contrast strain FA1090, however the frequency of conjugation remains the same (10^{-6}) through out all the mating times tested (**Table 22**). From these data, 5 hours was selected as the standard mating time for GC strain FA1090.

III- Construction of a suicide vector to generate mutations in specific gonococcal genes

A suicide vector based on plasmid vector pSup201-1was constructed (Simon et al., 1983). This vector, pSUP201-1 possesses two antibiotic resistance markers and the Mob region contained in a 1.9 kb *Bam* HI fragment, which allows mobilization from *E. coli* to GC by conjugation. The pSup201-1 vector was cut with *Eco* RI and blunt-ended as described in the material and methods. A 700 bp internal *kat* gene fragment was PCR amplified from pCRAAS-700 I and ligated into the blunt-ended *Eco* RI site of pSUP-201.1. The ligation was transformed into DHαMCR and putative clones were selected on LB-Amp. The presence of the *kat* internal fragment was determined by PCR using primers 22-98F and 23-98B. Clone 26 was selected for further characterization

Table 21. Conjugation frequencies of GC strain F62

Time (hours)	Number of donors	Number of recipients	Number of GC recovered	Frequency of conjugation
1	1.0×10^{8}	1.0×10^{8}	1.6×10^{9}	3.2×10^{-3}
2	1.0×10^{8}	1.0×10^{8}	2.7×10^{9}	4.8×10^{-3}
3	1.0×10^{8}	1.0×10^{8}	1.5×10^{9}	8.2×10^{-3}

Table 22. Conjugation frequencies of GC strain FA1090

Time (hours)	Number of donors	Number of recipients	Number of GC recovered	Frequency of conjugation
2	8.0×10^{7}	4.0×10^{8}	8.5×10^{8}	1.5×10^{-6}
3	1.2×10^{8}	9.7×10^{8}	1.8×10^{9}	4.5×10^{-6}
4	1.2×10^{8}	9.7×10^{8}	1.1×10^{9}	8.4×10^{-6}
5	1.9×10^{8}	1.3×10^{8}	2.2×10^{8}	8.1×10^{-6}
6	1.9×10^{8}	1.3×10^{8}	3.6×10^{8}	8.9×10^{-6}
7	1.9×10^{8}	1.3×10^{8}	1.6×10^{8}	1.7×10^{-6}
24	1.2×10^{8}	9.7×10^{8}	ND	ND

(pAAS201-*kat* 700, **Fig. 84**). This vector cannot replicate in GC due to the lack of a plasmid origin of replication recognized by GC, and therefore isolation of the desired mutant should be the result of the plasmid integrating into the chromosome.

A plasmid prep of pAAS202-*kat*700 I was prepared and the resultant DNA was transformed into *E. coli* S17-1. The suicide vector was transferred by conjugation as described before into GC strains FA1090 and F62 using different concentrations of ampicillin ranging from 5 to 25 μg/ml on GC agar plates to select for the desired recombinants. After various attempts, no *kat* mutant-Amp resistant transconjugants were obtained.

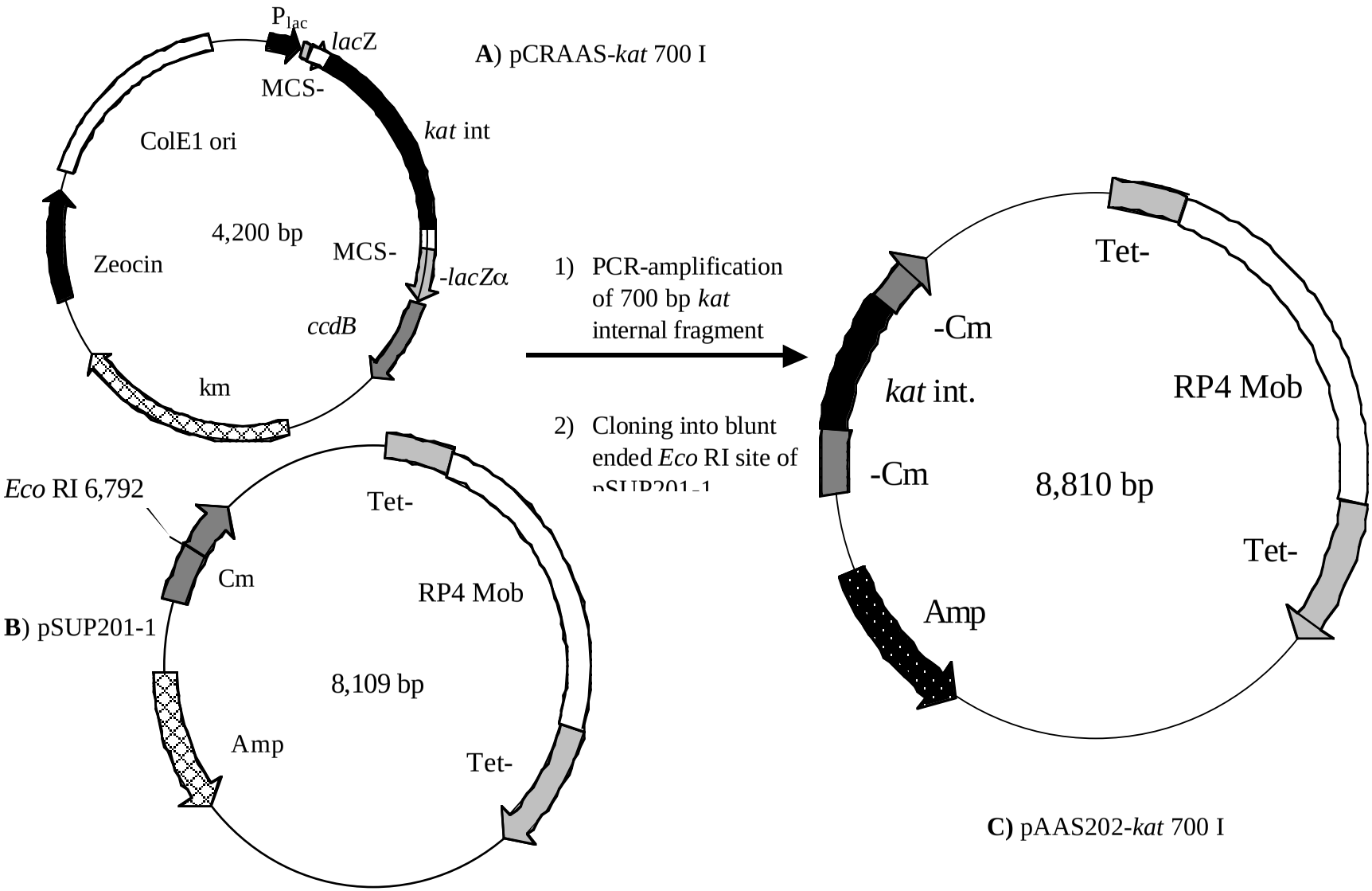
IV- Reporter vectors for identification of transcriptionally regulated genes

A. Construction of pAAS20-UPS, a versatile plasmid to transfer GC cloned genes by transformation and conjugation.

Primers 5-97 and 6-97 were designed to include the uptake sequence required for DNA competence in GC. Both primers included an *Eco* RI site on one end to facilitate cloning. The uptake sequence dimer was phosphorylated by mixing 1.6 μg of each primer and heating the mixture for 5 min at 70^{o}C. The mixture was left to cool at room temperature. The phosphorylation of the formed dimers was performed following instruction of the manufacturer.

The shuttle vector pLEE20 (erm^{R}) which is capable of replication in both *E. coli* and GC was the basis for the reporter gene shuttle vector (Nassif et al., 1991; Stein et al., 1983 a, b) (**Fig. 6**).

Figure 84. Construction of pAAS201-*kat* 700 I. A 700 bp conserved internal fragment from the gonococcal catalase gene was cloned into the unique *Eco* RI site of the chloramphenicol gene present in pSUP201-1. Suicide vector was transfered into GC by conjugation. Putative transcojugants were selected by ampicillin.


P$_{lac}$
lacZ
MCS-
kat int
ColE1 ori
4,200 bp
MCS-
Zeocin
-*lacZ*α
ccdB
km
A) pCRAAS-*kat* 700 I
1) PCR-amplification of 700 bp *kat* internal fragment
2) Cloning into blunt ended *Eco* RI site of pSUP201-1
Eco RI 6,792
Tet-
Cm
RP4 Mob
B) pSUP201-1
8,109 bp
Amp
Tet-
Tet-
-Cm
kat int.
RP4 Mob
-Cm
8,810 bp
Tet-
Amp
C) pAAS202-*kat* 700 I

The shuttle vector pLEE20 was cut with *Eco* RI and dephosphorylated with CIP following instructions of the manufacturer. The Plasmid vector was dephosphorylated to decrease self ligation and to increase cloning of the dimer. The phosphorylated dimer was cloned into the *Eco* RI site of the vector creating pAAS20-UPS (**Fig. 85**). The presence of the UPS in the plasmid was determined by sequencing using primer 17-97 F.

The transformation efficiency of the plasmid was tested following a standard protocol. Transformation was performed using plasmid DNA extracted from *E. coli* DH5αMCR. Although GC transformants were obtained, plasmid preps from transformants revealed degradation of the plasmid as evidenced of the smaller size of the plasmid as compared to *E. coli* DH5αMCR (**Fig. 86 A**). To verify that the observed smaller fragments correspond to degradation products of pAAS20-UPS and not to the GC cryptic plasmid (Korch et al., 1985; Sarandopoulos and Davies, 1993), a Southern blot was performed using *erm* probe. The 560 bp erythromycin probe was generated by PCR using primers 13-97 F and 14-97 B, and simultaneously labeled with digoxigenin followed the manufacturer instructions. Southern blots analysis revealed that the putative products of degradation belonged to pAAS20-UPS and not to GC cryptic plasmid (**Fig. 86 B**, lanes 2, 4 and 6).

Three species of plasmid DNA were observed in GC transformants based on size (**Fig. 86 A**). To identify the possible changes suffered by the plasmid

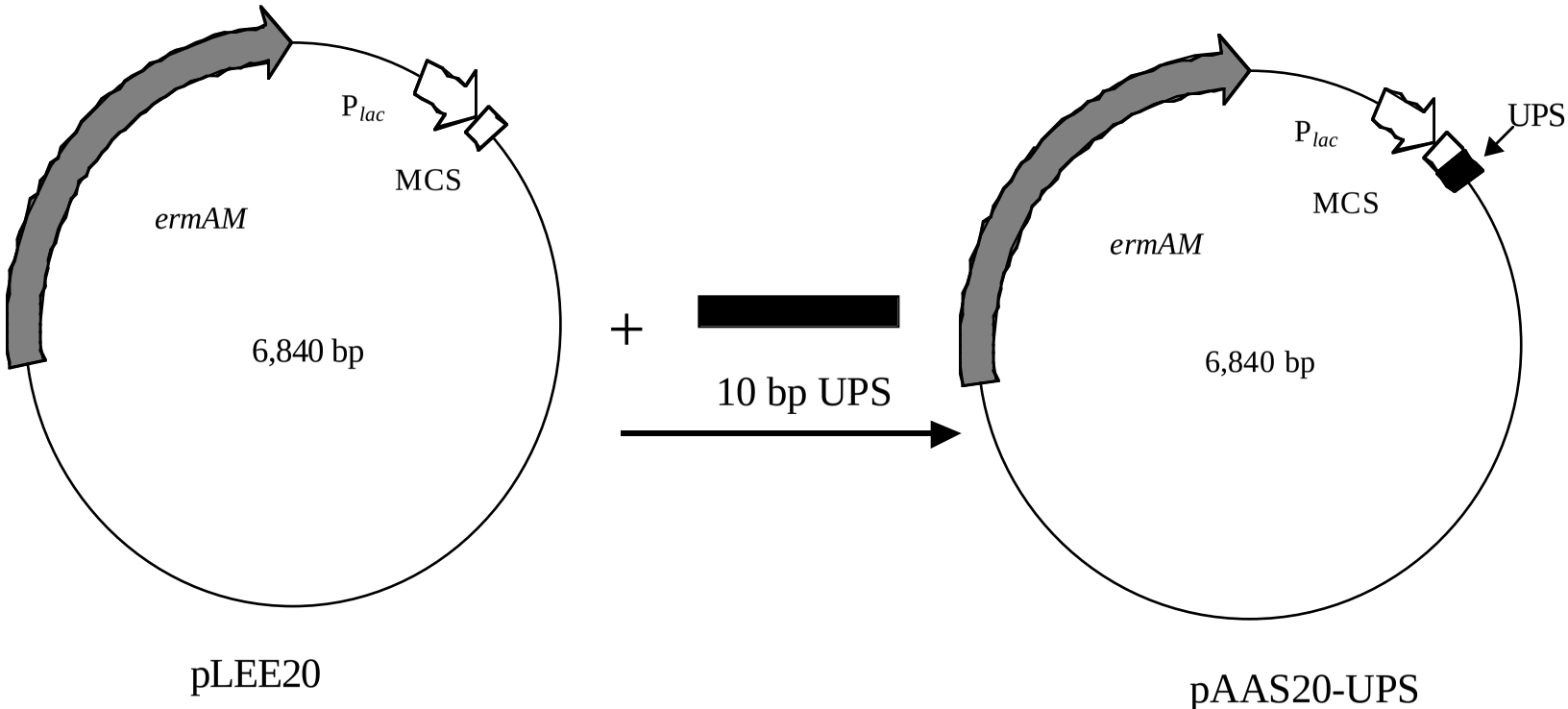


Figure 85. Construction of pAAS20-UPS. The 10 bp uptake sequence was cloned into the unique *Eco* RI site of pLEE20 to create pAAS20-UPS. The presence of the uptake sequence as determined by PCR.

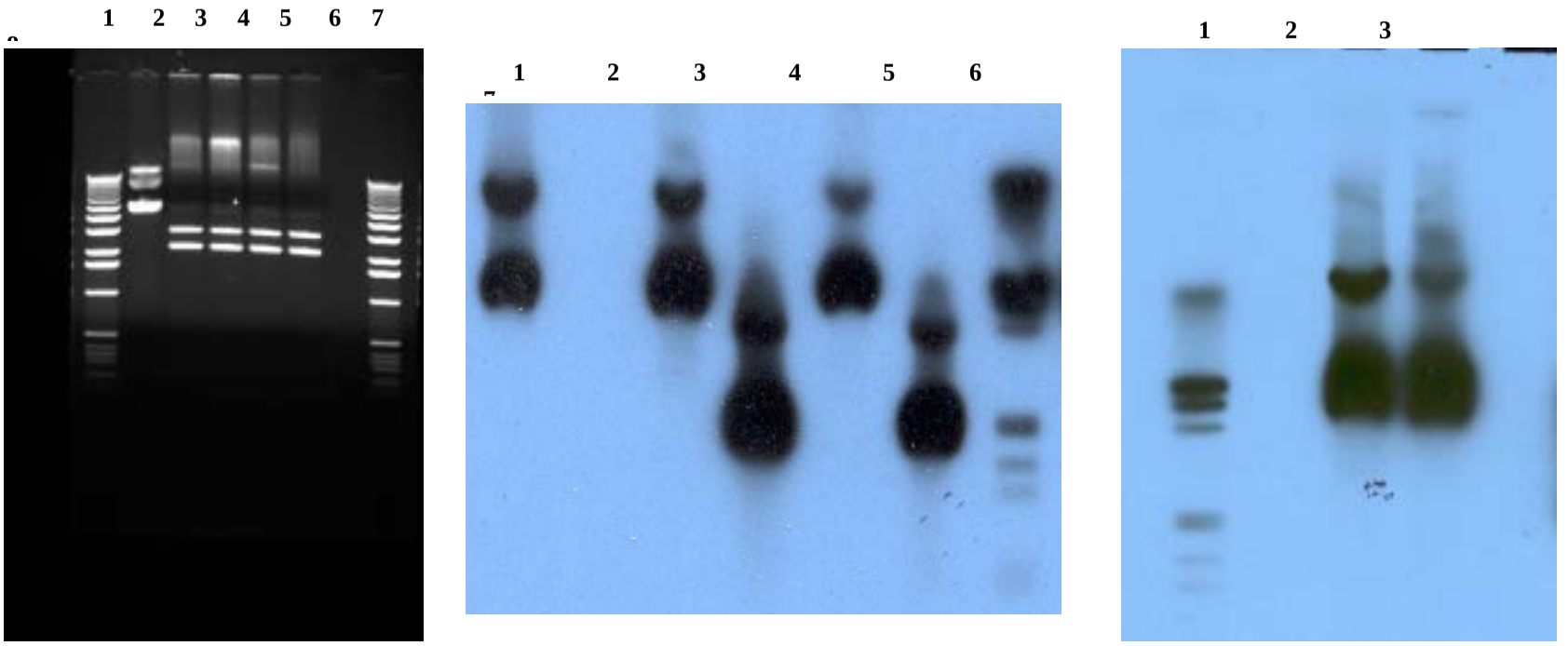


Figure 8b. Genetic characterization of pAAS20-UPS transferred into GC by transformation and conjugation. (**A**) Ethidium bromide stained agarose gel of GC (pAAS20-UPS) plasmids after transformation. Lanes represent plasmid preps: **1**and **8**, 1 kb ladder; **2**, pAAS20-UPS from S17-1; **3-6**, pAAS20-UPS plasmid extracted from GC after transformation. Plasmids in lanes **3-6** exhibited a different pattern as compared to lane **2** plasmid DNA extracted form *E. coli*. (**B**). Southern blot using 560 bp erm probe against plasmid DNA extracted from GC (pAAS20-UPS) after transformation. Lanes represent plasmid preps: **1**, S17-1 (pAAS20-UPS0; **2**, GC F62 cryptic plasmid; **3** and **5**, *E.coli* DH5αMCR (pAAS20-UPS); 4, GC F62 (pAAS20-UPS) after transformation; **6**, GC FA1090 (pAAS20-UPS) after transformation and **7**, λ *Eco* RI-*Hind* III ladder. (**C**) Southern blot using 560 bp erm probe against plasmid DNA extracted from GC (pAAS20-UPS) after conjugation. Lanes represent plasmid preps: **1**, λ *Eco* RI-*Hind* III ladder; **2** , GC F62 cryptic plasmid; **3**, S17-1 (pAAS20-UPS) and **4**, GC F62 (pAAS20-UPS) after conjugation.

pAAS20-UPS after transformation into GC, the different plasmid species were purified from the agarose gel, transformed into DH5aMCR, and tested for ability to be transferred by conjugation, and the presence structural elements such as the multiple cloning size and the erythromycin gene. First, the capacity of the plasmid species to be transferred by conjugation was tested by transformation of the plasmids into *E. coli* S17-1, and then transferred into GC by conjugation as described in the materials and methods.The GC transconjugants obtained in GC agar plates containing erythromycin indicated, **1**) successful conjugal transfer of the plasmid, **2**) the presence of a functional antibiotic marker, erythromycin and **3**) the ability of the species to serve as a shuttle vector given that plasmid DNA could replicate in both GC and *E. coli*. The frequency of conjugation was lower as compared to the transfer of pLEE20 into GC F62 (**Table 23**).

The presence of the multiple cloning site was determined by restriction digestion of the different plasmid species with restriction enzymes present in the pLEE20. Only digestion with *Hind* III led to linear plasmid DNA suggesting loss of the multiple cloning site. The lack of multiple cloning site let us to think that the loss region in the plasmid species could be flanked by the *lac* promoter in front of the multiple cloning site and the erythromycin gene. To determine if this is the case, PCR analysis was performed with primers annealing in the *lac* promoter region and the erythromycin gene. Two PCR products of approximate 900 bp and 1.6 kb in size were obtained from each of the three species of plasmid. The same pattern was observed in pAAS20-UPS plasmid DNA before transformation. This finding suggests that the lost region is not found between the *lac* promoter region.

Table 23. Conjugation frequencies of deleted species of pAAS20-UPS

Donor	Num. of donor (cfu)	Num. of recipients (cfu)	Efficiency (transconjugants/ recipient)
E. coli **S17-1 (pLEE20)**	**1.1×10^{8}**	**1.0×10^{8}**	**4.7×10^{-3}**
E. coli **S17-1 (pAAS20-UPS-LGH)**	**2.4×10^{8}**	**1.0×10^{8}**	**3.5×10^{-7}**
E. coli **S17-1 (pAAS20-UPS-LGL)**	**1.6×10^{8}**	**1.0×10^{8}**	**2.3×10^{-7}**
E. coli **S17-1 (pAAS20-UPS-LLL**	**1.3×10^{8}**	**1.0×10^{8}**	**4.7×10^{-7}**

and the erythromycin gene. The combined data do not allowed us to be conclusive about the nature of the deletion suffered by pAAS20-UPS after transformation into GC. On the other hand, a better characterization of the deleted plasmid species could lead to the development of an improved shuttle vector. The occurrence of deletions in plasmids transferred into GC by transformation has been reported before. Sox et al. (1979), found that 25% of the GC transformed with a penicillinase encoding plasmid carried a larger or smaller version of the original plasmid. The nature of the deletion and/or insertions was determined by restriction digestion analysis of modified plasmids as compared to the original plasmid. The deletion and/or insertion events can be triggered during processing of the circular plasmid at the time of transformation. It has been shown that after transformation of the circular plasmid the molecule is processed to a linear double-stranded DNA and the transformants that arise are due to circularization of some of the DNA linear molecules (Biswas et al., 1986). It is important to remember that double-stranded DNA is the substrate for the innate restriction enzymes in the recipient bacteria. Given that the plasmid DNA used for GC transformation was extracted from *E. coli*, one can conclude that the restriction-modification systems present in GC played a role in the plasmid DNA degradation (Hill, 1999; Stein et al., 1988; Stein et al., 1995).

It has been speculated that one reason for the origin of plasmid re-arrangements such as deletion and/or insertion can be the presence of similar sequences shared by the cryptic plasmid and the GC genome. The shuttle vector utilized is a derivative of the cryptic plasmid (Korch et al., 1985; Sarandopoulos and Davies, 1993a,b) and a spontaneous deletion of a conjugative plasmid (Nassif et al.,

1991;Stein et al., 1983a; Stein et al., 1983b). Hagblom et al. (1986) found cryptic plasmid integrated in the GC genome in cryptic plasmid bearing and plasmid free strains of GC. In at least two GC strains an intact copy of the cryptic plasmid was found integrated in the GC genome. Integration of the cryptic plasmid led to recombination given that GC DNA genome sequences were found intercalated into the cryptic plasmid sequences. It is important to mention that this type of recombination happened at low frequency. In contrast, Sarandopoulos and Davies (1993) found a limited number of sequences shared by the plasmid and the GC genome by Southern blot using subcloned fragments of the cryptic plasmid as probes, as evidenced by the low stringent hybridization between the probes and the GC genome. The latter support in a way the findings of Hagblom et al. (1986) in which the frequency that the cryptic plasmid integrated in the genome was low.

To overcome the restriction barrier that might prevent successful transfer of the plasmid from *E. coli* to GC, pAAS20-UPS plasmid DNA was transformed into *E. coli* S17-1 and transferred to GC by conjugation. Transconjugants were selected on GC-erm-colistin agar plates. Plasmid preps from putative transconjugants were prepared and hybridized with the erythromycin probe by Southern blot as before. The results showed that no degradation of the plasmid DNA after conjugation occurred (**Fig. 86 C**). Therefore conjugation was shown to be the preferable way to transfer DNA into GC using pLEE20.

B. Construction of pAAS20-UPS-*gfp* and pAAS20-UPS-*lacZ*

A *Neisseria*- reporter gene shuttle vector with by which transcriptionally-regulated GC genes can be isolated and characterized was designed. Two reporter genes were selected as candidates, and the advantages and disadvantages of the selected reporter genes are presented in **Table 24**.

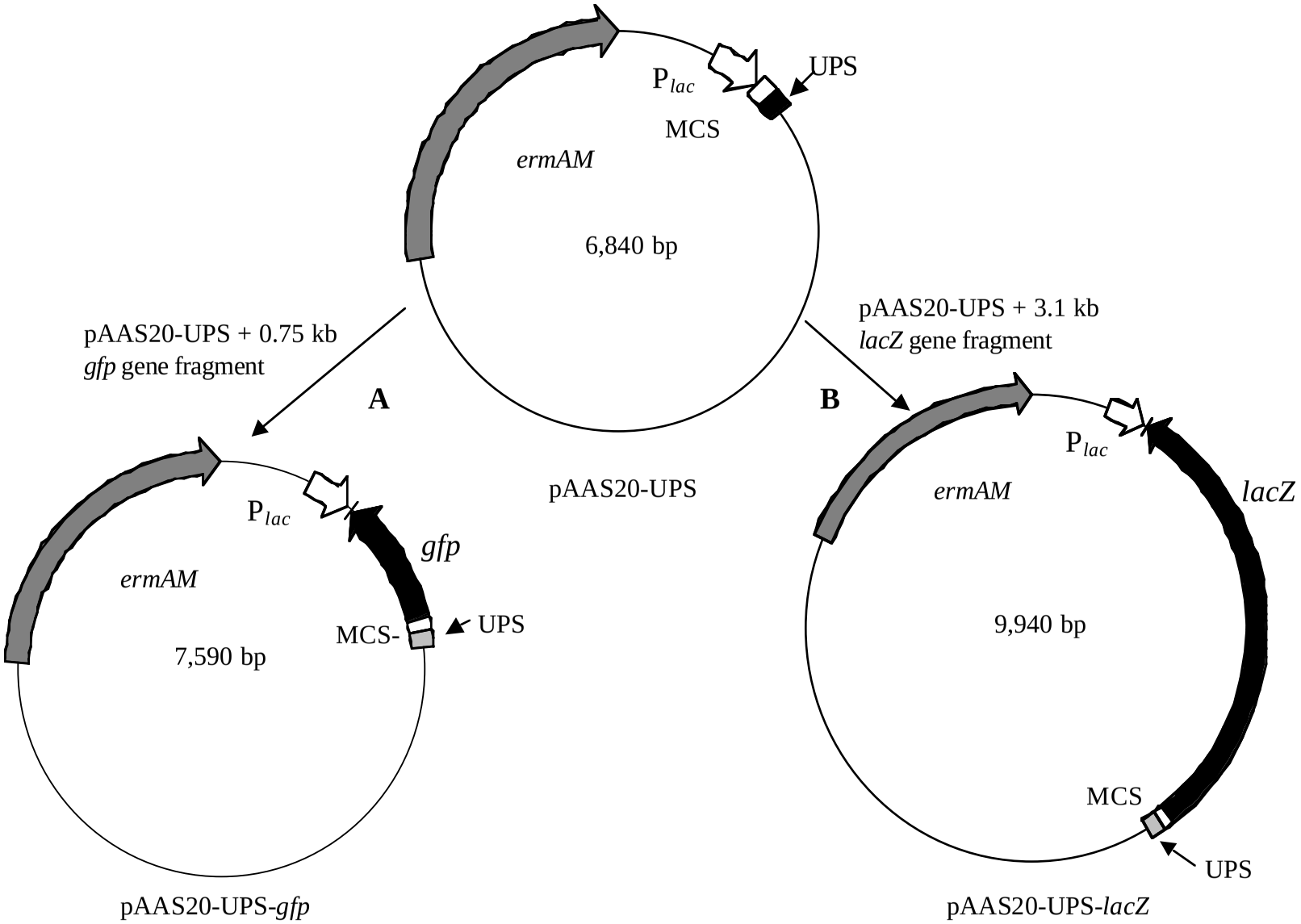
GFP has become a highly effective reporter molecule for monitoring gene expression (Dhandayuthapani et al., 1995). It has been expressed in both eukaryotes (Clark et al., 1994) and prokaryotes. In prokaryotes, GFP has been a very successful tool for studying the pathogenesis of *Salmonella* (Valdivia and Falkow, 1997), *Mycobacterium* (Dhandayuthapani et al., 1995; Kramer et al., 1995), and *E. coli* (Chalfie et al., 1994). Of the many *gfp* genes available, we selected the version by Cormack et al. (1996), *gfp*-mutant 2, since it is engineered so that its excitation maximum is at 488 nm, making it ideal for FACS detection and fluorescence microscopy. Epifluorescence microscopy, laser scanning confocal microscopy, fluorescence spectroscopy, and flow cytometry are other techniques that can be used to detect organisms expressing Gfp (Dhandayuthapani et al., 1995).

The *gfp* gene was isolated on a *Bam* HI-*Pst* I fragment from pKEN-*gfp* mut2 (Cormack et al., 1996) (obtained from Stanley Falkow, Stanford Univ.) and cloned in the same restriction sites of pAAS20-UPS giving pAAS20-UPS-*gfp* (**Fig. 87**). The *lacZ* gene was obtained as a *Bam* HI- *Sca* I from pQF50 (Farinha and Kropinski, 1990).

Table 24. Comparison of reporter genes *gfp* and l*acZ*

Reporter gene	Advantages	Disadvantages
Green fluorescence protein	1) Allows monitoring gene expression and protein localization *in vivo*, *in situ* and in real time 2) Does not require a co-factor or invasive treatment. 3) Detection can be made by FACS, fluorescence microscopy, etc.	1) Requires high number (10^5-10^6) of molecules in order to be detected.
β-galatosidase	1) Very easy to detect (enzymatic activity or stains for expression) 2) Previous success in GC 3) Only one copy is required in order to be detected 4) Chromosomal fusions can be constructed	1) Invasive treatment is required for detection (depending on the technique bacteria needs to be lysed)

Figure 87. Construction of pAAS20-UPS-*gfp* or *lacZ*. The *gfp* or *lacZ* gene were cloned into the *Bam* HI-*Pst* I sites of pAAS20-UPS. Ligated DNA was transformed into *E. coli* DH5αMCR and putative clones selected on LB with erythromycin. Putative clones carrying the *gfp* gene were selected by restriction digestion with *Bam* HI- *Pst* I. Putative clones carrying the *lacZ* gene were selected by blue/white selection on XGal**,** and then by *Eco* RI restriction digestion.


P lac
UPS
MCS
ermAM
6,840 bp
pAAS20-UPS
pAAS20-UPS + 0.75 kb
gfp gene fragment
A
B
pAAS20-UPS + 3.1 kb
lacZ gene fragment
P lac
gfp
ermAM
MCS-
UPS
7,590 bp
pAAS20-UPS-gfp
P lac
ermAM
lacZ
9,940 bp
MCS
UPS
pAAS20-UPS-lacZ

The fragment was gel isolated and purified. The isolated 3.1 kb band was blunt-ended with Vent polymerase according to the manufacturer and cloned into PCR-blunt. Clones containing the *lacZ* gene were selected by white/blue screening. Plasmid preps were prepared and restriction digestion with *Eco* RI was done to verify the presence of the insert.

The construction of pAAS20-UPS-*gfp* was successful. In contrast, although the *lacZ* version was obtained, its size made its general manipulation difficult, and therefore, only pAAS20-UPS-*gfp* was further characterized.

C. Construction of pAAS20-UPS-*gfp* control plasmid

A control plasmid was constructed to test the ability of pAAS20-UPS-*gfp* to support the transcription of *gfp* from a gonococcal promoter and to test the stability of Gfp in GC (**Fig. 88**). The plasmid contained the constitutively expressed promoter of the gene encoding the major GC porin P. I, *porIB* (Carbonneti et al., 1988). The P.I promoter from strain FA1090 was amplified using primers 3-97 F and 4-97 B based on published sequences (Carbonneti et al., 1988). The resultant PCR product was cloned into PCR-blunt and verified by nucleotide sequencing before cloning into pAAS20-UPS-*gfp*. The constructs were transformed into *E. coli* S17-1 and transferred into GC by conjugation (Seifert and So, 1991). To test the expression of the *gfp* gene driven by the P.I promoter, wet mount slides of S17-1 and GC F62 carrying pAAS20-UPS-*gfp* were prepared and analyzed under the fluorescence microscope. Green fluorescent bacteria were only observed in wet mount preps from S17-1 carrying the plasmid (**Fig. 8 9 A**).

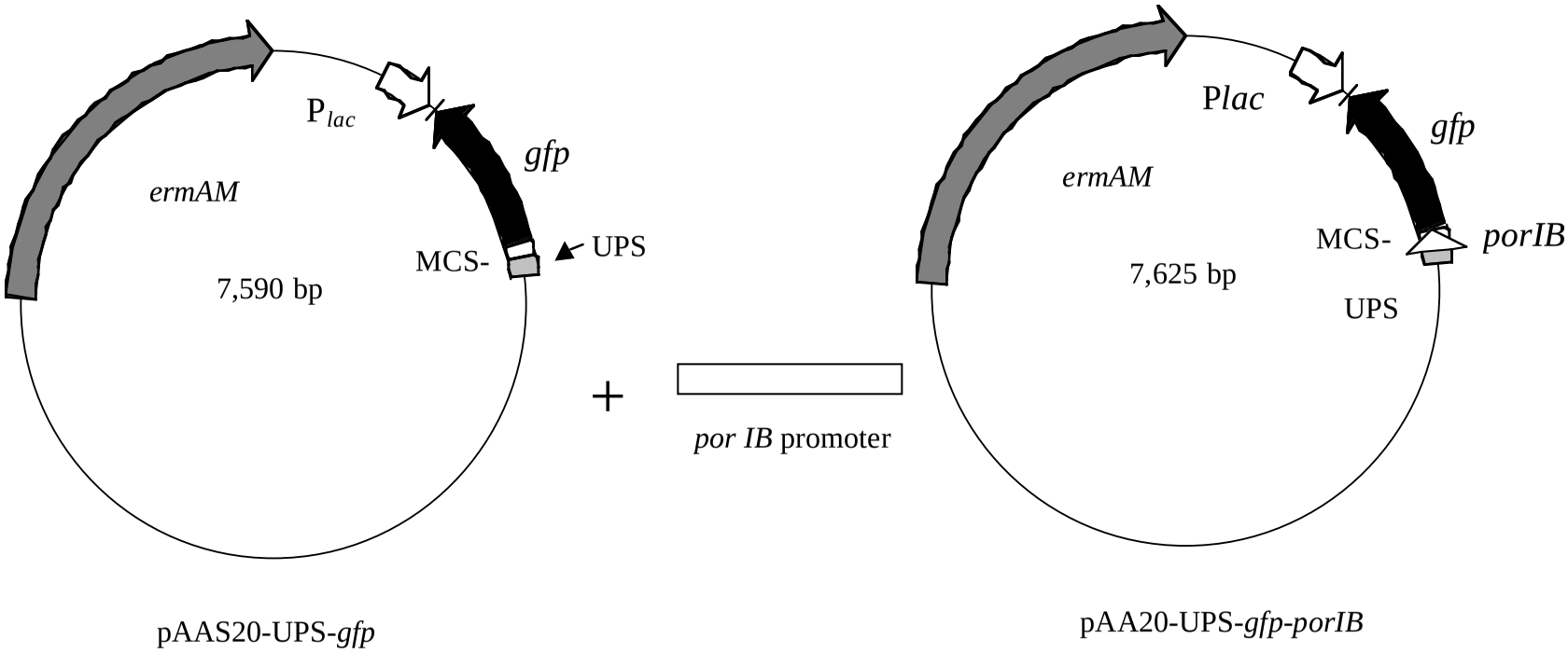
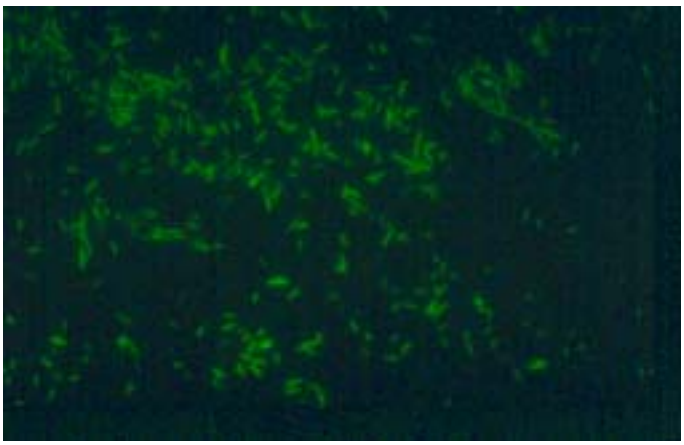


Figure 88. Construction of pAA20-UPS-*gfp-porIB* control plasmid. The *por IB* promoter was cloned into the *Not* I –*Bam* I sites of pAAS20-UPS. Ligated DNA was transformed into *E. coli* DH5αMCR and putative clones were selected by PCR-screening. The control plasmid was transformed into *E. coli* S17-1 and then transferred by conjugation into GC.

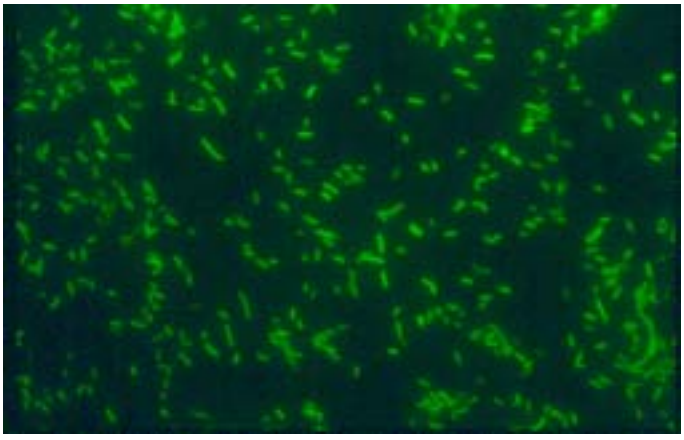
Figure 89. Fluorescence microscopy of bacteria carrying pAAS20-UPS-*gfp*. (**A**) *E. coli* S17-1, (**B**) GC F62 and (**C**) *E. coli* S17-1 carrying plasmid extracted from GC F62.

(**A**) S17-1 (pAAS20-UPS-*gfp*)



(**B**) GC F62 (pAAS20-UPS-*gfp*)

(**C**) S17-1 (pAAS20-UPS-*gfp* from GC F62)



No green fluorescent bacteria was obtained from GC F62 (**Fig. 89 B**). Based on this results we hypothesize that the pAAS20-UPS-*gfp* plasmid suffered a modification during the conjugation transfer or simply that the Gfp protein was not been expressed.

To rule out that a plasmid modification after conjugal transfer occurred, pAAS20-UPS-*gfp* was extracted from GC and transformed first into *E. coli* DH5αMCR, and then into *E. coli* S17-1 to circumvent restriction barriers. Interestingly, green fluorescent *E. coli* were observed, suggesting that pAAS20-UPS-*gfp* did not suffer any modification after conjugation into GC (**Fig. 89 C**).

To determine if the Gfp protein was made in GC, a western blot was performed using anti-Gfp antibody as described by the manufacturer (Clontech, CA). Bacterial lysates were prepared as before and protein was fractionated on a 10% SDS-PAGE gel (**Fig. 90 A**). GFP protein was detected in supernatant and pellet fractions of *E. coli* S17-1 (pAAS20-UPS-*gfp*) (**Fig. 90 B**, lanes 4 and 5). In contrast no protein corresponding to the size of Gfp was detected in GC (**Fig. 90 B**, lanes 6 and 7), although a slightly higher band as compared to the native Gfp protein is observed in lane 7. These data suggest that Gfp protein was not expressed in GC. The reason for this lack of expression remains unknown.

V- Construction of suicide vector to create *lacZ* chromosomal fusion.

The construction of the following suicide vector was designed based on Ohlsen et al. (1997 and **Fig. 91**) to test the expression of promoters of interest in a

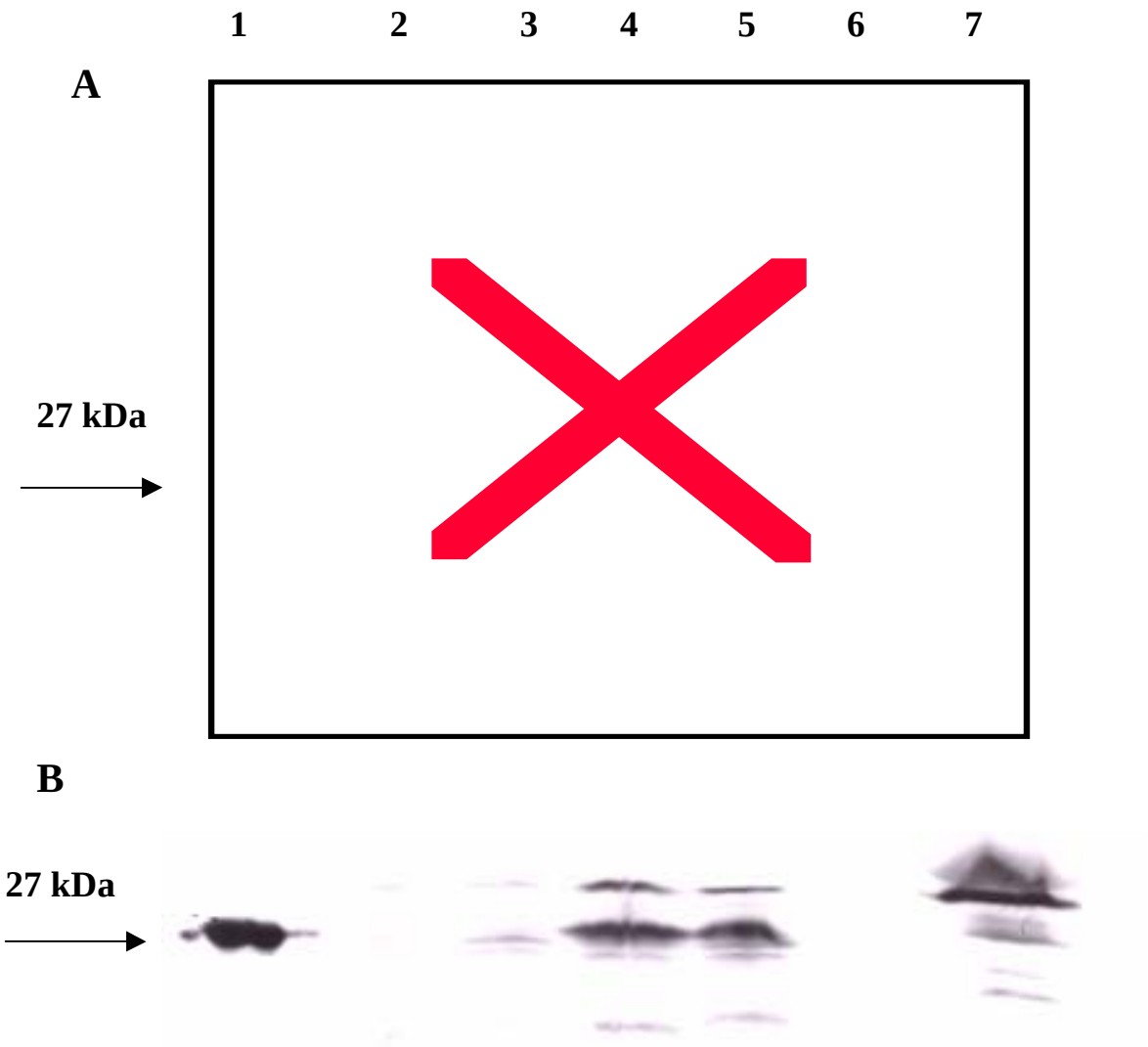
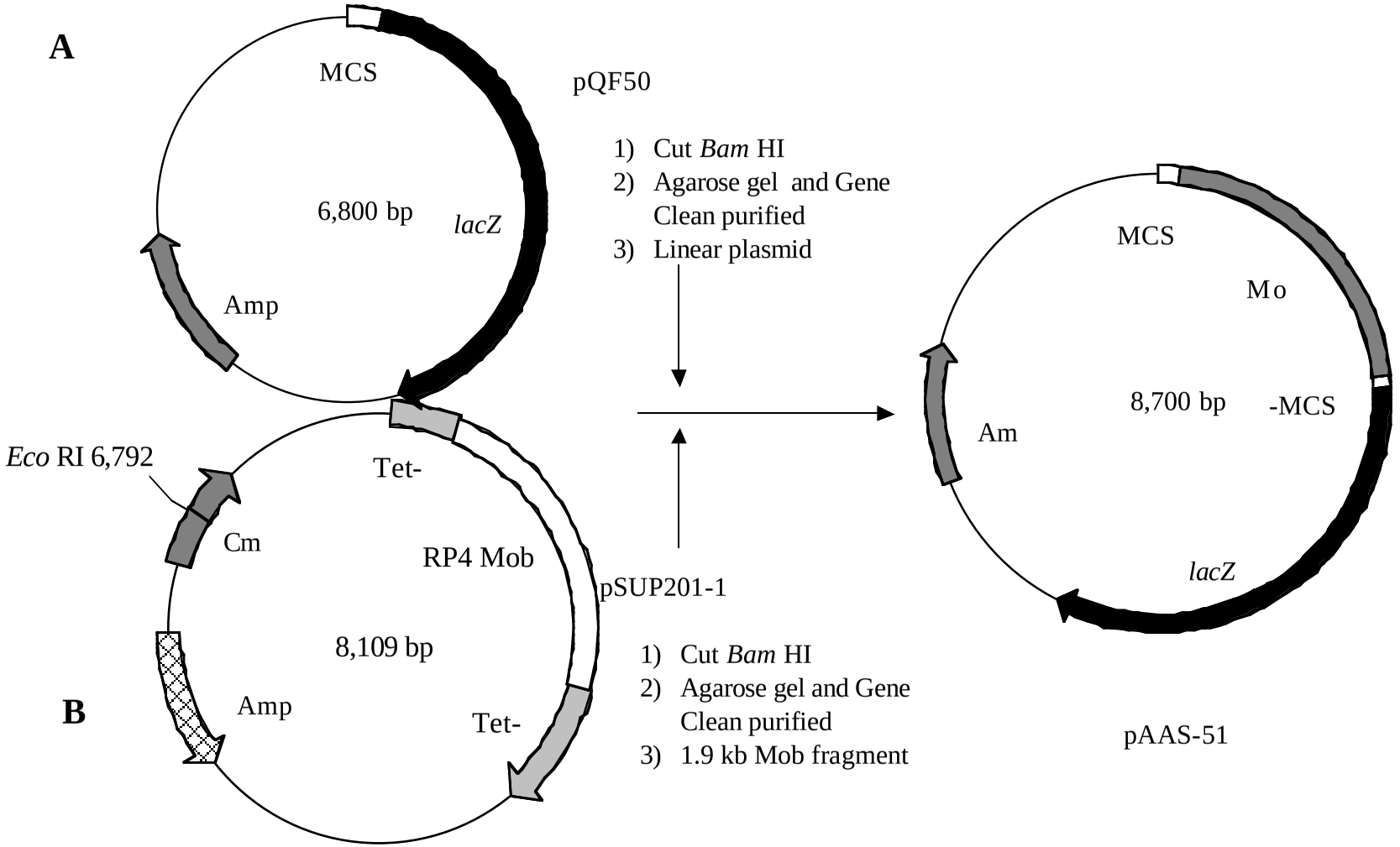


Figure 90. (A) SDS-PAGE gel. Lanes: **1**, purified Gfp protein; **2**, S17-1 supernatant; **3**, S17-1 pellet; **4**, S17-1 (pAAS20-UPS-*gfp*) supernatant; **5**, S17-1 (pAAS20-UPS-*gfp*) pellect; **6**, GC F62 (pAAS20-UPS-*gfp*) supernatant; and **7**, GC F62 (pAAS20-UPS-*gfp*) pellect. (**B**) No Gfp protein was observed in the GC F62 fractions by western blot.

Figure 91. Construction of suicide vector pAAS-51. (**A**) pQF50 and (**B**) pSUP201-1. The Mob region contained in the 1.9 kb *Bam* HI fragment was cloned into the *Bam* HI site of pQF50 to create pAAS-51.


A
MCS
pQF50
6,800 bp
lacZ
Amp
1) Cut Bam HI
2) Agarose gel and Gene Clean purified
3) Linear plasmid
MCS
Mo
8,700 bp
-MCS
Am
lacZ
pAAS-51
Eco RI 6,792
Tet-
Cm
RP4 Mob
pSUP201-1
8,109 bp
B
Amp
Tet-
1) Cut Bam HI
2) Agarose gel and Gene Clean purified
3) 1.9 kb Mob fragment

way that mimics the frequency of the gene on the GC genome. Using this method the wild-type gene is not disrupted, thereby allowing the study of essential genes (**Fig. 92 and 93**). Plasmid vector pQF50 (Farinha and Kropinski, 1990), which possess a promoterless *lacZ* gene was used as backbone for the suicide vector. The *lacZ* gene was used as the reporter since β-galactosidase it is easier to detect than Gfp in single copy fusions. First, a 1.9 kb *Bam* HI fragment from pSUP-201-1 containing the Mob region form pRT733 required for conjugation (Taylor et al., 1989) was cloned into pQF50 *Bam* HI site. After digestion, both cut pQF50 plasmid and fragment were agarose gel purified as describeb in the materials and methods. Fragments were ligated and transformed into DH5αMCR. Putative clones were screened by restriction digestion of plasmid DNA using *Bam* HI. Three putative clones were selected for further characterization. To test the ability of the suicide vector to be transferred by conjugation, plasmid DNA of the three selected clones were transformed into *E. coli* S17-1and mated with *E. coli* HS-4. Transconjugants were selected on LB-Amp-NaI.

Clone pAAS51-11 was selected as the plasmid vector to generate a chromosomal fusion in GC due to its high frequency of conjugation (**Table 25**). This plasmid was not used further due the presence of restriction sites in the 1.9 kb Mob fragment that were also present in the multiple cloning site.

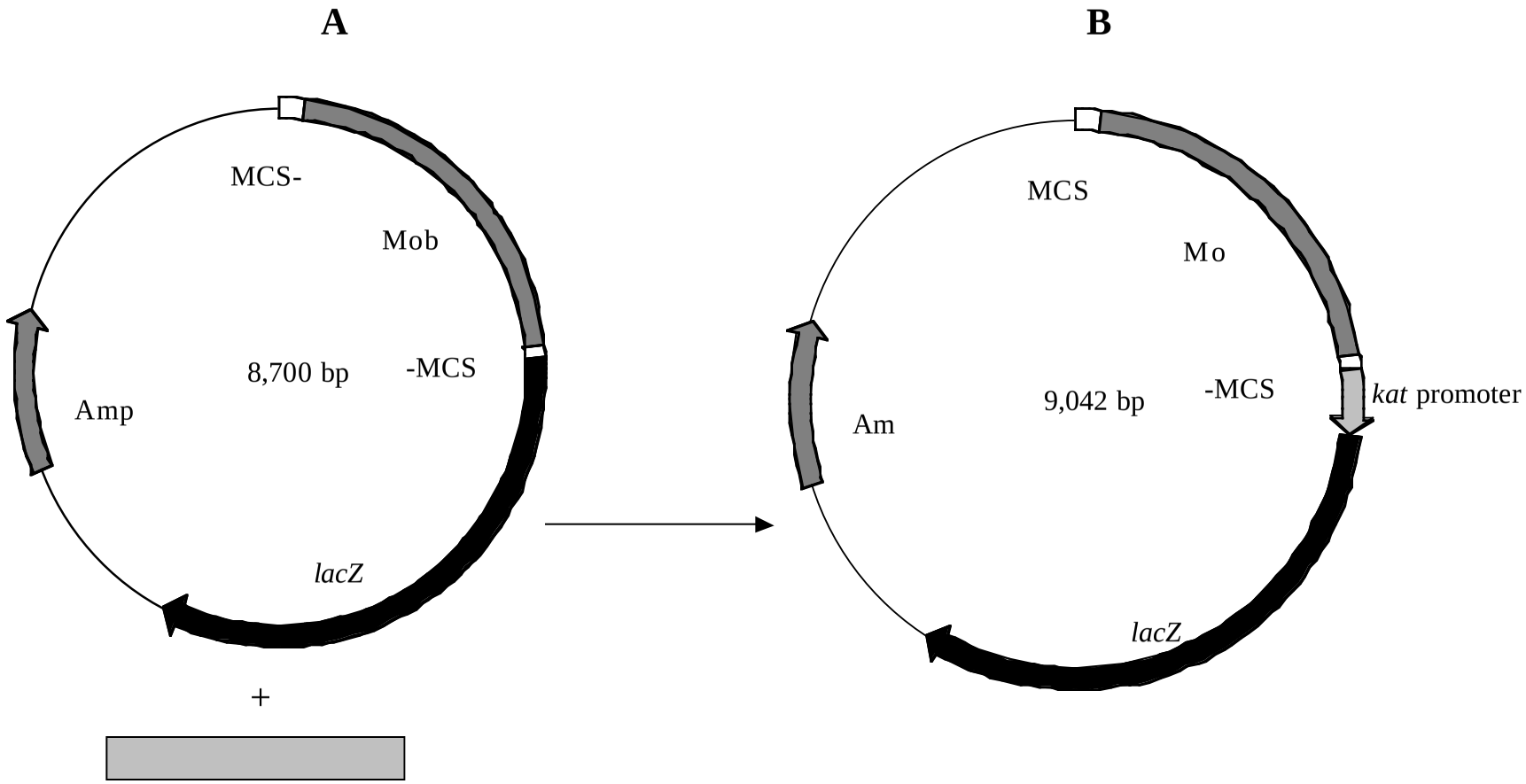


Figure 92. Construction of gene fusion using pAAS-51 suicide vector. The promoter of interest is PCR amplified and cloned into the *Sma* I site of pAAS-51. Ligated DNA is transformed into *E. coli* DH5αMCR and then selected by blue/white selection.

Figure 93. Recombination of suicide vector pAAS-51 carrying a promoter into GC chromosome.

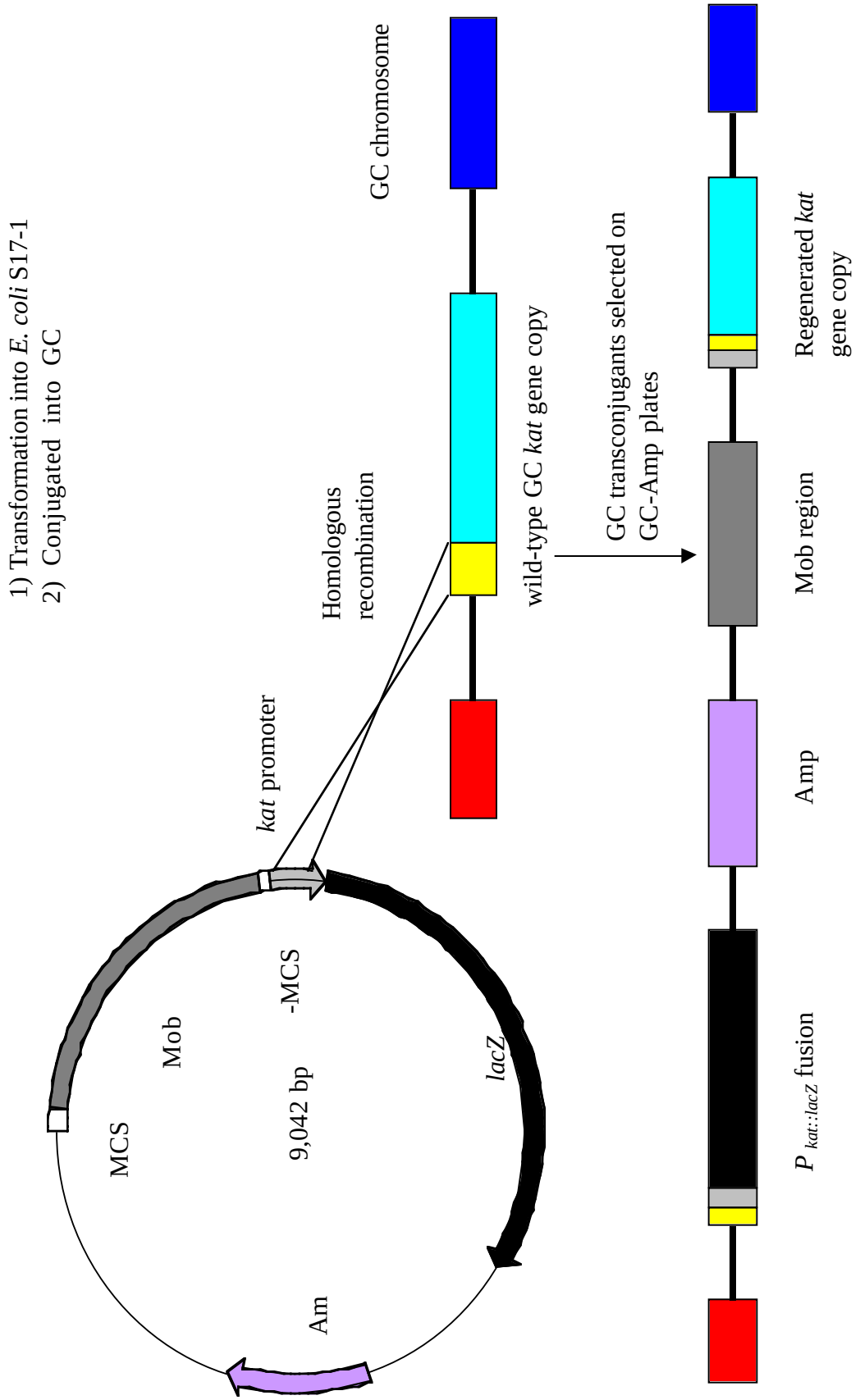

1) Transformation into E. coli S17-1
2) Conjugated into GC
GC chromosome
Homologous recombination
kat promoter
wild-type GC kat gene copy
GC transconjugants selected on GC-Amp plates
MCS
Mob
-MCS
9,042 bp
lacZ
Am
Amp
Mob region
Regenerated kat gene copy
P kat::lacZ fusion

Table 25. Conjugation frequencies of selected pAAS-51 constructs

Donor	Num. of donor (cfu)	Num. of recipients (cfu)	Efficiency (transconjugants/ recipient)
***E. coli* S17-1 (pAAS51-4)**	1.6×10^{7}	4.0×10^{8}	1.1×10^{-4}
***E. coli* S17-1 (pAAS51-11)**	3.3×10^{7}	4.0×10^{8}	1.4×10^{-3}
***E. coli* S17-1 (pAAS51-12)**	2.9×10^{7}	4.0×10^{8}	4.6×10^{-8}

VI- *Opa J*, a silent locus for complementation and the generation of chromosomal fusions

Opa J belongs to the family of phase and antigenic variable opacity proteins expressed in the surface of GC strain FA1090. The gene encoding for Opa J is permanently mutated and by consequence, no protein is expressed. We hypothesized that this site might therefore be a good region for introducing gene fusions and genes to complement mutations in GC. The *opa J* gene was cloned before by PCR amplification using primers F36 and R1 into PCR-blunt creating plasmid pOJ4. Then the cloned gene was released from pOJ4 by digesting the plasmid with *Bam* HI and *Eco* RI and into the same restriction sites in pBR322 generating pOJ41.1 (**Fig. 94 A**). Because a unique *Cla* I site was identified in between the two HV regions, pOJ41.1 was digested with *Ssp* I and *Eco* RV to eliminate the *Cla* I site of the pBR322 generating pOJ41.1. The km cassette was released from pUC18-Km with *Sma* I and cloned into pOJ41.1 blunt-ended *Cla* I site generating pOJ4.2. (**Fig. 94 B**) To test the capacity of pOJ4.2 to target the Opa J region, a plasmid prep was prepared and the Opa J-km fragment was released by digesting the plasmid DNA with *Bam* HI and *Sca* I. The linear fragment was agarose gel purified and transformed by spot dilution transformation as before. Transformants were selected on GC-km agar. The insertion of Opa J-km into the *opa J* locus was determined by PCR using primers specific to different Opas (**Table 19**).

Although transformants were obtained at high frequency, PCR analysis of putative GC transformants revealed that the antibiotic marker was not inserted in

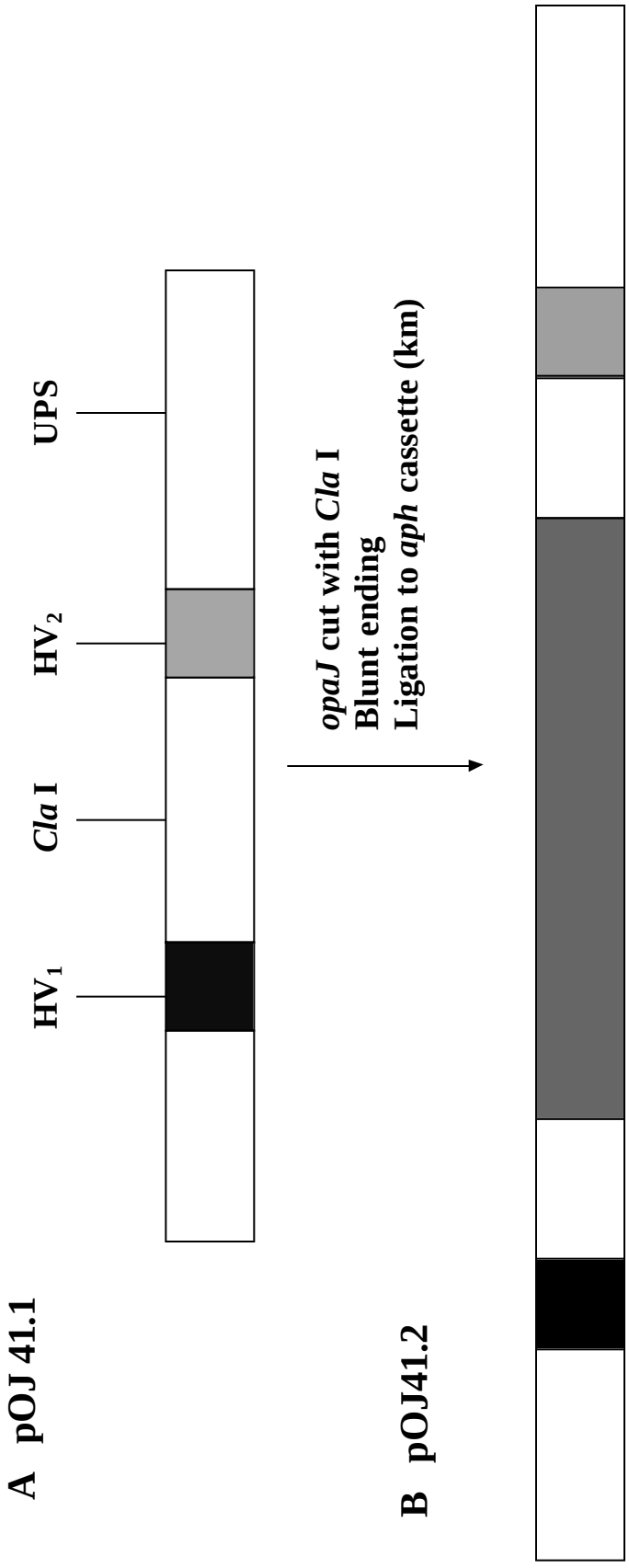


Figure 94. Schematic representation of pOJ4.1 and introduction of *aphA*-3 cassette into *opa J*

any the *opa* genes tested (**Fig. 95**). Due to the random insertion of the system, it was concluded that Opa J locus would not be useful as a target for chromosomal integration system.

VII- Construction of *kat* and *aniA* lacZ fusions

A second approach to develop chromosomal fusions was designed by using the plasmid pQF50 as a backbone. The promoters from the gonococcal *kat* and *aniA* genes were cloned into pQF50. The *kat* gene was released by cutting with *Bsr* BI and *Nla* IV and cloned into *Sma* I site of pQF50. The *ani* A promoter was isolated in a *Hind* III- *Sma* I site and cloned into the same restriction sites into pQF50 (**Fig. 96**). The promoters were ligated as before and transformed into DH5aMCR. The *kat::lacZ* and *ani A:: lacZ* expressing clones were selected by blue/white selection. The presence of the promoters were verified by PCR using the primers 50-00 F and 51-00 B for *kat* promoter; and F7 and R10 for *ani A* promoter.

A 145 bp PCR fragment containing the region of the Mob (145 bp) required for conjugational transfer and the uptake sequence necessary for DNA competence in GC was amplified. These modifications were done to increase the versatility of the vector. The specific region of the Mob required for conjugal transfer (99 bp) region was amplified using the primers 54-00 F and 55-00 B and using the 1.9 kb region containing the Mob sequence from pSUP201-1. The uptake sequence was included at each end of the PCR product by the primers. The Mob-UPS fragment was cloned into pQF50 *Bam* HI-*Xba* I sites (**Fig. 97**). The plasmid can be transferred by conjugation or transformation.

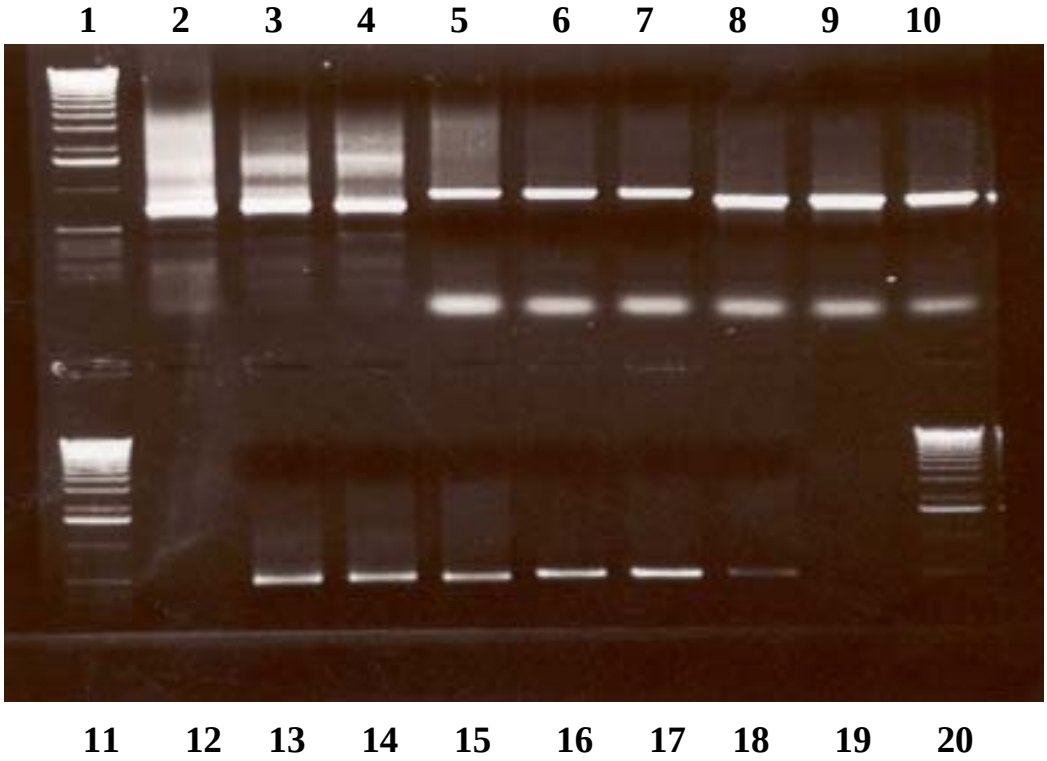


Figure 95. PCR analysis of chromosomal recombination of *opaJ-aph*A-3 in GC FA1090 and NGAAS-501. In order to identify the site of insertion, genomic DNA from GC FA1090 and NGAAS-501 transformed with *opaJ-km* linear fragment was analyzed by PCR using primers specific to the different Opa genes. Lanes **1**, **11** and **20**, 1 kb ladder; **2**, **5**, **8**, **13** and **16**, FA1090; **3**, **6**, **9**, **14** and **17**, FA1090-*opaJ-km*-8; and **4**, **7**, **10**, **15** and **18**, NGAAS-501 *opaJ-km*-13. PCR products specific to each opa gene are: **2-4**, *opaJ*; **5-7**, *opa I*; **8-10**, *opa B/D*; **13-15**, *opa C* and **16-18**, *opa F*. No shift in size was observed suggesting that *opaJ-km* did not recombine in either of the opa genes tested.

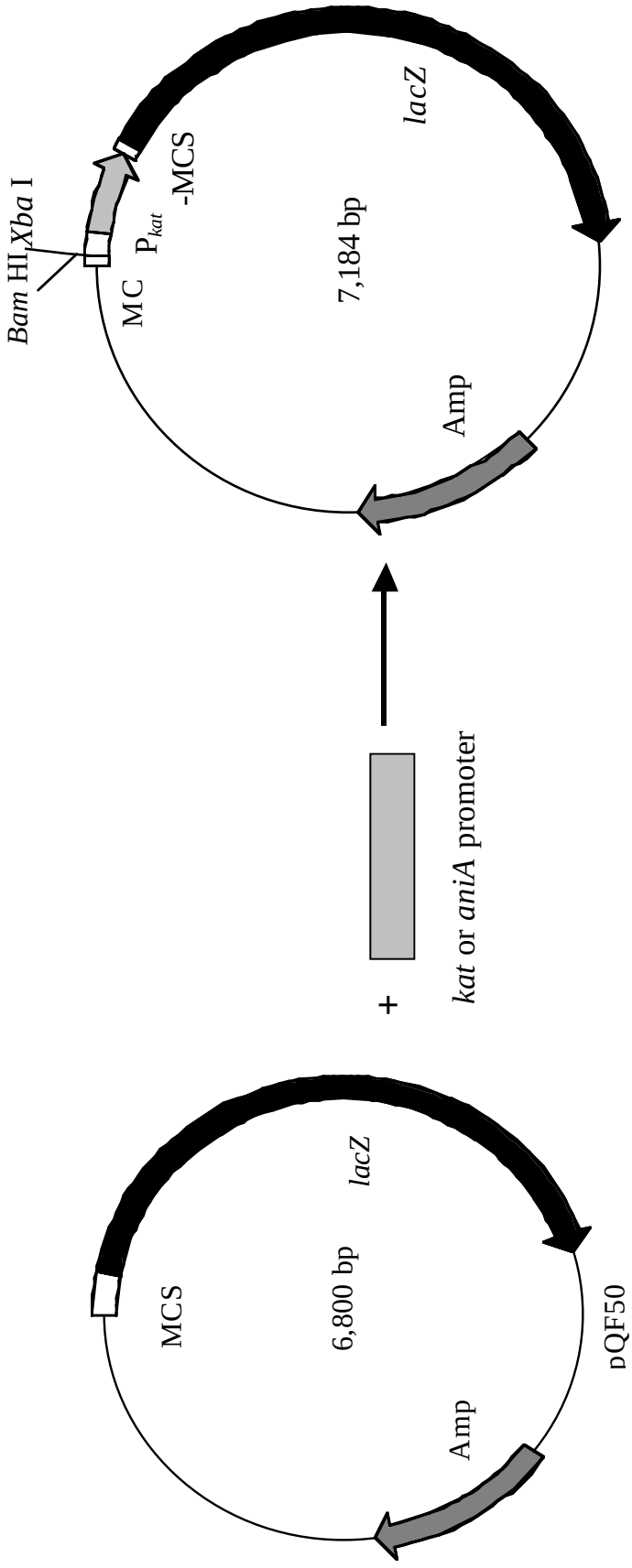


Figure 96. Construction of *kat::lacZ* and *aniA:: lacZ* fusions. Both promoters were cloned into the *Sma* I site of pQF50. The presence of the promoters was determined by blue/white selection in *E. coli* DH5αMCR ,PCR and sequencing.

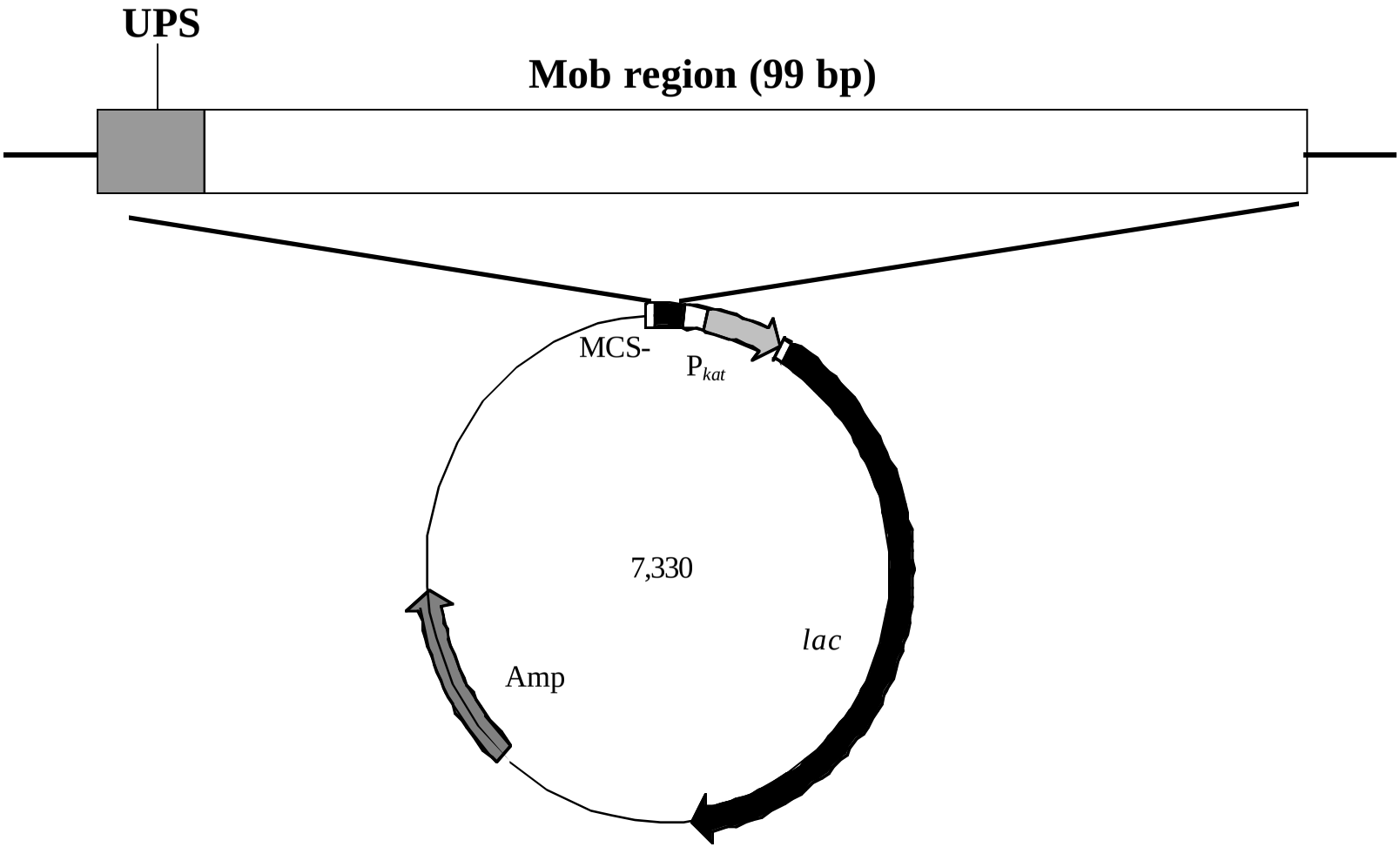


Figure 97. Cloning of the Mob-UPS fragment into *kat::lacZ* or *aniA:: lacZ* fusions.

Transformation and conjugation of GC was done as described in the material and methods. The amplified PCR product was cloned into PCR-blunt and the putative clones were screened by PCR using M13 primers. Two putative clones were selected for sequencing. The ability to be transferred by conjugation was tested by using *E. coli* strains S17-1 and HS-4 as donor and recipients, respectively.

The functionality of the cloned Mob region was demonstrated by the high conjugation frequency between *E. coli* strains (**Table 26**). Transfer of fusions from *E. coli* S17-1 to GC, however were unsuccessful in that no recipients containing the pQF50 *kat::lacZ* or *aniA::lacZ* integrated in the chromosome.

VIII- *proBA*, a silent locus for complementation and the generation of chromosomal fusions

The *pro BA* gene was amplified using primers 58-01 B and M13 reverse and pLES 94 plasmid as a template (**Fig. 98**). The 2.3 kb PCR fragment was agarose gel purified, and cloned into PCR-Blunt and transformed into DH5aMCR. Putative transformants were screened by *Eco* RI and *Bam* HI-*Hind* III restriction digestion of plasmid DNA. Clone 8.3 was selected for sequence confirmation (**Fig. 99**). Initial sequencing was performed by using forward and reverse M13 primers and completed with primers 59-01 F and 60-01 F. For further manipulation the cloned *proBA* gene was released form PCR-Blunt by *Eco* RI and cloned into pACYC184 in the same site. The fragments were ligated and then transformed into DH5αMCR. The putative clones were screened first by disruption of the chloramphenicol gene

Table 26. Conjugation frequency of pQF50-based plasmids carrying *lacZ* fusions transferred between *E. coli* strain S17-1 and HS-4

Donor	Num. of donor (cfu)	Num. of recipients (cfu)	Efficiency (transconjugants/ recipient)
***E. coli* S17-1 (pCRAAS-MobUPS)**	1.7×10^{7}	8.5×10^{7}	1.7×10^{-7}
***E. coli* S17-1 (pAAS52-P*kat::lacZ*)**	1.6×10^{7}	9.9×10^{7}	1.4×10^{-3}
***E. coli* S17-1 (pAAS52-P*aniA::lacZ*)**	1.6×10^{7}	8.8×10^{7}	1..3 $\times 10^{-3}$

Figure 98. Schematic representation of pLES94 (**A**) and PCR-amplification of 2.3 kb *proBA* fragment (**B**).

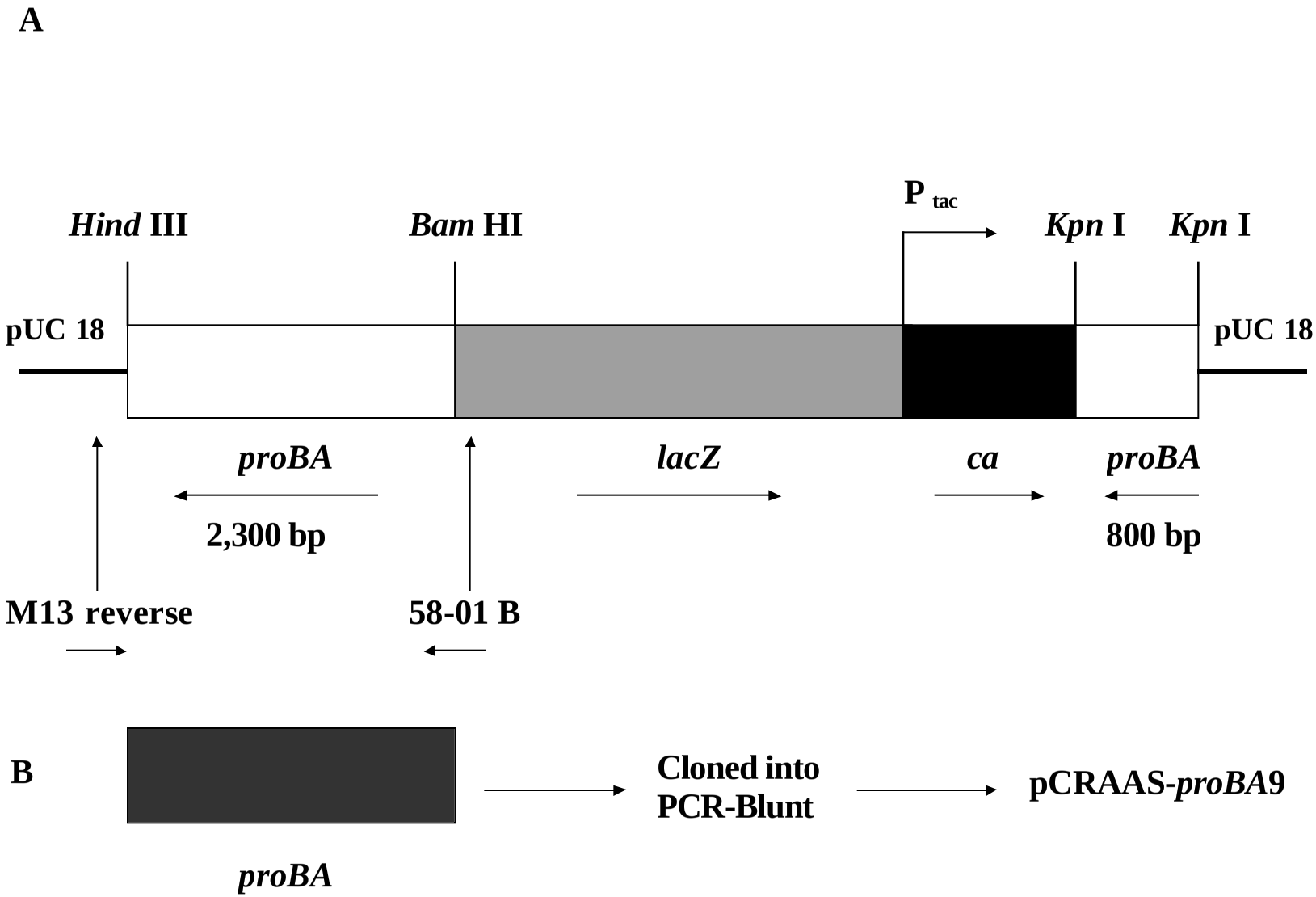
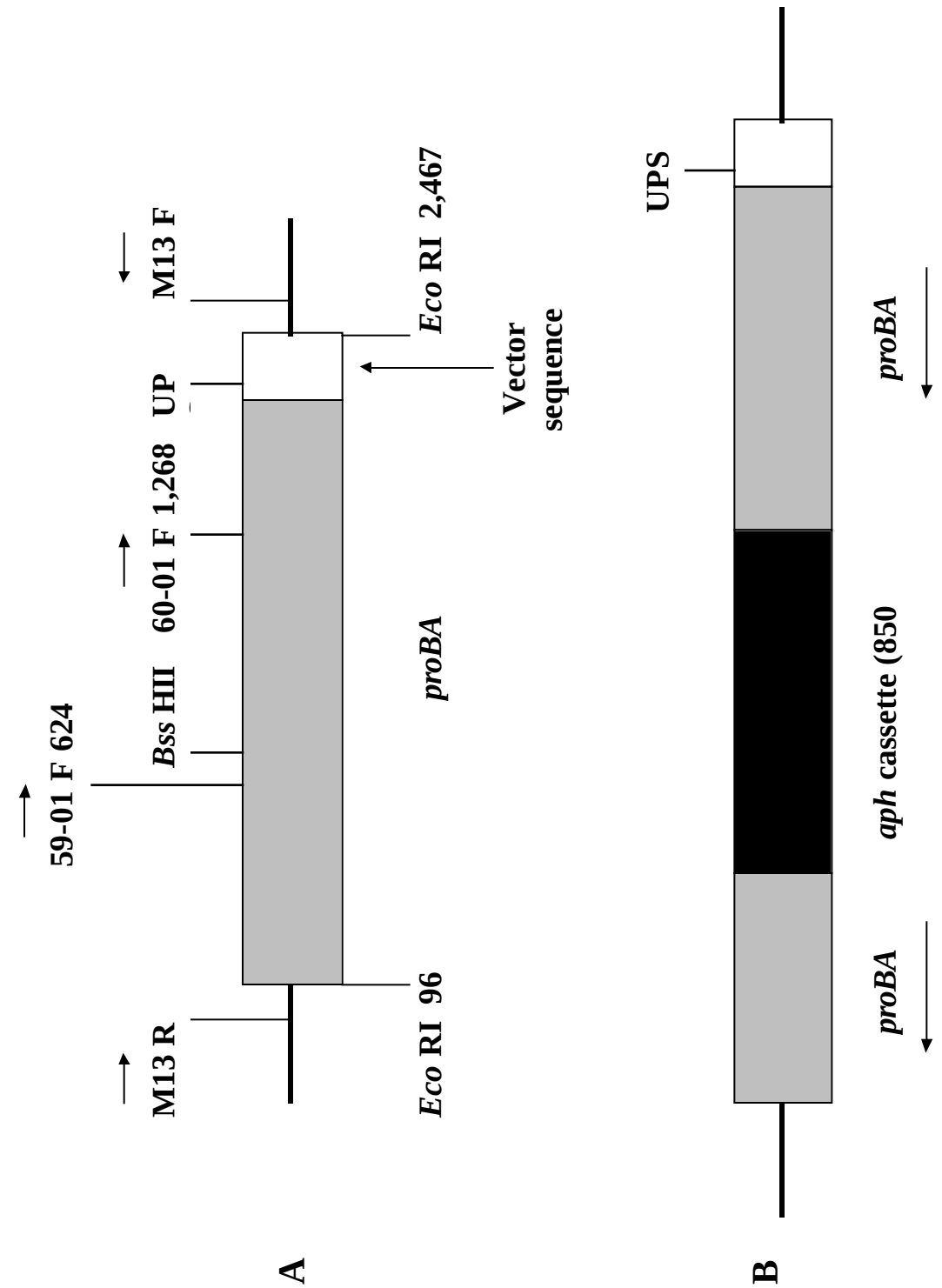

A
P tac
Hind III
Bam HI
Kpn I
Kpn I
pUC 18
pUC 18
proBA
lacZ
ca
proBA
2,300 bp
800 bp
M13 reverse
58-01 B
B
Cloned into
PCR-Blunt
pCRAAS-proBA9
proBA

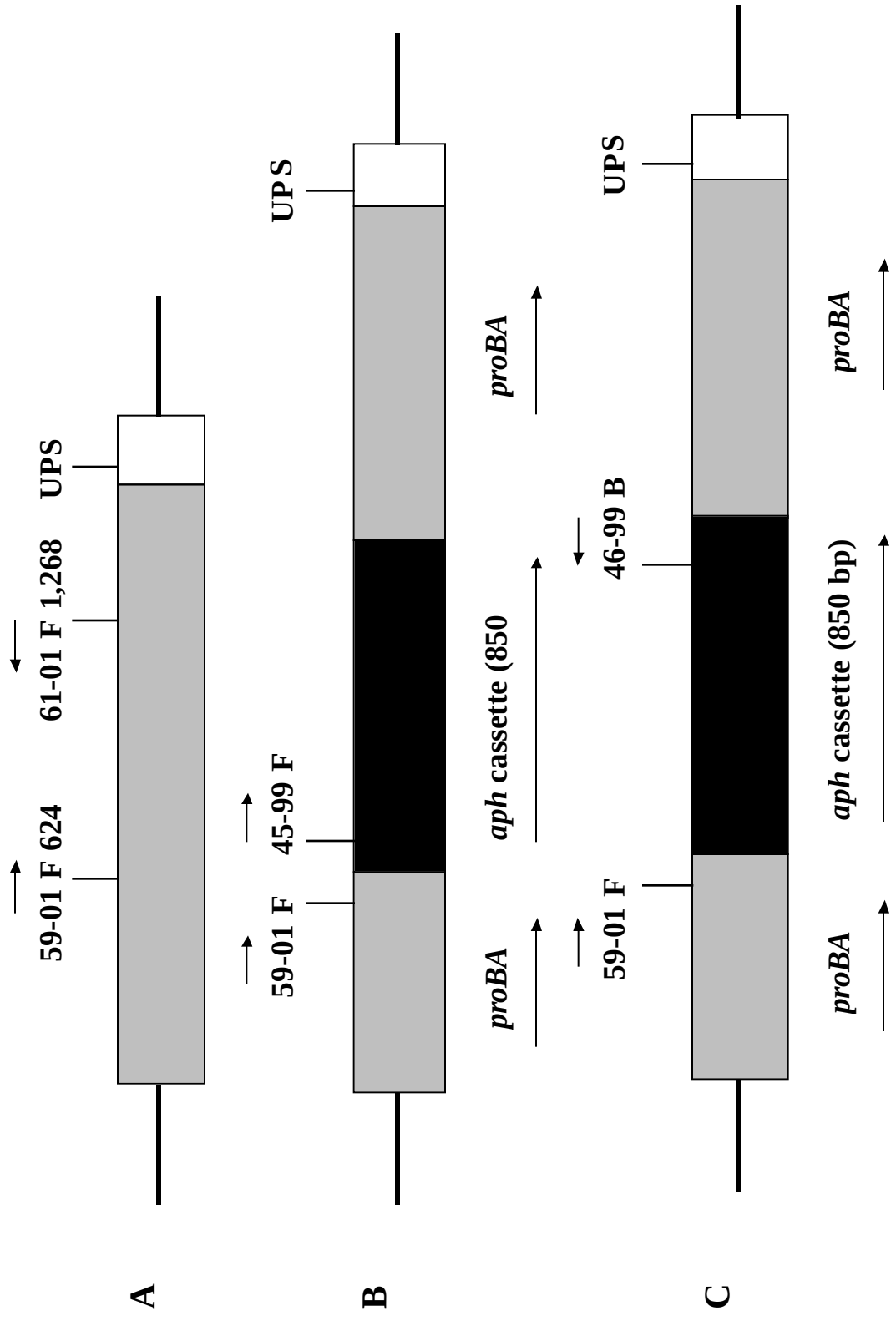
Figure 99. Sequencing strategy and features of the *proBA* cloned region (**A**), and insertion of *aph*A-3 cassette into *proBA* gene (**B**).


A
M13 R
59-01 F 624
Bss HII
60-01 F 1,268
UP
M13 F
Eco RI 96
proBA
Eco RI 2,467
Vector sequence
B
UPS
proBA
aph cassette (850
proBA

and then by restriction digestion with *Eco* RI. To follow GC chromosomal recombination in this locus, the *aph*A-3 cassette was released by cutting pUC18-km with *Sma* I and introduced into blunt-ended *BssH* II site of *proBA* gene (**Fig. 99 B**). The *proBA-km* fragment was released from pACYC184 by restriction digestion with *Eco* RI. The fragment was purified and isolated as before. The orientation of the antibiotic marker was determined by PCR. The modified fragment was then transfer into GC FA1090 and NGAAS-501 by spot dilution transformation. Transformants were selected in GC-km agar plates. The presence of the km cassette in the genome was determined by PCR using primers 45-99 F and 46-99 B.

PCR analysis confirmed that the km cassette was in the same orientation of the *proBA* promoter (**Fig. 100 and 101**). Transformants were obtained on GC-Km plates. After transformation into GC FA1090 and NGAAS-50, PCR analysis of the genomic DNA revealed the presence of the km cassette in the GC genome confirming specific insertion (**Fig. 102**). Among all the genetic systems designed the *proBA* locus based integrating system seems to be promising.

Figure 100. Schematic illustration of cloned PCR-amplified *proBA* (**A**) and possible orientations of *aph*A-3 cassette in the gene (**B** and **C**). (**A**) Predicted PCR product 664 bp; (**B**) no PCR product expected and (**C**) predicted PCR product 864 bp.


A
59-01 F 624
61-01 F 1,268
UPS
B
59-01 F
45-99 F
UPS
proBA
aph cassette (850
proBA
C
59-01 F
46-99 B
UPS
proBA
aph cassette (850 bp)
proBA

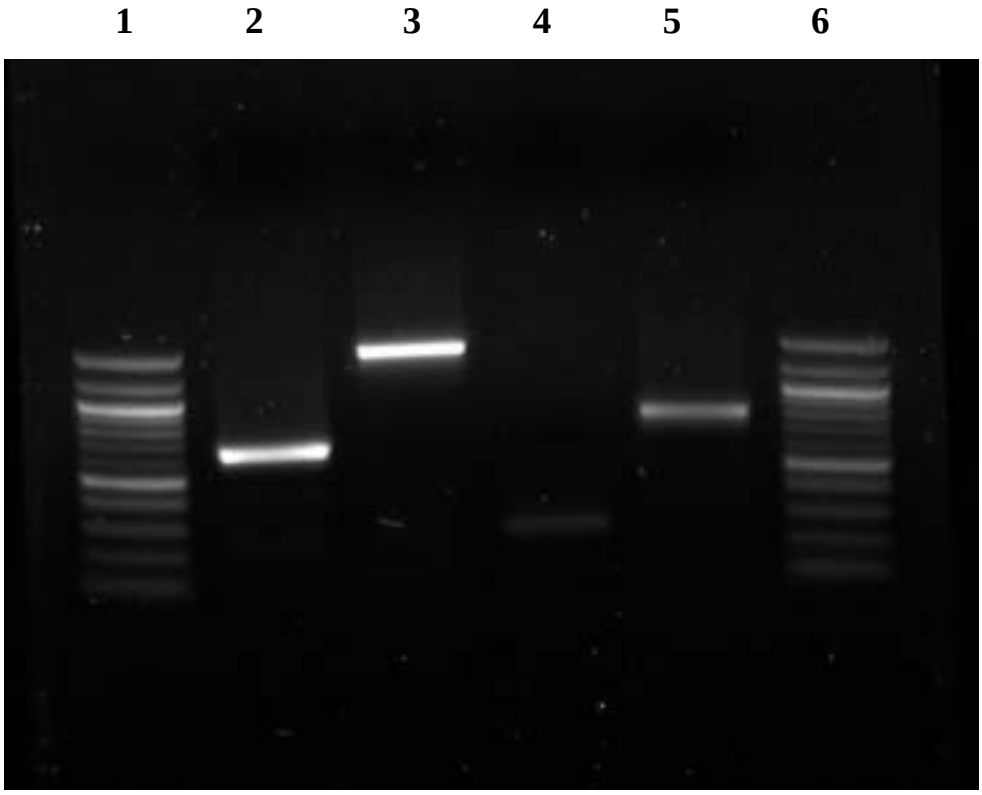


Figure 101. PCR determination of *aph*A-3 cassette insertion and orientation with respect to *proBA* promoter. Plasmid DNA was used as a template. Lanes: **1** and **6**, 100 bp ladder; **2**, pAAS184-*proBA*9; **3**-**6**, pAAS184-*proBA*-*km*. PCR products were generated by the combination of different primers. Lanes **2**-**3**, primers 59-01 F and 61-01 B; **4**, 59-01 F and 45-99 F; and **5**, 59-01 F and 46-99 B. PCR products exhibited the expected sizes.

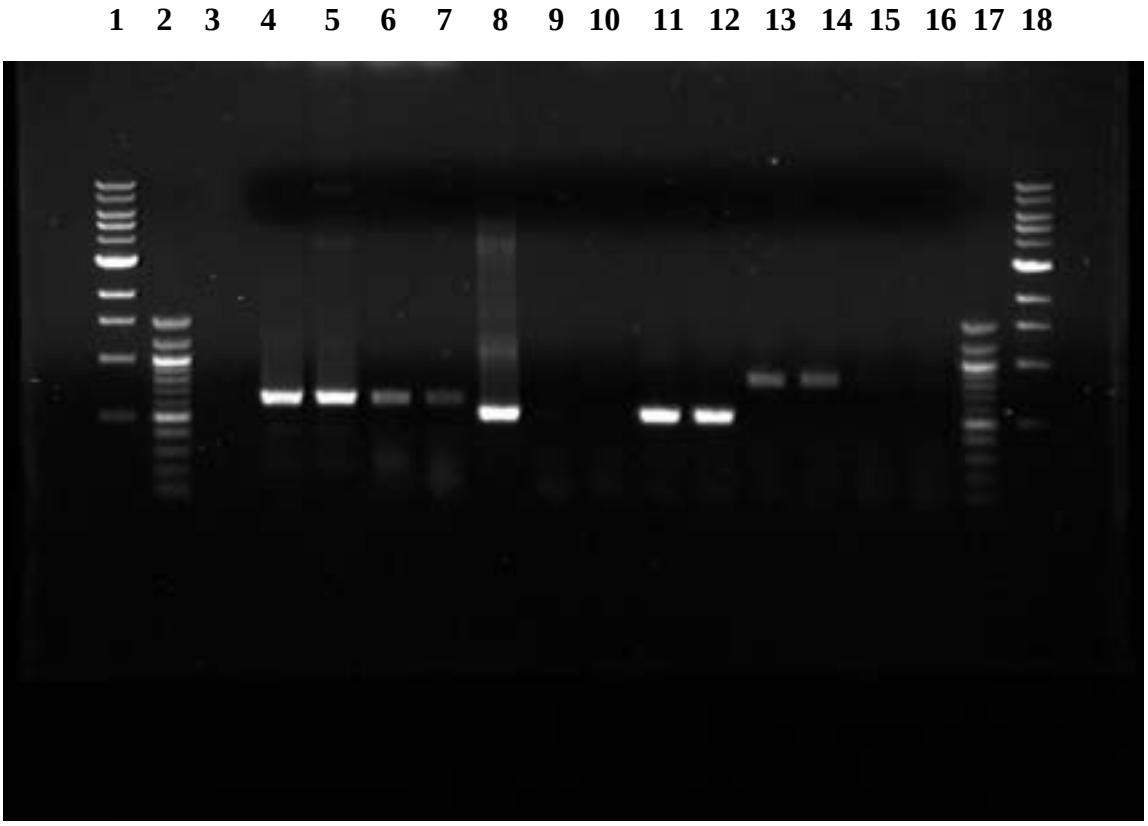


Figure 102. Determination of *aph*A-3 cassette in the GC chromosome and confirmation of recombination into the *proBA* gene in FA1090 and NGAAS-501. Lanes **1** and **18**, I kb ladder; **2** and **17**, 100 bp ladder; **3**, empty; **4**, pLES94; **5** pAAS184-*proBA*9; **6** and **9**, FA1090; **7** and **10**, NGAAS-501; **8**, pUC18-km; **11**, **13** and **15**, FA1090 *proBA-km*; **12**, **14** and **16**, NGAAS-501-*proBA-km*. PCR products were generated with: lanes **4-7**, *proBA* primers 59-01 F and 61-01 B; lanes **8-14**, km primers 45-99 F and 46-99 B; **13** and **14**, primers 59-01 F and 46-99 B; **15** and **16**, primers 59-01 F and 45-99 F.

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