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Lactate dehydrogenases from *Neisseria gonorrhoeae*: molecular analysis and role in host cell/bacterial interactions

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Abstract

Neisseria gonorrhoeae is an obligate human pathogen. It causes the sexually transmitted infection gonorrhoea that occurs on mucosal epithelia of the male urethra and female uterine cervix. Globally, there are more than sixty million cases of gonococcal infections reported every year that creates a high burden to the healthcare system. There are already cases reported of multi-drug resistant *N. gonorrhoeae*, where commonly used antibiotics for treatment of gonorrhoea cannot cure the infection.

Amongst different carbon sources *N. gonorrhoeae* can utilise only glucose, pyruvate and lactate efficiently. Studies have shown that phagocyte derived lactate, which accumulates as a consequence of glucose catabolism, stimulates oxygen consumption by *N. gonorrhoeae*. In addition, lactate is present at high concentrations in the female genito-urinary tract. This leads to the possibility that lactate metabolism is critical for the growth of *N. gonorrhoeae*. *N. gonorrhoeae* possesses at least three lactate dehydrogenase (Ldh) enzymes based on genome sequence analysis. However, the organisation and functional properties of these enzymes have not been fully defined. It was shown that two of the enzymes, D-lactate dehydrogenase (LdhD) and L-lactate dehydrogenase (LldD), are membrane-bound, independent of NAD^+, and linked to respiratory electron transport. The third lactate dehydrogenase is a cytoplasmic NAD^+ dependent D-lactate dehydrogenase (LdhA). It was observed that there was residual L-lactate dehydrogenase activity in the *lldD* mutant, suggesting the presence of an additional enzyme capable of oxidising lactate. A *lutABC* operon was identified in *N. gonorrhoeae*. Although homologous Lut enzymes in other bacteria have been shown to oxidise L-lactate analysis of *lutABC* mutants did not provide evidence for activity of LutABC towards L-lactate.

Mutants lacking the two respiratory lactate dehydrogenases were not able to survive in primary cervical epithelial (pex) cells under hypoxic conditions. Similarly, the *lldD* and *ldhD* mutants showed greatly reduced survival in neutrophils conpared to wild-type cells. Taken together these data show that

host-derived lactate is critical for the growth and survival of *N. gonorrhoeae* in host cells.

Lactate is transported into the gonococcus by a lactate permease (LctP) transporter. Previous work in the murine model of infection, showed that a mutant lacking *lctP* failed to cause infection. The importance of the LctP transporter was confirmed by construction and analysis of an *lctP* mutant; this mutant was unable to grow with L-lactate as sole carbon source and showed reduced survival in neutrophils compared to wild-type gonococcus.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

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Prof. Alastair McEwan provided input into the design of the project and critically reviewed the project and thesis so as to contribute to interpretation of data.
Dr. Cheryl Ong provided input into the drafting of significant parts of the work.
Dr. Jennifer Edwards performed primary cervical epithelial cells infection assay that is non-routine technical work in McEwan laboratory.

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Keywords

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Abbreviations

Abs	absorbance
Akt	protein kinase B
bp	base pair
CFU	colony-forming unit
Cm	chloramphenicol
CMP-NANA	N-acetyl neuraminic acid
^{o}C	degrees celcius
DCPIP	2,6-dichlorophenolindophenol
DNA	deoxyribonucleic acid
g	gram
HBSS	human serum albumin
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
Km	kanamycin
l	litre
LB	Luria-Bertani
LPS	lipopolysaccharide
M	molar
mM	millimolar
MOI	multiplicity of infection
μM	micromolar
mg	milligram
μg	microgram
min	minutes
ml	millilitre
μl	microlitre
NAD^{+}	β-Nicotinamide adenine dinucleotide
nm	nanometer
PCR	polymerase chain reaction
PMN	polymorphonuclear
pex	primary cervical epithelial
PMS	phenazine methosulfate
psi	pound-force per square inch
v	volume
w	weight
wt	wild type

Chapter 1

Introduction

1.1 Neisseria gonorrhoeae, the gonococcus

Neisseria gonorrhoeae is a Gram-negative diplococcal bacterium that causes the sexually transmitted infection gonorrhoea. Gonorrhoea is one of the oldest diseases known to human; (Handsfield 1990) has reported that Galen used the term gonorrhoea in the year 130 A. D. . However, the causative agent of gonorrhoea, *Neisseria gonorrhoeae*, was not described until 1879. With the introduction of sulfonamide (in 1936) and penicillin (in 1943) antibiotic therapies for the treatment of infection led to decrease in gonorrhoea prevalence (Handsfield 1990, Hook 1999, Sparling 1999). In 1960's the onset of oral methods of contraception reversed this trend and *N. gonorrhoeae* infections reached over 1 million reported cases in the USA in 1978 (Handsfield 1990, Knapp 1995). The HIV epidemic in the late 1980's caused wider use of barrier contraceptives and the incidence of gonococcal infection again declined (Knapp 1995). *N. gonorrhoeae* is transmitted more efficiently from an infected male to female (50 to 73% probability, independent of number of exposures) (Handsfield 1990, Lin, Donegan et al. 1998, Bolan 1999) than from an infected female to male (20 to 35% probability with one exposure). Even though sexual contact is the main mode in transmitting this disease, pregnant women infected with *N. gonorrhoeae* can transmit their children upon the delivery.

N. gonorrhoeae was previously successfully treated with antibiotics as sulfonamides, penicillins, cephalosporins, tetracyclines, macrolides, and

fluoroquinolones. Recently there has been an emergence of *N. gonorrheae* strains resistant to nearly all therapeutically available antibiotics (Unemo and Shafer 2011, Bolan, Sparling et al. 2012, Ison 2012, Unemo and Nicholas 2012, Whiley, Goire et al. 2012). There is great concern that gonorrohea might become untreatable using antimicrobial monotherapy. In response to this concern, recommendations to use dual-antimicrobial therapy, i.e., mainly ceftriaxone and azithromycin, have been introduced in the United States (Maldonado and Takhar 2013), the United Kingdom (Bignell and Fitzgerald 2011), and all of Europe (Unemo 2012). Resistance to antibiotics in gonococci develops due to spontaneous mutation and/or gene acquisition, which are effectively selected due to antibiotic pressure in patients. The resistance determinants then can be donated to other gonococci largely by transformation, which significantly enables spread of resistance alleles. *N. gonorrhoeae* uses these mechanisms to adapt to and survive at different sites in the human host. In this way it has evolved nearly all known physiological mechanisms of antimicrobial resistance to all antimicrobials recommended and/or used for treatment: (a) antimicrobial destruction or modification by enzymatic means (b) target modification or protection that reduces affinity for the antimicrobials (c) decreased influx of antimicrobials (d) increased efflux of antimicrobials (Unemo and Shafer 2014). In *N. gonorrhoeae* most of the acquired or developed antimicrobial resistance mechanisms do not appear to significantly lower biological fitness but can, in certain strains, enhance its fitness (Warner, Folster et al. 2007, Warner, Shafer et al. 2008, Kunz, Begum et al. 2012). As a result, in recent years, the susceptibility to the recommended first-line cefixime and ceftriaxone has decreased worldwid

(Tapsall, Ndowa et al. 2009, Lewis 2010, Kirkcaldy, Ballard et al. 2011). The cefixime treatment failures are identified and *N. gonorrhoeae* isolates are now displaying high-level clinical resistance to ceftriaxone, which is the last remaining option for first-line treatment (Unemo, Golparian et al. 2010, Ohnishi, Golparian et al. 2011, Ohnishi, Saika et al. 2011, Unemo and Nicholas 2012). Recently, a strain with high resistance to ceftriaxone was isolated in Australia. Only after dual therapy with ceftriaxone and azithromycin was the patient reportedly culture-negative for gonorrhoea. This once more arouses concerns over the ongoing efficacy of ceftriaxone monotherapy for treatment of gonorrhoea (Lahra, Ryder et al. 2014).

The number of estimated cases of gonorrhoea worldwide is ~106 million annually ((WHO) 2012). However, the numbers of reported cases, especially from low-resource settings, are substantially smaller. This is due to lack of appropriate methods or access to testing and use of syndromic management or incomplete case reporting and epidemiological surveillance (Unemo and Shafer 2014). In Australia, gonorrhoea is relatively rare, apart from among some populations of Aboriginal people and men who have sex with men. The largest increase in notifications between 2007 and 2012 was observed in both men and women in New South Wales (2.9- and 3.7-fold greater in 2012 than 2007, respectively) and Victoria (2.4- and 2.7-fold greater in 2012 than 2007, respectively), men in the Australian Capital Territory and women in Queensland (Roberts-Witteveen, Pennington et al. 2014). The highest notification rates remained in Indigenous people in the Northern Territory and Western Australia, and particularly in women. There is an ongoing gonorrhoea epidemic affecting

Aboriginal and Torre. Strait Islander people in Australia, but the increases in notifications have occurred primarily in non-Aboriginal populations in the larger jurisdictions (Roberts-Witteveen, Pennington et al. 2014).

Public health control of gonorrhoea relies totally on appropriate antimicrobial treatment, together with generalized and targeted prevention efforts, use of effective diagnostics, partner notification processes, and epidemiological surveillance. Therapy should cure individual cases to reduce the risk of complications and prevent further transmission of the infection (Unemo and Shafer 2014).

1.2 Virulence factors produced by Neisseria gonorrhoeae

The gonococcus (GC) produces virulence factors that can enable the bacterium to interact and colonise host cells. Key virulence factors include pilus, opacity associated-protein (Opa) outer membrane proteins and lipooligosaccharide (LOS)(Merz and So 2000). Type IV pili are filamentous structures composed of protein subunits. Their role is to promote initial adhesion to epithelial and endothelial cells (Swanson 1973, Brodeur, Johnson et al. 1977, McGee 1981, Kent 1992, Cohen, Cannon et al. 1994, Ohnishi, Golparian et al. 2011). The pilus fibre consists of 15 proteins (known as Pil proteins) that are involved in the biogenesis, assembly and disassembly of this adhesion (Carbonnelle, Helaine et al. 2006). Numerous PilE (major subunit) are arranged in helical configuration and minor subunits (PilC, PilX, PilV) can be incorporated in the fibre and modulate its function. The variable domains and post-translational

modifications are exposed on the surface of the assembled pilus. Glycosylation is one of the post-translational modifications (Virji, Saunders et al. 1993, Parge, Forest et al. 1995, Virji 1997) that can indirectly have effect on cellular interaction, perhaps by affecting the self-agglutination of pili (Parge, Forest et al. 1995, Nassif, Marceau et al. 1997). Pili contain positively and negatively charged regions with the glycans situated within negative regions of the pilus. They undergo phase and antigenic variation and therefore likely modulate adherence properties of the pilus (Craig, Volkmann et al. 2006). The complement regulatory protein CD46, a human specific membrane protein, was thought to serve as a receptor for *N. gonorrhoeae* pilus (Kallstrom, Islam et al. 1998, Kallstrom, Blackmer et al. 2001).

Neisseria type IV pili are retractile fibres that promote neisserial adhesion and epithelial cell signaling (Merz and So 2000, Merz, So et al. 2000). Type IV pili-mediated *N. gonorrhoeae* infection induces the downregulation of surface and intracellular CD46 (Gill, Koomey et al. 2003). Type IV pili-induced downregulation of CD46 is dependent on PilT, the type IV pili retraction motor (Gill, Spitzer et al. 2005). Newer studies implicates that *N. gonorrhoeae* induces CD46 processing. This is accomplished by presenilin/γ-secretase (PS/γS) and requires type IV pili (Weyand, Calton et al. 2010). Pili are involved in several additional functions. They enable uptake of foreign DNA from extracellular environment and this increases transformation frequency and maintains genetic diversity among the population (Fussenegger, Rudel et al. 1997). Coordinate action of PilC and ATPase PilT results in rapid assembly and disassembly of pili

resulting in twitching mobility (Maier, Potter et al. 2002). This may provide one mechanism by which *N. gonorrhoeae* is able to colonise and ascend mucosal surfaces (Wall and Kaiser 1999, Henrichsen 1983).

Opa proteins were originaly identified by their ability to confer opacity and colour change of *N. gonorrhoeae colonies* (James and Swanson 1978, Swanson 1978). They are devided in two classes based on their their differential ability to recognize host cell molecules: the Opa_{HS}-type proteins (the term `$Opa_{HS}$' denotes heparansulphate-recognising Opa proteins such as OpaA/$Opa_{30}$ of gono-coccal strain MS11), and the large group of Opa-type proteins (the term `Opa ' com- CEA CEA describes all Opa proteins recognising carcinoembryonic antigen (CEA) or related molecules such as Opa_{52} of gonococcal strain MS11) (Beauchemin, Draber et al. 1999). Opa-CEACAM interactions have been demonstrated in cell cultures (Virji, Makepeace et al. 1996, Virji, Watt et al. 1996, Chen, Grunert et al. 1997, Gray-Owen, Lorenzen et al. 1997) but not in imortalized cell lines (Neumaier, Paululat et al. 1993, Hixson, McEntire et al. 1995, Hsieh, Luo et al. 1995, Kleinerman, Troncoso et al. 1995, Kunath, Ordonez-Garcia et al. 1995, Nollau, Scheller et al. 1997, Screaton, Penn et al. 1997). Taking both in consideration, leads to conclusion that Opa-CEACAM interaction may or may not occur *in vivo*. Some studies suggest that Opa-CEACAM association can occur *in vivo* (Virji, Watt et al. 1996, Virji, Evans et al. 1999, Fedarovich, Tomberg et al. 2006, Sintsova, Wong et al. 2015). Opa-HSPG interaction has been demonstrated (Grassme, Gulbins et al. 1997) and this interaction leads to cascade of reactions which modulate cytoskeletal reorrganisation required for endocytosis of cell-adhered *N. gonorrhoeae* (Grassme, Ireland et al. 1996, Grassme, Gulbins et al.

1997). *N. gonorrhoae* posesses LOS in its outer membrane. LOS lacks repeating O-antigen sugar that comprises the polysaccharide side chain of LPS (Mandrell and Apicella 1993, van Putten and Robertson 1995, Preston, E et al. 1996, Giardina 1999). The presence or absence of available substrates for LOS biosynthesis and enzymes involved in LOS biosynthesis dictate spontaneous interconversion of LOS oligosaccharide (Schneider, Hammack et al. 1988, Kerwood, Schneider et al. 1992, Danaher, Levin et al. 1995, Yang and Gotschlich 1996, Burch, Danaher et al. 1997). LOS oligosaccharide side chains terminate in epitopes that mimic sugar moieties of mammalian glycosphingolipids (Mandrell, Griffiss et al. 1988, Mandrell 1992, Mandrell and Apicella 1993, van Putten and Robertson 1995, Yamasaki, Koshino et al. 1999, Harvey, Swords et al. 2001). This provides the bacterium with a method to avoid innate immune system of the host. Some LOS structures can serve as acceptor molecules for sialic acid deposition. LOS sialylation is mediated by gonococcus-encoded sialyltransferase (Mandrell and Apicella 1993, Mandrell, Smith et al. 1993) that is present in the gonococcal outer membrane (Shell, Chiles et al. 2002). The gonococcus lacks the ability to synthesize CMP–*N*-acetylneuraminic acid, substrate required for LOS sialylation, and must scavenge it from its human host (Parsons, Patel et al. 1988, Apicella, Mandrell et al. 1990, Frangipane and Rest 1993). The presence of sialic acid on gonococcal LOS confers unstable resistance to the bactericidal action of normal human serum, i.e., serum resistance (Nairn, Cole et al. 1988, Parsons, Patel et al. 1988, Patel, Martin et al. 1988, Parsons, Cole et al. 1990, Parsons, Curry et al. 1992). The lipid A to which oligosaccharide core is attached requently contains phosphoethanolamine (PEA) (Lewis, Choudhury et al. 2009,

Lewis, Shafer et al. 2013). The presence or absence of PEA-decorated lipid A (PEA-lipid A) influences inflammatory signaling (Liu, John et al. 2010) and bacterial susceptibility to host innate immune response, including the bactericidal activities of normal human serum, complement, and cationic antimicrobial peptides (CAMPs) (Lewis, Choudhury et al. 2009, Balthazar, Gusa et al. 2011). The presence of a single PEA group on lipid A enhances its ability to induce TNF-α in monocytes. Thus, the occurrence of a PEA on lipid A from is correlated with increased inflammatory cytokine signaling.

N. gonorrhoeae undergoes extensive phase and genetic variation (van der Woude and Baumler 2004). These processes often aid the bacteria in immune evasion and adaptation to new host niches (Kline, Sechman et al. 2003, van der Woude and Baumler 2004). Phase variation in general refers to a reversible switch between an "all-or-none" (on/off) expressing phase, resulting in variation in the level of expression of one or more proteins between individual cells of a clonal population. What distinguishes this variation from genetic noise and classical gene regulation is that there is a genetic or epigenetic mechanism that allows the variability to be heritable. This means that a daughter cell will inherit the expression phase of the parent. However, the phase of expression must also be reversible between generations (van der Woude and Baumler 2004, Bayliss 2009). Antigenic variation refers to the expression of functionally conserved moieties within a clonal population that are antigenically distinct. The genetic information for producing a family of antigenic variants is available in the cell, but only one variant is expressed at a given time. Barbour listed three criteria that must be fulfilled for variation to be considered as antigenic variation

(Barbour 2002). These are (i) that the antigenic change must be involved i avoidance of immune or niche selection, (ii) that it is a multiphasic change, and (iii) that the mechanism is consistent with gene conversion.

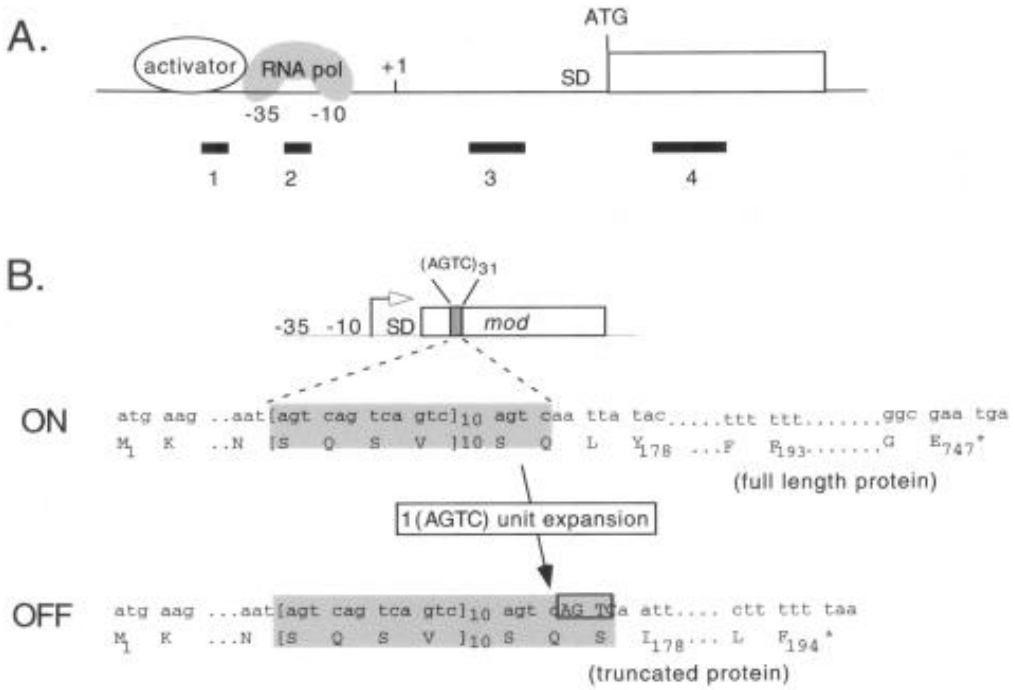


Figure 1.1 Phase variation as a result of SSM at short sequence repeats. (A) Schematic of the four positions, relative to a gene, at which short sequence repeats can cause phase variation. Indicated are a coding sequence (open rectangle), promoter (−10, −35) with RNA polymerase (RNA pol), the +1 transcription start site, the Shine-Dalgarno sequence for ribosome binding (SD), and the ATG translation start codon. Repeat sequences at regions 1 through 4 can lead to phase variation by affecting transcription initiation (regions 1 and 2), translation (region 4), and as yet unidentified means (region 3) (see the text). (B) Effect on the translation product of a one-unit insertion due to SSM at the tetranucleotide repeat sequence (AGTC) in the coding sequence of *mod* of *H. influenzae* (HI056). Partial nucleotide and amino acid sequences and numbering are indicated for 31 (on) and 32 (off) tetranucleotide repeats. Note that as a result of the insertion, the reading frame changes at amino acid 177, which leads to the formation of a premature stop codon (*) following amino acid 194.

Pili undergo phase variation during the course of infection. *N. gonorrhoeae* switches between pilated and non-pilated phenotype depending of the site and the state of infection. Several studies have shown that formation of pili is necessary for efficient adherence of *N. gonorrhoeae* (Swanson 1973, Brodeur, Johnson et al. 1977, McGee 1981, Kent 1992, Cohen, Cannon et al. 1994) and that the absence of pili in otherwise isogenic backgrounds is crucial for invasion thus has important implications for the development of invasive gonococcal disease (Faulstich, Bottcher et al. 2013).

N. gonorrhoeae has a single copy of *pilE* and 19 unique *pilS* cassettes located in six discrete chromosomal loci (Hamrick, Dempsey et al. 2001). The *pilS* donor copy is never lost while the *pilE* locus undergoes gene conversion. The divergence in genomic organization and increased number of *pilS* cassettes in *N. gonorrhoeae* may account for the higher frequency and rate of pilin antigenic variation observed in *N. gonorrhoeae* or may be due to a need for more antigenic diversity within a sexually transmitted infection (Cahoon and Seifert 2011).

opa genes also undergo phase and antigenic variation. In *N. gonorrhoeae* there are eleven genes encoding for different Opa proteins (Bhat, Gibbs et al. 1991, Dempsey, Litaker et al. 1991). *opa* genes contain CTCTT repeats in the DNA encoding the signal sequence (Stern, Brown et al. 1986). There are two to twenty CTCTT repeats in every *opa* gene (Stern, Brown et al. 1986, Ball and Criss 2013). Every *opa* gene can independently phase vary ON/OF. Thus, each individual Opa

ON/OFF variation is mediated by a phase variation event (in combination these result in the expression of up to 11 different variants) and therefore represents an antigenic variation process mediated through phase variation (Rotman and Seifert 2014). *N. gonorrhoeae* phase vary between *opa*$^{+}$ and *opa*$^{-}$ strains. Recent data has demonstrated the importance of Opa protein expression for persistence of *N.gonorrhoeae* in the female genital tract (Cole, Fulcher et al. 2010). In contrast, *opa*$^{-}$ *N. gonorrhoeae*, survives exposure to primary human PMNs and suppresses the PMN oxidative burst (Ball and Criss 2013). Switch between *opa*$^{+}$ and *opa*$^{-}$ phenotype enables *N. gonorrhoeae* to avoid innate immune response and proliferate within the host.

LOS undergoes phase variation as a consequence of expression of different combinations of glycosyltransferases, which produce different LOS structures (Jennings, Srikhanta et al. 1999). There are twelve recognized LOS immunotypes in *Neisseria* (Scholten, Kuipers et al. 1994) and these have different sensitivities to human serum. The *lgt* genes of *N. gonorrhoeae* encode different LOS biosynthesis genes that transfer sugars to the LOS core. The subset of the *lgt* genes contains homopolymeric repeats in their 5′ ends (Frangipane and Rest 1993, Jennings, Srikhanta et al. 1999). LOS confers unstable resistance to the bactericidal action of normal human serum (Nairn, Cole et al. 1988, Parsons, Patel et al. 1988, Patel, Martin et al. 1988, Parsons, Cole et al. 1990, Parsons, Curry et al. 1992, Smith, Parsons et al. 1995). Phase variable changes in genes *lgtA* and *lgtC* within the *lgtABCDE* operon of *N. gonorrhoeae* can modulate gonococcal susceptibility to normal human serum (Shafer, Datta et al. 2002). Some studies show that association between LOS and C' protein C3 occurs on cervical epithelia

(Edwards and Apicella 2002).

N. gonorrhoeae infection of men most commonly occurs as an acute urethritis, which develops from the concomitant inflammatory response (Ramsey, Schneider et al. 1995), although a small percentage will develop asymptomatic gonococcal infection (Hook 1999, Sparling 1999). Symptomatic *N. gonorrhoeae* infection is characterized by purulent discharge, which is associated with polymorphonuclear leaukocyte (PMN) influx and shedding of urethral epithelial cells. Studies with human volunteers indicate that there is an incubation period from the infection to the onset of the disease. (Schneider, et al. 1995). Experimental infection of men also demonstrates that the chemokine interleukin-8 (IL-8) and the cytokines IL-6 and tumor necrosis factor alpha (TNF-α) are prevalent within the urethral lumen with progressive gonococcal disease (Ramsey, Schneider et al. 1995). Release of cytokines and chemokines from the urethral epithelium may initiate the inflammatory response by triggering PMN influx. PMN influx in conjunction with cytokine release from the urethral epithelium subsequently might potentiate the clinical symptoms associated with disease. *N. gonorrhoeae* is found within PMNs and urethral epithelial cell, which is documented by microscopic examinations of urethral exudes from men infected with this bacterium (King, James et al. 1978, Apicella, Ketterer et al. 1996). The interaction of gonococci with PMNs is not dependent upon the presence of Opa, and it does not require pili (Ball and Criss 2013). Analysis of primary male urethral epithelial cells demonstrates that the observed intimate association between the urethral epithelium and the gonococcus is probably achieved through the interaction of the asialoglycoprotein receptor (ASGP-R)

and gonococcal LOS (Harvey, Jennings et al. 2001). (ASGP-R) is present on sperm too. Interaction between (ASGP-R) present on sperm and *N. gonorrhoeae* could facilitate disease transmission (Harvey, Porat et al. 2000).

In contrast to *N. gonorrhoeae* infection in men where the inflammatory response is recruited, 50-80% of women with lower genital tract *N. gonorrhoeae* infection are asymptomatic (Densen 1989, Hook 1999). Analysis of cervical secretions obtained from uninfected women and from women infected with the *N. gonorrhoeae* also reveal that an antibody response is not generated with uncomplicated infection (Hedges, Mayo et al. 1999). These clinical findings are consistent with the ability of the gonococcus to evade and subvert host immune function. CR3 serves as the primary receptor for *N. gonorrhoeae* adherence to and invasion of the ectocervix and endocervix (Edwards and Apicella 2002). Binding of the gonococcus to CR3 requires the cooperative action of iC3b bound to the gonococcal surface in conjunction with gonococcal porin and pilus (Edwards and Apicella 2002). *N. gonorrhoea* releases phospholipase D (NgPLD) upon the infection of primary cervical epithelial cells. This enzyme enhances signaling events that trigger CR3 mobilization to the surface of primary cervical cells (Edwards, Entz et al. 2003). Cervical cells signal transduction events that lead to membrane ruffling are modulated by NgPLD (Edwards, Entz et al. 2003, Edwards and Apicella 2006). Isolates obtained from men tend to express Opa proteins (Jerse, Cohen et al. 1994) as do cervical isolates obtained from women at the time of ovulation. Opa^- gonococci predominate within the fallopian tubes and in the cervix at the time of menses (Morse 1985).

N. gonorrhoeae can move from the lower to the upper genital tract. This can be achieved by twitching mobility in conjunction with hormonal changes which influence the expression of complement and those molecules serving as gonococcal receptors within the female genital tract (Edwards, Brown et al. 2001). Microscopic analysis of tissue biopsies indicates that the expression of CR3 progressively decreases in an ascending manner from the ectocervix to the fallopian tubes (Edwards, Brown et al. 2001). Gonococcal adherence to fallopian tube epithelia occurs selectively on non-ciliated cells (McGee, Johnson et al. 1976 , McGee, Stephens et al. 1983 , Gorby, Clemens et al. 1991). However, it is the ciliated cells of the fallopian tube epithelia that are subsequently shed (Stephens, McGee et al. 1987). Access to sub-epithelial tissue is also speculated to be obtained with invasion of non-ciliated cells, after which gonococci are transcytosed to the basal lateral surface of these cells and released (Handsfield 1990).

N. gonorrhoeae can subvert immune responses to co-pathogens, such as HIV-1 (Unemo M 2013). For example, studies have shown that gonococcal infection is associated with enhanced HIV-1 acquisition and impaired HIV-1-specific CTL responses (Kaul, Rowland-Jones et al. 2001), decrease in blood $CD4^+$ T cell count (Anzala, Simonsen et al. 2000) and increased semen and plasma viral loads (Cohen, Hoffman et al. 1997, Winter, Taylor et al. 1999, Anzala, Simonsen et al. 2000) in HIV-1 infected individuals, and these are often found to be return to normal with effective gonococcal therapy. The relationship between these pathogens appears to occur at multiple levels since *in vitro* studies indicate that *N. gonorrhoeae* can dramatically enhance HIV-1 replication in humans $CD4^+$ T

cells (Chen, Boulton et al. 2003). Moreover, gonococcal infection of genital epithelial cells promotes the release of pro-inflammatory cytokines that drive further HIV-1 expression (Ferreira, Nazli, Khan et al. 2011). Taking in consideration all of these observations, one can conclude that there is a close linkage between these two major sexually transmitted pathogens

1.3 *Interaction of gonococcus with host cells and the host bacterial flora*

1.3.1 Neutrophils

Neutrophils (polymorphonuclear leukocytes = PMNs) are the main phagocytic cells of the human immune system. As the first responders of the immune system to infection or injury, neutrophils possess cytoplasmic granules that contain many antimicrobial enzymes, peptides and reactive chemicals (Burg and Pillinger 2001, Borregaard 2010). These cells either take up microorganisms by phagocytosis into phagolysosomes, which then fuse with the granules to kill the microorganisms inside, or kill microorganisms extracellularly via granule exocytosis or release of DNA-rich neutrophil extracellular traps (Borregaard 2010, Urban, Lourido et al. 2006). Even though neutrophils posses this arsenal of defense, they cannot clear gonococcal infection. Viable bacteria are found in gonococcal secretions together with neutrophils (Wiesner and Thompson 1980). Bacteria in gonorrhoeal secretions are attached to and within PMNs (Ovcinnikov and Delektorskij 1971, Farzadegan and Roth 1975, Evans 1977, King, James et al. 1978, Apicella, Ketterer et al. 1996). Using different methods it was observed that *N. gonorrhoeae* not only can survive but also replicate within PMNs

(Simons, Nauseef et al. 2005, Criss, Katz et al. 2009). *N. gonorrhoeae* possess factors that promote attachment and phagocytosis by PMNs. Opsonic and non-opsonic interactions are basic means of phagocytosis and both are utilized by *N. gonorrhoeae* (Groves, Dart et al. 2008). Immunoglobulins and complement are involved in opsonic phagocytosis. They bind to Fc receptors and complement receptors such as CR3 (Groves, Dart et al. 2008). Patients with gonorrhoea produce antibodies IgG and IgA directed against *N. gonorrhoeae* surface-exposed components including porin, Opa proteins, pilin, iron-regulated outer membrane proteins, and LOS (Brooks, Israel et al. 1976, Tramont, Ciak et al. 1980, Rice and Kasper 1982, Siegel, Olsen et al. 1982, Lammel, Sweet et al. 1985). Many of the Gc surface-exposed components that promote immune responses are phase and antigenically variable and thus evade the innate immune response (Virji 2009).

The complement system can be activated by three ways: the classical, the alternative, and the lectin pathway. All three ways normally proceed to proteolytic activation of the major complement protein C3 and assembly of the membrane attack complex (Ram, Lewis et al. 2010). *N. gonorrhoeae* resists the bactericidal activities of complement in normal human serum by binding the complement regulatory proteins C4b-binding protein (C4BP) and factor H (fH) on its surface via porins and sialylated LOS (Ram 1998, Ram 1998, Gulati, Cox et al. 2005). C4BP and fH also increase deposition of iC3b on *N. gonorrhoeae* surface. The iC3b is a ligand for CR3, which in PMNs drives actin-dependent phagocytosis and production of reactive oxygen species (ROS) (Groves, Dart et al. 2008).

Non-opsonic uptake of *N. gonorrhoeae* is achieved via Opa proteins. Opa proteins bind HSPG and CEACAM molecules on the surface of PMNs. Only those Opa proteins that bind CEACAMs are reported to influence *N. gonorrhoeae* interactions with PMNs (Sadarangani, Pollard et al. 2011). However, Opa$^-$ can be internalized too, but this is achieved via an actin-dependent manner. Studies show that Opa$^-$ cells survive better after exposure to PMNs (Rest, Fischer et al. 1982, Virji and Heckels 1986, Criss, Katz et al. 2009, Ball and Criss 2013). There is also a difference in sensitivity to PMN killing between pilated and non-pilated strains of *N. gonorrhoeae*. Non-pilated *N. gonorrhoeae* showed decreased resistance to PMN killing than pilated bacteria (Ofek, Beachey et al. 1974, Dilworth, Hendley et al. 1975, Gulati, Cox et al. 2013). Clearly, expression of surface structures modulates interaction and internalization of *N. gonorrhoeae* by PMNs.

PMNs produce reactive bactericidal chemicals or reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide, and hydroxyl radical. ROS induce DNA, protein, and cell membrane damage that can lead to cell death (Fang 2004). PMN NADPH oxidase transports electrons across the phagosomal or plasma membrane to generate superoxide, which in turn generates hydrogen peroxide. Hydrogen peroxide is then used as a substrate by azurophilic enzyme myeloperoxidase to generate hypochlorous acid (Roos, van Bruggen et al. 2003). *N. gonorrhoeae* has evolved different strategies to avoid PMNs oxidative burst. Lactate released by PMNs stimulates oxygen consumption of *N. gonorrhoeae*, which consequently leads to less amount of oxygen that can be used as a substrate by PMNs NADPH oxidase (Britigan, Klapper et al. 1988).

N. gonorrhoeae possesses superoxide dismutase (SodB), which catalyzes conversion of superoxide to hydrogen peroxide, but the activity of this protein is low in gonococci and does not provide protection against ROS *in vitro* (Tseng, Srikhanta et al. 2001). Another protein involved in protection against ROS is cytoplasmic catalase (KatA). *N. gonorrhoeae* defective in *katA* is more sensitive to hydrogen peroxide and superoxide anion (Johnson, Steiner et al. 1993, Soler-Garcia and Jerse 2004, Stohl, Criss et al. 2005). *N. gonorrhoeae* also imports Mn(II) into its cytoplasm via the MntABC transporter, where it scavenges superoxide and hydrogen peroxide by a mechanism independent of SodB and catalase (Tseng, Srikhanta et al. 2001). The M23B family zinc metallopeptidase, Ngo1686, with dual activities (dd-carboxypeptidase and endopeptidase activities), helps protect *N. gonorrhoeae* from hydrogen peroxide and PMN killing (Stohl, Criss et al. 2005, Stohl EA 2013). However, mutants lacking *sodB*, *katA* or *mntABC* are not impaired in ability to survive PMN killing (Seib, Simons et al. 2005, Criss, Katz et al. 2009). It can be concluded that *N. gonorrhoeae* is remarkably resistant to PMN killing and that killing occurs independently of neutrophil ROS production (Criss, Katz et al. 2009).

The non-oxidative antibacterial factors in neutrophils include degradative enzymes (such as lysozyme, elastase and cathepsin G), cationic peptides (such as defensins and the cathelicidin LL-37) and the vesicular proton-ATPase, which lowers the pH of the phagosome (Levy 2004, Kinchen and Ravichandran 2008). Cathepsin G is a highly cationic serine protease which, cleaves *N. gonorrhoeae* outer membrane proteins including porin and Opa proteins (Rest and Pretzer 1981 , Shafer and Morse 1987) but studies show that its action against *N.*

gonorrhoeae is independent of this process (Shafer, Onunka et al. 1986). Instead, cathepsin G may impede peptidoglycan biosynthesis (Shafer, Onunka et al. 1990). The antigonococcal mechanism of action of LL-37 is not well known. It may be related to its ability to form pores that disrupt the integrity of bacterial membranes (Brogden 2005). The multiple transferable resistance system (MtrCDE) and fatty acid resistance system (FarAB) efflux pumps play a prominent part in conferring resistance of *N. gonorrhoeae* to antimicrobial peptides (Shafer, Qu et al. 1998, Lee and Shafer 1999).

1.3.2 Lactic acid bacteria (LCB)

Lactic acid bacteria (LCB) constitute a group of Gram-positive bacteria united by a constellation of morphological, metabolic and physiological characterization (Axelsson 2004). The general description of bacteria included in the group is Gram-positive, non-sporulating, non-respiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. The LAB term is associated with bacteria involved in food and food fermentation including related bacteria normally associated with the mucosal surfaces of humans and animals (Axelsson 2004, Ljung and Wadstrom 2009)

Lactobacilli have a high capacity to ferment carbohydrates and related compounds. The end product is lactic acid (Axelsson 2004). In the 1960's lactobacilli were devided in three groups: 1) the obligately homofermentative lactobacilli, lacking both glucose-6-phosphate and 6-phosphogluconate dehydrogenases (*Thermobacterium*); 2) the facultatively hormofermentative lactobacilli, having both dehydrogenases, but degrading glucose preferentially

via the Embden-Meyerhof–Parnas (EMP) pathway (*Streptobacterium*); and 3) the oligately heterofermentative lactobacilli lacking fructose-1,6-bisphospate-aldolase (*Betabacterium*) (van der Hamer 1960). The development and application of modern molecular techniques brought new insight in the taxonomy of the genus. However, for practical reasons lactobacilli are still today divided in the same three major groups.

Lactose fermentation is the most studied metabolism in lactic acid bacteria. *Lactobacillus casei* takes up lactose via the phosphoenolpyruvate-dependent phosphotransferase system (PTS) and enters the cytoplasm as lactose phosphate (Chassy and Alpert 1989). Lactose phosphate is cleaved to glucose and galactose-6-phosphate. Glucose phosphorylated and metabolized through either the glycolytic pathway or the pentose phosphate pathway (Bissett and Anderson 1974). Glucose is fermented by two major pathways: the Embden-Mayerhof–Parnas pathway (EMP) and the pentose phosphate pathway. The Embden-Mayerhof–Parnas (EMP) is used by the homofermentative lactobacilli and the pentose phosphate pathway is used by the heterofermentative lactobacilli (Axelsson 1998).

LCB are aerotolerant anaerobes and when oxygen is present it is reduced via enzymes such as pyruvate oxidase to generate hydrogen peroxide. The hydrogen peroxide produced has bactericidal effects (Muench, Kuch et al. 2009).

Lactobacilli are found in a wide variety of habitats. Four species, *Lactobacillus*

crispatus, *L. gasseri*, *L. iners*, and *L. jensenii*, are common and abundant in the human vagina and absent from other habitats (Eschenbach, Davick et al. 1989, Giorgi, Torriani et al. 1989, Reid, McGroarty et al. 1996, Antonio, Hawes et al. 1999, Ravel, Gajer et al. 2011). These species are thought to play key protective roles by lowering the environmental pH through lactic acid production (Boskey, Cone et al. 2001, Graver MA 2011) by producing various bacteriostatic and bactericidal compounds, or through competitive exclusion (Klebanoff, Hillier, Eschenbach et al. 1991, Boskey, Cone et al. 2001, Kaewsrichan, Peeyananjarassri et al. 2006, Voravuthikunchai, Bilasoi et al. 2006). Lactobacilli are strongly associated with reduced risks of infections by reproductive tract pathogens, including HIV-1 (Sewankambo, Gray et al. 1997, Martin, Richardson et al. 1999), HSV-2 (Cherpes, Meyn et al. 2003), *Trichomonas vaginalis* (Brotman, Bradford et al. 2012), *N. gonorrhoeae* and *Chlamydia trachomatis* (Wiesenfeld, Hillier et al. 2003) as well as the multiple species of bacteria associated with bacterial vaginosis (BV) (Zariffard, Saifuddin et al. 2002, Srinivasan, Liu et al. 2010).

Several clinical studies have reported an inverse relationship between gonorrhoea and vaginal colonization by lactobacilli (Saigh, Sanders et al. 1978, Hillier, Krohn et al. 1992, Martin, Richardson et al. 1999). Some studies support the hypothesis that commensal lactobacilli in the lower genital tract reduce the risk of gonococcal infection in women through the production of H_2O_2 (Antonio, Hawes et al. 1999, St Amant, Valentin-Bon et al. 2002). The hypothesis that H_2O_2-producing lactobacilli are an innate defense against *N. gonorrhoeae* is consistent with reports that H_2O_2-producing *Lactobacillus* species commonly isolated from the vagina and cervix inhibit *N. gonorrhoeae in vitro*

(Antonio, Hawes et al. 1999, St Amant, Valentin-Bon et al. 2002). It is speculated that the inability of gonococcal catalase to neutralize H_2O_2 -mediated lactobacillus inhibition *in vitro* could be due to the production of overwhelming amounts of H_2O_2 or to a lactobacillus-encoded factor that interferes with the ability of gonococcal catalase to cleave H_2O_2 (St Amant, Valentin-Bon et al. 2002). However, more recent studies show that lactobacilli do not inhibit *N. gonorrhoeae* in mouse genital tract model (Muench, Kuch et al. 2009). *N. gonorrhoeae* is an obligate human pathogen and mouse genital tract differ from the human, hence, this studies outcome could be influenced by that fact. Interaction between lactobacilli and *N. gonorrhoeae* were investigated under anaerobic co-cultivation. This study shows that lactobacillus inhibitory action appears to be primarily due to acidification rather than H_2O_2 production (Graver MA 2011). Moreover, to successfully infect the female genital tract, *N. gonorrhoeae* must first adhere to the epithelia to avoid clearance by the continual flow of vaginal fluid. A reduction in the ability to adhere would significantly affect the outcome of an infection following exposure. This has been demonstrated by *in vitro* model of *N. gonorrhoeae* infection, in which, pre-colonization with lactobacilli reduced *N. gonorrhoeae* adherence to epithelial cells by 40 to 50% (Spurbeck and Arvidson 2008).

1.3.3 Infection of human cervical epithelial cells

N. gonorrehoeae is capable of infecting and invading primary human epithelial cells derived from both from the endo- and the ectocervix (Edwards, Shao et al. 2000). Invasion was found to occur primarily in an actin dependent manner

(Edwards, Shao et al. 2000). *N. gonorrhoeae* induced membrane ruffling and inflammation suppression are consistent with ability of this bacterium to enter cervical epithelial cells by interaction with complement receptor 3 (CR3) (Edwards, Brown et al. 2001), a receptor that does not trigger inflammatory response (Caron and Hall 1998). Binding of the gonococcus to CR3 requires the cooperative action of iC3b bound to the *N. gonorrhoeae* surface in conjunction with *N. gonorrhoeae* porin and pilus (Edwards, Brown et al. 2002) (Fig.1:1). Upon infection of primary cervical epithelial cells, gonococci release a phospholipase D homolog that gains access to the cervical intracellular environment nonspecifically through macropinocytosis of gonococci (Edwards, Entz et al. 2003, Edwards and Apicella 2006). Gonococcal phospholipase D (NgPLD) appears to promote infection of primary cervical epithelial cells in several ways. Recent data indicate that this secreted gonococcal protein augments signaling events that trigger CR3 mobilization to the surface of primary cervical cells (Edwards, Entz et al. 2003). NgPLD also modulates cervical cell signal transduction events leading to membrane ruffling. Mutant gonococci that lack functional NgPLD activity do not elicit membrane ruffling, and they are impaired in their ability to associate with and to invade primary human cervical cells (Edwards, Entz et al. 2003). These data suggest that NgPLD might modulate host cell signaling events required for successful, progressive, cervical infection.

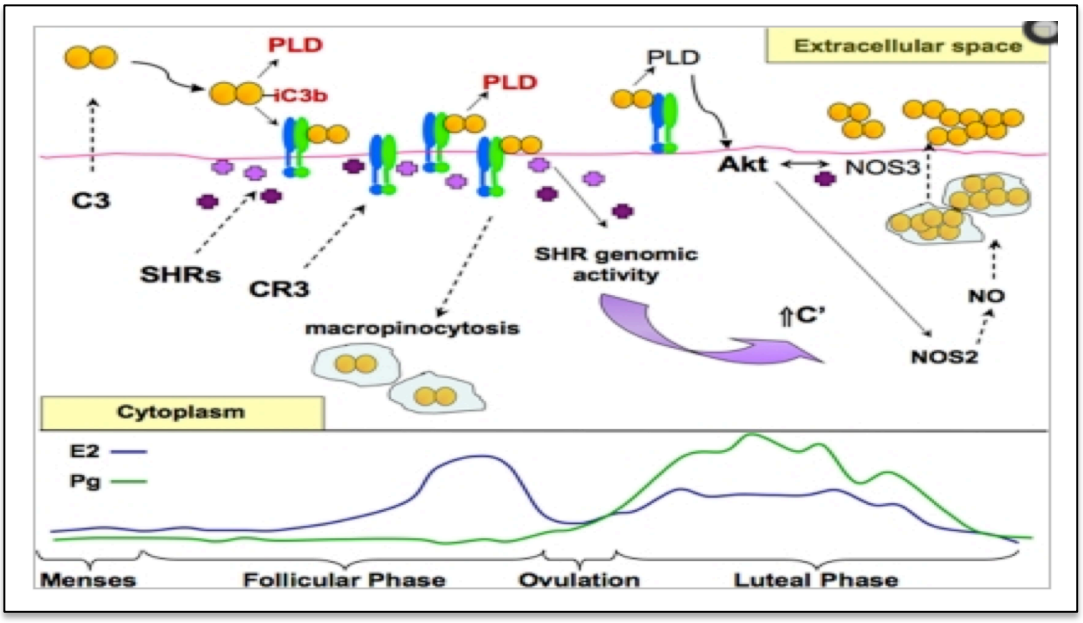


Figure 1.2 A working model of cervical infection by gonococci. Based on published, as well as unpublished data, a putative, working model of the dynamic interactions mediating gonococcal disease of cervical epithelia is presented in context of the menstrual cycle. The direct interaction of (Ng)PLD with (host) Akt, triggers ruffle formation and the internalization of gonococci within macropinosomes. It is expected that Akt activity would be increasingly maintained as more PLD is released by gonococci in the presence of increased C3 (Binnicker, Williams et al. 2003).

Other factors that influence infection and survival of gonococci in primary cervical epithelial cells (pex cells) is the serine/threonine kinase, protein kinase B (Akt) (Edwards and Apicella 2006). The ability of Akt to inhibit apoptosis as well as regulate cell cycle, gene transcription, glucose (Fig. 1.2) and nutrient uptake and metabolism and endocytosis make this regulator an attractive target by which pathogens could subvert normal host cell function (Wilkowsky, Barbieri et al. 2001, Yilmaz, Jungas et al. 2004, Huang, Li et al. 2005, Kierbel, Gassama-Diagne et al. 2005, Lee, Higashi et al. 2005). Akt is present in most tumour cells where it is regulator of cellular energy metabolism (Bhaskar and Hay 2007). Those cells have increased glycolysis with excess lactate accumulation in both in presence or absence of oxygen commonly referred as

Warburg effect (Robey and Hay 2009).

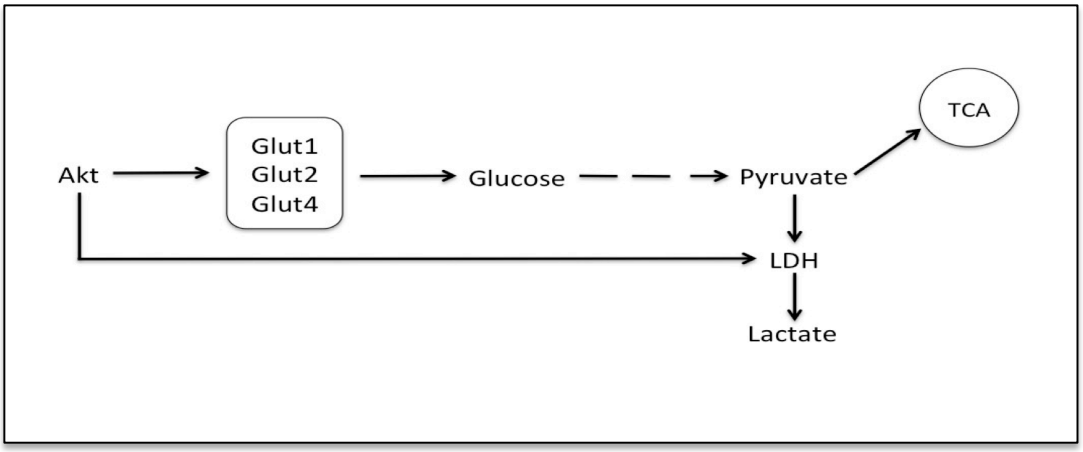


Figure 1.3 Akt increases glucose uptake by increasing the expression of glucose transporters (GLUT1, GLUT2, GLUT4) and increases the amount of lactate dehydrogenase (LDH), which leads to conversion of pyruvate to lactate. Modified from (Robey and Hay 2009).

Akt activation is a sequential process initiated by PI3-K-independent phosphorylation on Thr450, which does not con- tribute to its kinase activity but may serve as a signal indicating proper protein folding (Chan, Rittenhouse et al. 1999). Akt is then translocated to the plasma membrane by a process that is dependent on its plecktrin homology (PH) domain (Fukuda, Guo et al. 2003). Bacterial and human PLDs can bind to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (Kusner, Barton et al. 2003). PtdIns(4,5)P_2 plays a critical role in regulating the actin cytoskeleton and membrane trafficking. Edwards suggest that *N. gonorrhoeae* subvert cervical cell signaling pathways by competing with PtdIns(3,4,5)P_3 (and possibly other phosphoinositides) for Akt binding. Akt activation augments CR3 recruitment to the cervical cell surface in a PtdIns(4,5)P_2 and myosin light chain kinase (MLCK)- dependent manner and plays a further role in gonococcus invasion of, and survival within, the host cervical cell (Edwards and Apicella 2006).

Another consequence of the Akt pathway is the production of nitric oxide (NO) through the actions of nitric oxide synthases (NOS). NO is present in cervical secretions (Vaisanen-Tommiska, Nuutila et al. 2003), which are capable of supporting *N. gonorrhoeae* growth. Nitric oxide induction of *N. gonorrhoeae* nitric oxide reductase (NorB) could confer a survival advantage to these bacteria *in vivo* by serving as an energy source under conditions of oxygen limitation (Overton, Whitehead et al. 2006). Estradiol (E2) and progesterone (Pg) represent the major form of estrogens and progestagens, respectively, found in women. Pg-enhances NOS activity with subsequent NO production (Edwards 2010). Pg functions in an additive manner with NgPLD to induce Akt activity that, in turn, regulates NOS expression and NO production (Edwards 2010).

1.4 Aerobic and anaerobic respiration *in N. gonorrhoeae*

N. gonorrhoeae is Gram-negative cocci that typically appear in pairs (diplococci). It is non-spore forming and does not posses capsule. Its cell wall is typical of Gram-negative bacteria, with a peptidoglycan layer and an outer membrane containing endotoxic glycolipid complexed with protein. *N. gonorrhoeae* is oxidase positive and non-fermentative bacterium.

1.4.1 Aerobic respiration

N. gonorrhoeae has a high aerobic respiratory capacity and it has been suggested that this may enable gonococci to minimize oxidative damage from reactive oxygen species generated both from its own respiratory metabolism and by other bacteria that share its environment (Li, Hopper et al. 2010).

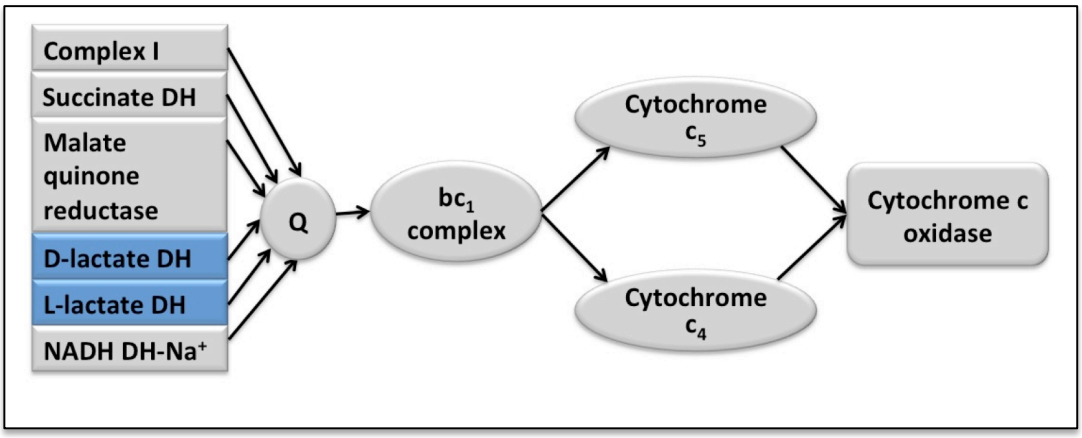


Figure 1.4 Organisation of the *Neisseria gonorrhoeae* aerobic respiratory chain. Description of the components appears in the text.

N. gonorrhoeae has only a single cytochrome oxidase, cytochrome cbb_3, which has a very high affinity for oxygen (Pitcher and Watmough 2004, Snyder, Davies et al. 2005, Chung, Yoo et al. 2008, Deeudom, Koomey et al. 2008). There are also the cytochrome bc_1 complex, and two cytochromes, designated *c*4 and *c*5, that transfer electrons between the bc_1 complex and the terminal oxidase (Fig. 1:5) (Li, Hopper et al. 2010). These cytochromes are tightly associated with the cytoplasmic membrane. Until recently it was not known about how electrons are transferred from the cytochrome bc_1 complex to the cytochrome oxidase cbb_3. Parallel pathways for electron transfer from the cytochrome bc_1 complex to the terminal oxidase through cytochrome *c*4 and *c*5 was proposed first for *Azotobacter vinelandii* (Downs and Jones 1975). Recent study in *N. gonorrhoeae* shows that gonococcal cytochromes *c*4 and *c*5 are involved in electron transfer to

oxygen and that one of these two cytochromes is essential for survival when the gene for the other has been mutated (Li, Hopper et al. 2010). Single mutants in either *c*4 or *c*5 have a small loss of the respiratory capacity but were far more sensitive to excess oxygen than the parental strain (Li, Hopper et al. 2010). This implies that the high respiratory capacity of *N. gonorrhoeae* prevents the accumulation of oxygen, which could generate toxic oxygen species such as hydrogen peroxide, superoxide, and hydroxyl radicals. Even though ROS is a key element of the innate immune response it can also be produced as a by-product of the bacterium's own metabolic processes. Based on studies of *E. coli* and eukaryotic mitochondria, NADH dehydrogenase (Nuo), succinate dehydrogenase, and the cytochrome bc_1 complex of the respiratory chain of *N. gonorrhoeae* are likely to be the main sites of endogenous ROS generation (Seib, Wu et al. 2006).

In contrast to the simplicity of the aerobic respiratory chain that oxidises ubiquinol there are a large number of dehydrogenases feed electrons into the ubiquinone pool.

1.4.2 Respiratory dehydrogenases

NADH:ubiquinone oxidoreductase (Nuo) also known as NADH dehydrogenase or Complex I in mitochondria, is a multiple subunit enzyme complex embedded in the cytoplasmic membrane (Yagi and Matsuno-Yagi 2003). This enzyme represents the first step of the respiratory chain and links the electron transfer from NADH to quinone with the translocation of protons from

the cytoplasmic phase to the periplasmic phase (Yagi and Matsuno-Yagi 2003). This protein complex is the first of the respiratory chain complexes which generate a proton motive force required for energy consuming processes like the synthesis of ATP (Anraku 1987).

The overall reaction is:

$$NADH + Q + 5H^{+}_{n} \rightarrow NAD^{+} + QH_2 + 4H^{+}_{p}$$

Where Q refers to ubiquinone, and H^{+}_{n} and H^{+}_{p} to the protons taken up from the negative inner and delivered to the positive outer side of the membrane.

Although mammalian mitochondrial complex I is composed of 46 subunits, bacterial counterparts contain 14 different subunits where the NuoBCDEFGI constitute the hydrophilic part harboring all known prosthetic groups, and NuoAHJKLMN make up the hydrophobic domain, embedded in the membrane. (Friedrich, Abelmann et al. 1998, Yagi, Yano et al. 1998, Carroll 2003). Recent studies show that the c-terminal end of the NuoA polypeptide is localized in the bacterial cytoplasm, in contrast to what was previously reported for the homologous NQO7 subunit from *Paracoccus denitrificans* Complex I (Virzintiene, Trane et al. 2011)

N. gonorrhoeae contain NuoA-NuoM based on sequence comparison with other bacterial species. The crystal structure of the hydrophilic domain (NuoBCDEFGI) of complex I from *Thermus thermophilus* was determined, revealing the location of the FMN and nine iron-sulfur clusters. Seven of these nine iron-sulfur clusters

are involved in electron transfer accepting the electrons from flavin mononucleotide (FMN), the primary electron acceptor, and sending them to the quinone-binding site (Q-site) at the interface with the membrane domain. Two additional iron-sulfur clusters are not part of the main redox chain (Berrisford and Sazanov 2009)

Na^+-translocating NADH dehydrogenase (Nqr)

NQR is distinguished from other protein complexes of electron-transport chains not only by the amino acid sequence but also by its set of cofactors employed. The main electron transferring centers of this enzyme are flavins (Verkhovsky and Bogachev 2010). In addition to flavin adenine dinucleotide (FAD) and two flavin mononucleotides (FMN), the protein was found to contain also a non-covalently bound riboflavin (Barquera, Zhou et al. 2002). NqrF subunit was also predicted to contain an iron–sulfur cluster (Rich, Meunier et al. 1995). In accordance with this prediction, a [2Fe–2S] cluster was detected in both the Na^+-NQR complex and and in an isolated fragment of the NqrF subunit (Barquera, Hellwig et al. 2002, Barquera, Zhou et al. 2002, Turk, Puhar et al. 2004).

During *in vivo* function Nqr oxidizes NADH and transfers two electrons to ubiquinone with production of ubiquinol:

$$NADH + H^+_{in} + Q + 2Na^+_{in} \rightarrow NAD^+ + QH_2 + 2Na^+_{out}$$

This redox reaction is coupled with a vectorial transfer of two sodium ions across the membrane.

In the absence of sodium ions Nqr is unable to translocate protons

(Tokuda, Udagawa et al. 1985, Zhou, Bertsova et al. 1999). The transfer of sodium by the enzyme is associated with generation of a trans-membrane electrical potential (Tokuda, Udagawa et al. 1985, Zhou, Bertsova et al. 1999). Protons released on oxidation of NADH during the catalytic cycle of Na^+-NQR enter the cytoplasm, whereas protons required for the formation of ubiquinol are also taken up from the cytoplasmic side of the membrane (Zhou, Bertsova et al. 1999).

Succinate dehydrogenase (Sdh)

Succinate dehydrogenase (SDH) is a hetero-tetrameric enzyme complex that catalyzes the oxidation of succinate to fumarate with the concomitant reduction of ubiquinone to ubiquinol (Hagerhall 1997). Formally, the enzyme can be described as a succinate:ubiquinone oxidoreductase, and the classic oxidation-reduction reaction it catalyzes is dependent on a flavin adenine dinucleotide (FAD) cofactor in subunit 1 (SdhA). The second subunit of Succinate dehydrogenase subunit 2 (Sdh2) contains additional cofactors; three distinct iron-sulfur clusters whose function is to transfer the two electrons in one electron increments resulting from the dehydrogenation of succinate at the active-site FAD (Oyedotun, Sit et al. 2007, Tran, Rothery et al. 2007, Maklashina, Rajagukguk et al. 2010 , Kim, Khalimonchuk et al. 2012). FAD is covalently bound to the polypeptide structure. The catalytic chemistry of succinate oxidation yielding fumarate is absolutely dependent on this covalent bond (Blaut, Whittaker et al. 1989, Robinson, Rothery et al. 1994).

The overall reaction is:

succinate $\rightleftharpoons$ fumarate + $2H^+$ + $2e^-$

quinone + $2H^+$ + $2e^-$ $\rightleftharpoons$ quinol

Malate quinone reductase (Mqr)

Malate quinone reductase (Mqr) is a membrane-bound protein. It is a flavin adenine dinucleotide (FAD)- and lipid-dependent peripheral membrane protein catalyzing the oxidation of L-malate to oxaloacetate (van der Rest, Frank et al. 2000). The electrons are donated to the electron transfer chain at the level of quinone. This reaction is essentially irreversible (Kather, Stingl et al. 2000).

D-lactate dehydrogenase (D-iLDH)

D-lactate dehydrogenase (D-iLDH) is a peripheral membrane respiratory enzyme involved in electron transfer, located on the cytoplasmic side of the inner membrane (Johnston and Gotschlich 1974). In *N. gonorrhoeae* D-lactate dehydrogenase catalyzes the oxidation of D-lactate to pyruvate. It uses D lactate as a carbon source and subsequently transfers two electrons and two protons to the electron transfer chain. It consists of three domains: the flavin adenine dinucleotide (FAD)-binding domain, the cap domain, and the membrane-binding domain (Fischer, Martin et al. 1994). The FAD-binding domain contains the site of D-lactate oxidation by a non-covalently bound FAD cofactor and has an overall fold similar to other members of a recently discovered FAD-containing family of proteins. This structural similarity extends to the cap domain as well. The most prominent difference between LdhD and the other members of the FAD-containing family is the membrane-binding domain, which is either absent in

some of these proteins or differs significantly (Dym, Pratt et al. 2000). D-iLDH from *E. coli* was characterised (Vanderpuye, Labarrere et al. 1992) and crystal structure determined (Fig. 1.4). D-iLDH from *N. meningitidis* was purified and used D-lactate as a substrate. Mutant lacking D-iLDH was not able to grow on D-lactate as a single carbon source (Caron and Hall 1998).

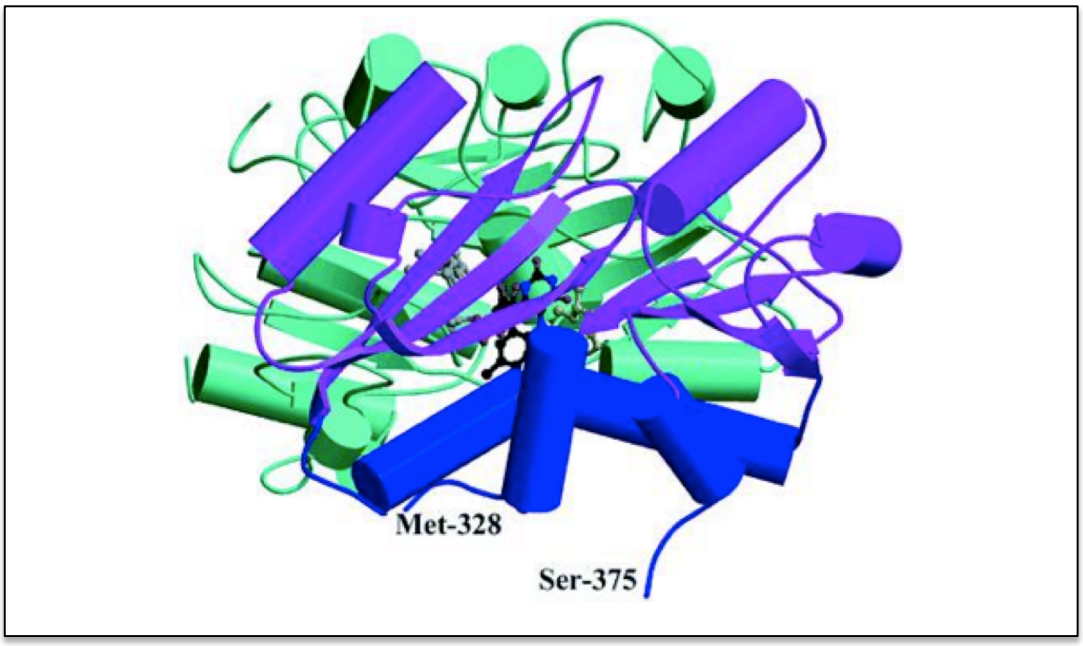


Figure 1.5 Ribbon representation of the D-iLDH molecule from *E coli* complexed with FAD. The three domains are: in cyan, the FAD-binding domain; in purple, the cap domain; and in blue, the membrane-binding domain.

L-lactate dehydrogenase (L-iLDH)

L-lactate dehydrogenase (L-iLDH) is the membrane bound protein located at the cytoplasmic side of the inner membrane just like D-iLDH. It converts L-lactate to pyruvate transferring the electrons to ubiquinone. It is an FAD containing protein (Garvie 1980). The activity of this enzyme can be measured in intact bacteria and in some membrane fractions by lactate-dependent oxygen uptake. *E. coli* possesses single L-iLDH and mutation in the gene coding for this protein, renders it unable to grow on L-lactate as a single carbon source

(Dong, Taylor et al. 1993). Unlike *E. coli*, a *N. meningitidis* mutant strain defective in L-iLDH was still able to grow on L-lactate, thus it could possess two L-lactate dehydrogenases (Gotschlich 1996). Moreover, Fu and co-workers found that mutant strain defect in L-iLDH consumed O_2 at a rate considerably slower than the parental strain with L-lactate as a carbon source (Fu, Hassett et al. 1989). Both L-iLDH and D-iLDH from *N. gonorrhoeae* (annotated as LldD and LdhD in this study) have previously been purified and characterised (Fischer, Martin et al. 1994). L-iLDH and D-iLDH showed affinity for L- and D-lactate respectively. With this study it was concluded that *N. gonorrhoeae* possesses two respiratory lactate dehydrogenases, one oxidising L- and the other oxidising D-lactate and possibly second L-lactate dehydrogenase.

1.4.3 Anaerobic respiration

N. gonorrhoeae was long considered to be an obligate aerobe until it was discovered that anaerobic growth was possible when nitrite or nitric oxide (NO) was used as a terminal electron acceptor (Knapp and Clark 1984, Barth, Isabella et al. 2009). Anaerobic growth is accomplished through utilization of a truncated denitrification pathway, which consists of a nitrite reductase (AniA) and a nitric oxide reductase (NorB) (Isabella, Wright et al. 2008). AniA was identified in a screen for anaerobically regulated genes, as one of three outer membrane proteins whose expression is induced under anaerobic growth conditions (Clark, Campbell et al. 1987). AniA is a lipoprotein that functions as a nitrite reductase (Fig. 1:5) and is subsequently required for anaerobic growth (Hoehn and Clark 1992, Mellies, Jose et al. 1997 , Householder, Belli et al. 1999). NorB is a heme protein responsible for reducing AniA-generated NO to nitrous oxide

(Fig. 1:5) (Householder, Fozo et al. 2000). Unlike most NO reductases that are found in denitrifying organisms (Zumft 1997), NorB is composed of a single functional subunit (Householder, Fozo et al. 2000). AniA is a major antigen recognized in sera from patients with gonococcal disease (Clark, Knapp et al. 1988). Anaerobic respiration probably occurs naturally during infection. Hence, *N. gonorrhoeae* forms biofilms during cervical infection, it uses anaerobic respiration in this oxygen-limited environment (Steichen, Shao et al. 2008, Falsetta, McEwan et al. 2010, Phillips, Steichen et al. 2012). Mutants in which the *aniA* or *norB* gene was interrupted are attenuated for biofilm formation over glass and transformed human cervical epithelial cells (THCEC) (Falsetta, Bair et al. 2009). Some evidence suggests that the *N. gonorrhoeae* reduction of host-produced NO may be responsible for the high incidence of asymptomatic disease (Cardinale and Clark 2005, Barth and Clark 2008). Studies have shown that regulation of the genes involved in denitrification and/or adaptation to anaerobic growth in the neisserial species involves the transcriptional regulators FNR and NsrR, and the two-component system NarQP (Clark 2009). FNR is the only regulator in the pathogenic *Neisseria* known to respond directly to anaerobic environmental conditions, and does so through a labile, oxygen-sensitive [4Fe–4S] cluster coordinated by four cysteine residues (Overton, Reid et al. 2003, Clark 2009). FNR activation is a requirement for efficient transcription of *aniA* and *fnr* mutations in *N. gonorrhoeae* result in the inability of *N. gonorrhoeae* to reduce nitrite (Householder, Belli et al. 1999, Clark 2009). NsrR is an NO-sensing rrf2-type transcriptional repressor. In *N. gonorrhoeae* NsrR regulates negatively *aniA* and *norB* and derepression occurs in the presence of NO (Isabella, Wright et al. 2008, Clark 2009, Isabella, Lapek et al.

2009). The NarQP two-component regulatory system is involved in regulation of the Neisserial denitrification pathway, and is known to activate AniA in *N. gonorrhoeae* (Clark 2009).

Cytochrome *c* peroxide, encoded by *ccp*, is a lipoprotein and is one of the proteins induced during anaerobic growth (Turner, Reid et al. 2003). Apart from *aniA* and *norB*, this is the protein that is also involved in respiratory metabolism and stress tolerance. It has been demonstrated that *ccp* was upregulated in biofilms grown on glass and transformed human cervical epithelial cells (Phillips, Steichen et al. 2012). These data suggest that biofilm formation by the gonococcus may represent a response that is linked to the control of nitric oxide steady-state levels during infection of cervical epithelial cells (Falsetta, Bair et al. 2009). Transcription of *ccp* is controlled by the fumarate and nitrate reductase regulator protein, which controls the expression of other important anaerobic metabolism genes in *N. gonorrhoeae* (Falsetta, Bair et al. 2009).

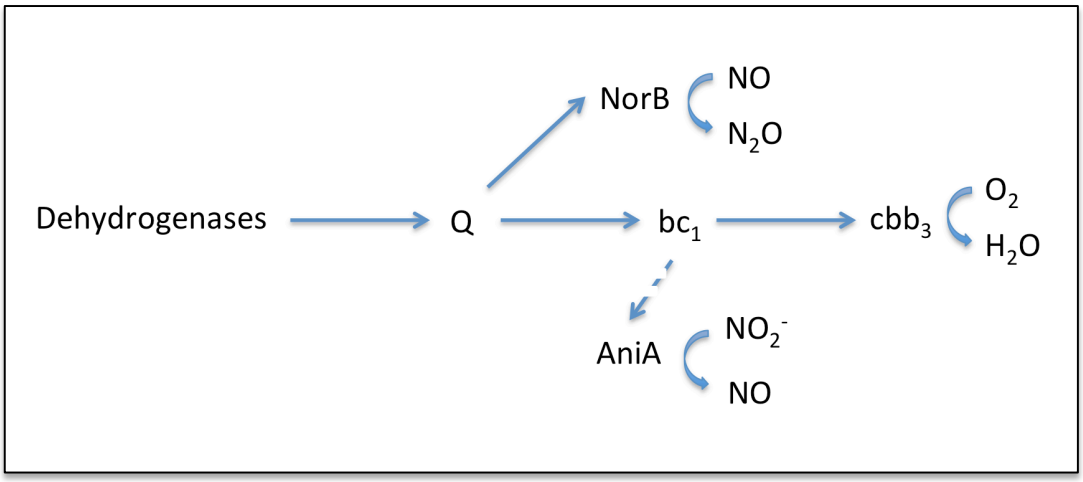


Figure 1.6 AniA (nitrite reductase) reduces nitrite to nitric oxide (NO), which is then reduced to nitrous oxide (N_2O) by NorB (NO reductase).

1.5 Lactate metabolism in N. gonorrhoeae

Carbon sources most effectively utilised by *N. gonorrhoeae* are lactate and glucose, and the acquisition of lactate has been implicated in the virulence of this species (Morse, Stein et al. 1974). The first indication that lactate metabolism might contribute to the pathogenesis of gonococcal infection was the demonstration that lactate from human neutrophils stimulated oxygen consumption by gonococci, which in turn could impair oxygen-dependent bactericidal mechanisms (Britigan, Klapper et al. 1988). Lactate stimulates gonococcal metabolism leading to a more rapid emergence from lag phase, a 20% increase in the rate of growth with enhanced LPS, and protein production (Gao, Parsons et al. 1998). The reason for the stimulation is that lactate does not have to contribute to gluconeogenesis in the bacterium in the presence of glucose and so is solely used as a source of energy (Yates, Gao et al. 2000, Yates and Smith 2003). Lactate permease (LctP) is the protein responsible for uptake of lactate, which is naturally present in the female genital. A mutant defective in *lctP* was markedly impaired in the colonisations capacity (Exley, Wu et al. 2007).

Female genital tract is inhabited by lactobacilli (Eschenbach, Davick et al. 1989, Giorgi, Torriani et al. 1989, Reid, McGroarty et al. 1996, Antonio, Hawes et al. 1999, Ravel, Gajer et al. 2011). Lactobacilli are the main source of lactate in the female lower genital tract. Lactate provides carbon source to *N. gonorrhoeae* (Catlin 1973) and is important in pathogenicity of this bacterium (Yates, Gao et al. 2000, Smith, Yates et al. 2001, Exley, Wu et al. 2007). Lactate stimulates overall metabolism evidenced by greater LPS production enhanced protein

synthesis and larger pentose contents (Gao, Parsons et al. 1998, Gao, Linden et al. 2000). When *N. gonorrhoeae* was grown in media containing both lactate and glucose there was more rapid emergence from lag phase with lactate and a 20% increase in the rate of logarithmic growth compared with growth in glucose alone (Gao, Parsons et al. 1998). The fate of the lactate carbon was followed by nuclear magnetic resonance (NMR) analysis of membrane lipids and LPS purified from gonococci grown in a defined medium with labeled glucose alone, with labeled lactate and unlabeled glucose, and with labeled glucose and unlabeled lactate. The result was that in the presence of glucose, lactate carbon was incorporated into fatty acid moieties and not into the ethanolamine/glycerol of membrane lipids or the carbohydrate of LPS (Yates, Gao et al. 2000, Yates and Smith 2003). When lactate is used alone by gonococci it is oxidized to pyruvate and then fulfils two functions: 1) gluconeogenesis to produce sugar, glycerol and ethanolamine moieties of gonococcal constituents and 2) formation of acetyl-coenzyme A (CoA), the precursor of fatty acid synthesis and constituents of the tricarboxylic acid (TCA) cycle. When glucose is present gluconeogenesis from lactate does not take place (Smith, Yates et al. 2001).

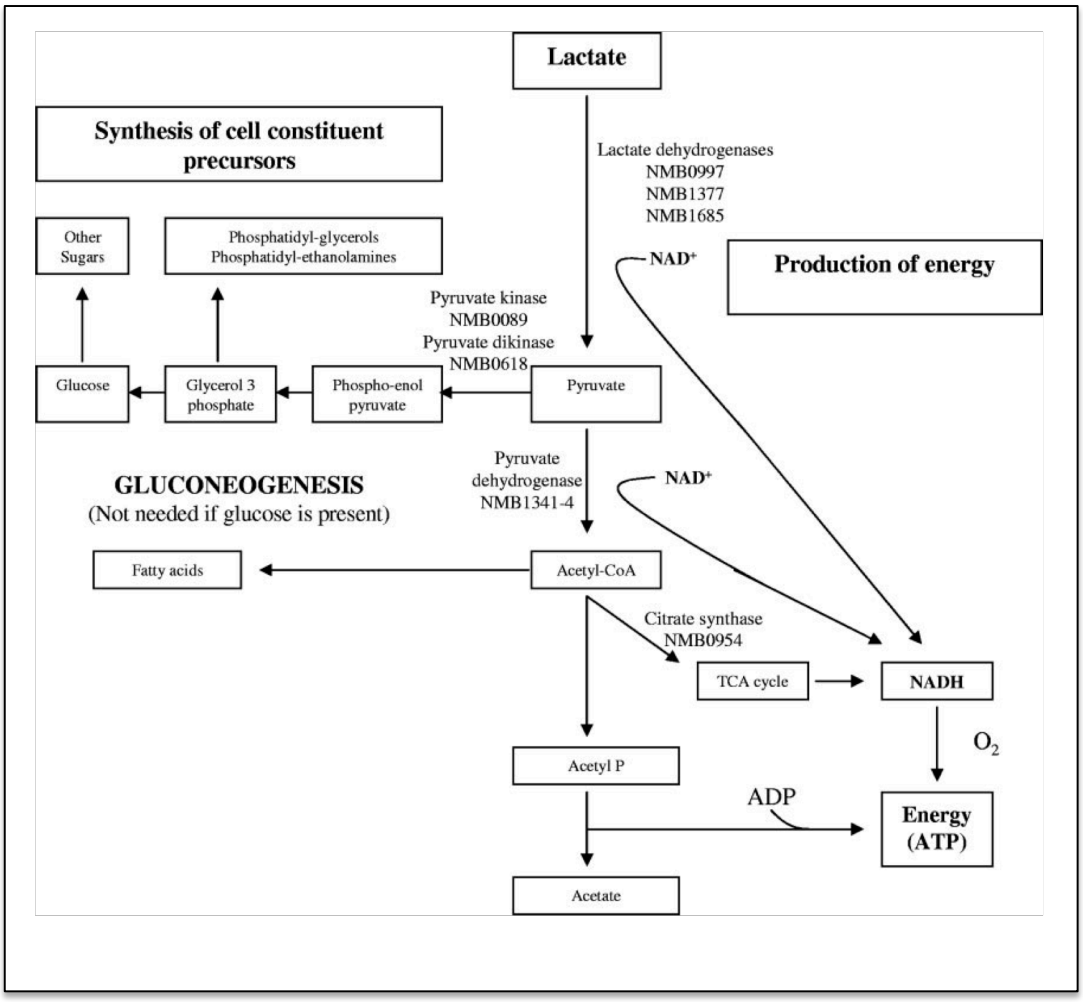


Figure 1.7 Utilization of lactate by gonococci for energy production and synthesis of cell constituent precursors. If glucose is present, gluconeogenesis from lactate is shut down. Relevant enzymes are shown and annotated according to the serogroup B *N. meningitidis* genome sequence (Smith, Tang et al. 2007).

In the female genital tract, both glucose and lactate are present in millimolar concentrations (Preti 1978). This shuts off the gluconeogenesis from lactate and consequently stimulates metabolism. Addition of lactate to gonococcal cultures containing physiological concentrations of glucose is associated with changes in the fatty acid and carbohydrate composition of LPS (Yates, Gao et al. 2000, Yates and Smith 2003). Alterations of both lipid and carbohydrate components of LPS have been associated with changes in serum resistance of pathogenic neisseria

(Vogel and Frosch 1999). From this one may conclude that lactate metabolism is crucial for both survival and pathogenicity of *N. gonorrhoeae*

1.6 Glucose metabolism in gonococci

Previous studies demonstrated that *N. gonorrhoeae* utilize glucose by involving the Entner–Doudoroff (ED) pathway and pentose phosphate pathway (PP) (Morse, Stein et al. 1974, Holten 1975, Hebeler and Morse 1976). pH affects participation of these two pathways during glucose catabolism. At pH 7.2 ED participates with 80%, while PP participates with 20%. ED is inferior to glycolysis in the mean of energy produced. At more acidic pH (pH 6), partition of glucose catabolized, is the same for both pathways (50%) (Morse and Hebeler 1978). At this pH cells have also active tricarboxylic acid cycle, which increases the amount of energy obtained from glucose. Taking in consideration that pH at the site of infection ranges from 5.4-8.2 (depending on the menstrual cycle) (Wilson 2008) with the median of 7 one can speculate that glucose is catabolized with different partitions of ED and PP during the menstrual cycle. Also, at the site of infection, glucose is ever present with lactate.

1.7 N. gonorrhoeae and iron

Iron plays an important role in different metabolic pathways, making it essential for life in almost all organisms. Iron is a component of heme, iron-sulfur cluster or iron centres in enzymes such as catalase, cytochromes, metalloflavoproteins, ribonucleotide reductase, and peroxidase (Andrews, Robinson et al. 2003). Most bacterial pathogens must compete with their hosts for iron. For many pathogens,

this process involves secretion of low-molecular weight chelators called siderophores, which sequester and solublize otherwise, inaccessible ferric iron from the environment within the host (Braun and Hantke 2011). *N. gonorrhoeae* is somewhat unusual in that they do not have the capacity to secrete siderophores. Instead, it express surface receptors that mediate direct extraction and import of iron from the human host iron-binding proteins hemoglobin, lactoferrin, and transferrin (Cornelissen and Hollander 2011). Lactoferrin and transferrin are present on the urogenital mucosal surface while hemoglobin is released into the environment during the menses (Aisen and Leibman 1972, Hallberg and Rossander-Hulten 1991). Tbp, Lbp and Hpu are the receptors responsible for acquisition of transferrin, lactoferrin and hemoglobin respectively (Lee and Schryvers 1988, Stojiljkovic, J et al. 1996). All three receptors require the TonB-ExbB-ExbD inner membrane complex to energize transport through the outer membrane (Biswas, Anderson et al. 1997, Stojiljkovic and Srinivasan 1997). Experiment with a murine model of infection demonstrated that gonococcal transferrin and hemoglobin receptor mutants are not attenuated in mice, thereby ruling out transferrin and hemoglobin as essential for murine infection. An increased frequency of phase variants with the hemoglobin receptor "on" (Hg(+)) occurred in ca. 50% of infected mice suggesting that the presence of hemoglobin in inflammatory exudates selects for Hg(+) phase variants during infection (Jerse, Crow et al. 2002). It is also demonstrated that expression of TonB is crucial for the survival of *N. gonorrhoeae* in cervical epithelial cells while expression of lactoferrin and

transferrin receptors was not necessary for intercellular survival (Hagen and Cornelissen 2006).

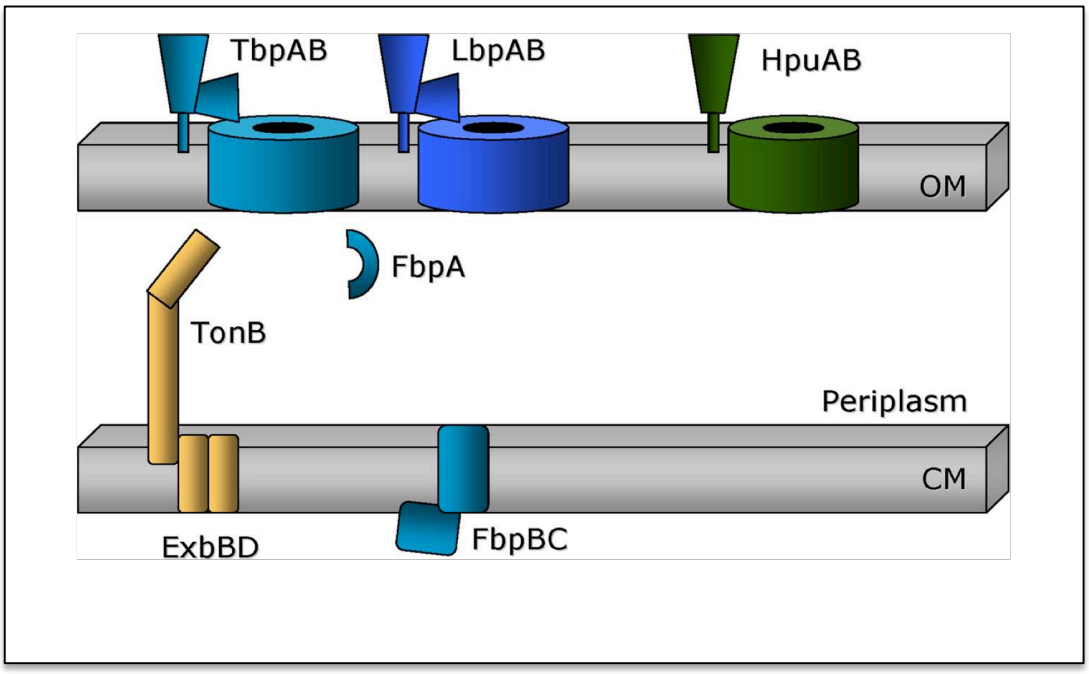


Figure 1.8 Two component gonococcal systems for acquisition of iron from host proteins. The TonB dependent outer membrane transporters are shown as barrels traversing the outer membrane (OM). The lipid-modified companion proteins are shown tethered to the outer membrane surface. TonB, ExbB, and ExbD (gold) are depicted as attached to or imbedded within the cytoplasmic membrane (CM).

Other studies show that ferric citrate, free heme and some xenosiderophores are acquired by the gonococcus in pathways that do not depend upon expression of TonB or any of the individual TonB-dependent transporters (Biswas, Anderson et al. 1997, Strange, Zola et al. 2011).

In the course of infection gonococcal genes involved in iron homeostasis are up-regulated including the ferric uptake regulatory protein (Fur) (Agarwal S 2008). Fur functions as a regulator of gene transcription through both direct and indirect mechanisms (Yu and Genco 2012).

Microarray data show that genes encoding proteins involved in protein synthesis, energy metabolism, and transcription were preferentially expressed when *N. gonorrhoeae* was grown under high-iron conditions (Ducey, Carson et al. 2005). This suggests an overall increase in cellular metabolism. The expression profile of these genes is consistent with an increased growth rate in iron-containing medium compared to iron-restricted medium (Ducey, Carson et al. 2005). Increased level of protein synthesis would dictate a higher energy requirement for the organism. This is reflected in the microarray data. The putative membrane-associated proteins involved in electron transport, *nqrC* and *nqrF*, *nqrA* and *nuoD* were induced by iron (Ducey, Carson et al. 2005, Jackson, Ducey, Day et al. 2010). Interestingly, expression of L-lactate dehydrogenase was induced under iron depleted conditions. As L-lactate dehydrogenase is involved in respiration too, this would help provide energy to cell when iron is present in lower concentrations.

Chapter 2

Materials and Methods

2 General methods

2.1 Growth conditions

N. gonorrhoeae was routinely grown on solid GC agar (Oxoid) supplemented with 1% (v/v) IsoVitalX (Becton Dickinson) or liquid GW medium or chemically defined medium (CDM) (Wade and Graver 2007, Spence, Wright et al. 2008). Solid medium was supplemented with appropriate antibiotics. Growth on solid medium was performed at 37°C with 5% (v/v) CO_2, whereas liquid cultures were grown at 37°C unless otherwise stated. GW consisted of normal strength M199 cell culture medium, ammonium bicarbonate 17 mM, sodium acetate trihydrate 4.9 mM, L-glutamine 3.4 mM, spermidine 919.0 μM, L-arginine 383.0 μM, hypoxanthine 99 μM, uracil 298 μM, oxaloacetate 252 μM, thiamine hydrochloride 99 μM, L-ornithine 39 μM, NAD^+ 10 μM. CDM was prepared according to the stated protocol in Laboratory Maintenance of *Neisseria gonorrhoeae* (Sun, Bakshi et al. 2000).

E. coli was routinely grown on solid or liquid Luria-Bertani (LB) medium supplemented with appropriate antibiotics. LB consisted of Tryptone (10 g), Yeast Extract (5 g) and NaCl (10 g) (Sambrook and Russell 2001).

The working concentrations of antibiotics are shown in Table 1D.

2.1.1 Bacterial strains, plasmids and primers

The strains, plasmids and primers used in this thesis are described in Tables 1A, 1B and 1C.

Table 1A. Bacterial strains used in this thesis

Strains	Description	Reference
1291	*N. gonorrhoeae*	
F62	*N. gonorrhoeae*	
ΔlldD	*N. gonorrhoeae* (1291) *lldD::kan*	Ch.1
ΔldhD	*N. gonorrhoeae* (1291) *ldhD::kan*	Ch.1
ΔldhA	*N. gonorrhoeae* (1291) *ldhA::kan*	Ch.1
ΔlldDΔlutABC	*N. gonorrhoeae* (1291) *lldD::kan, lutABC::cm*	Ch.2
ΔlutABC	*N. gonorrhoeae* (1291) *lutABC::cm*	Ch.2
ΔlctP	*N. gonorrhoeae* (1291) *lctP::kan*	Ch.3
DH5-α	*E. coli* laboratory cloning strain	Promega, USA

Strains *ΔldhD, ΔlldD* and *ΔldhA* were generated in the McEwan laboratory by Dr Rachel vanden Hoven (Atack, Ibranovic et al. 2014). Genotypes of all mutant strains were confirmed by sequencing.

Table 1B. Plasmids used in this thesis

Plasmids	Description	Reference (Chapter)
pGem T-Easy	Commercial cloning vector	Ch.3, 4
pUC4K	Deletion mutant template plasmid (*kan*)	Ch.3, 4
pUC19	Commercial cloning vector	Ch.5

Table 1C. Primers used in this study

Primer	Description	Sequence (5'-3')
Primers used in Ch.3		
DLNA F	*ldhD* knock out forward primer	CTCACCGGTATGCTTGCAGC
DLNA R	*ldhD* knock out reverse primer	CCGGGAAACACGCCTACC
DLD F	*ldhA* knock out forward primer	CAGCGAACGCATCCGCAC
DLD R	*ldhA* knock out reverse primer	GAACTGCCCTACCGCGTG
LLD F	*lldD* knock out forward primer	AAAACCATGGGGAAGGCTGATCAGG
LLD R	*lldD* knock out reverse primer	AAACTGCAGGGCAGTCATGGTATGG
Primers used in Ch.4		
lutA F	lutABC knock out forward primer	AAACGGAAGGTGCGTATCAG
lutA-Cm R	50 bp overhang tag reverse primer	AAAATACGCCCGGTAGTGGGAATGGTTGC GCTCAT
lutB-Cm F	50 bp overhang tag forvard primer	TAAGGCAGTTATTGGTGCCCGAAGAAAACCCTGCACGAA
lutB R	lutABC knock out reverse primer	AAAGGACTGAGCCCTGTTCA
Cm F	Cm cassette forward primer	CACTACCGGGCGTATTTTT
Cm R	Cm cassette reverse primer	GGCACCAATAACTGCCTTA
lutA screen F	screening forward primer	CAGCGAGAGGAAAATCCAAA
Cm screen R	screening reverse primer	GGGAAATAGGCCAGGTTTTC
Cm screen F	screening forward primer	AAACGTTTTCATCGCTCTGG
lutB screen R	screening reverse primer	CAGTTGAAAGGCAACGGTCT
Primers used in Ch.5		
Lctp-KO F1	lctP knock out primer	CCGGCGAATTCATAATCCGCCACGACATCC
Kan-KO R1	50 bp overhang tag reverse primer	TGAGACACAACGTGGCTTTCTTTCACCATCAGCCAAACA
Kan-KO F2	50 bp overhang tag forvard primer	AACACTGGCAGAGCATTACGCGGCAATGATTTTCTTCCTC
lctP-KO R2	lctP knock out primer	GCGCGGTCGACGTTTGCCGATTTTACGCTGT

Table 1D. Media additives: Working concentrations

Additive	Working concentration	Bacterium
Ampicillin	100 μg/ml	*E. coli*
Chloramphenicol	30 μg/ml	*E. coli*
Chloramphenicol	3 μg/ml	*N. gonorrhoeae*
Kanamycin	100 μg/ml	*E. coli*
Kanamycin	100 μg/ml	*N. gonorrhoeae*
Glucose	1% (w/v)	*N. gonorrhoeae*
L-lactate	6 mM	*N. gonorrhoeae*

2.2 DNA manipulation and genetic techniques

Plasmid DNA was isolated the QIAprep Spin Miniprep (Qiagen, Australia). Restriction endonucleases were used according to manufacturer's specification (New England Biolab, USA). Ligation was performed with T4 DNA ligase according to the manufacturer (New England Biolabs, USA). PCR was performed using Phusion Hot Start High- Fidelity polymerase (New England Biolabs, USA) according to the manufacturer's instructions. PCR products were purified using the QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen, Australia). Gel extraction was performed using the QIAquick gel extraction kit according to the manufacturer's instructions (Qiagen, Australia) DNA concentration was determined by the Nanodrop 1000 spectrophotometer (Thermo Scientific, USA) as per manufacturer's instructions. DNA sequencing was performed by the Australian Genome Research Facility (The University of Queensland, Brisbane, Australia).

Prior the transformation, *N. gonorrhoeae* 1291 was streaked on the GC plate (medium density, in 2-3 cm square) and grown overnight. 10-20 μg DNA (linearized plasmid) was added to the neisserial cells. DNA was allowed to soak and cells were replated on the GC plates containing the appropriate antibiotic and incubated over night (Dillard 2011).

E. coli DH5-α competent cells were transformed according to the Promega's manual (Promega, USA).

2.3 Methods specific to Chapter 3

2.3.1 *N. gonorrhoeae* membrane preparation

400 ml *N. gonorrhoeae* (1291 and isogenic mutant strains) cultures were grown microaerobically (400 ml in 500 ml flask, 100 rpm) to an OD_{600} of ~0.5 before the addition of 100 μg/ml chloramphenicol to inhibit protein synthesis. The culture was shaken for an additional 30 min and harvested by centrifugation at 6000 rpm (Beckman Avanti J20i XPi Centrifuge) for 30mins at 4°C. Pellets were washed twice with 50 mM HEPES-NaOH pH 7.0 + 10 mM $MgCl_2$ and resuspended in a final 2 ml volume of the same buffer. Cells were broken by passage through a French pressure cell at 2500 psi and cell debris was removed by centrifugation at 6000 rpm (Beckman Avanti J20i XPi Centrifuge) for 30mins at 4°C. The supernatant containing the cell membrane was removed and ultracentrifuged at 50,000 rpm (Beckman Optima XL/I) for 2 hours to collect the membrane pellet. This final membrane-containing pellet was resuspended in 1 ml of 50 mM HEPES-NaOH pH 7.0 + 10 mM $MgCl_2$.

2.3.2 Oxygen consumption assays

Respiratory studies were performed using membranes isolated from wild type *N. gonorrhoeae* 1291 and the isogenic mutant strains, *DldhA, DldhD,* and *DlldD* by using a Clark-type oxygen electrode and accompanying software (Hansatech), based on the method of Markwell & Lascelles (1978). The reaction mixture contained 25 mM Phosphate buffer pH 7.5, 5 mM substrate, D- or L-lactate

(Sigma-Aldrich, USA), plus 100µl of the indicated membrane preparation. Reactions were carried out in a total volume of 2 ml at 25°C. The experiment is performed at three different occasions in duplicates. Excel was used to calculate oxygen consumption.

2.3.3 Enzyme assays

Prior to carrying out enzyme assays, cells were grown microaerobically (200 ml in 250 ml flask, 100 rpm) to mid exponential phase and harvested. The cell pellet was lysed with BugBuster (Protein Extraction Reagent, Novagen, Australia), debris removed by centrifugation at 14000 rpm, and cell free supernatant collected and used in the assays. The LDH activity of wild type (1291) and mutants defective in each of the respiratory dehydrogenases (isogenic mutant strains *ΔldhD* and *ΔlldD*) was determined with L- or D-lactate (Sigma-Aldrich, USA), using cell free extracts containing ~0.5 mg total protein. 2,6-dichlorophenolindophenol (DCPIP) linked assays were performed aerobically (50 ml in 250 ml flask, at 200 rpm) 37°C in a 3 ml reaction mixture containing 20 mM potassium phosphate buffer pH 7.2, 2 mM $MgCl_2$, 30 mM sodium L- or D-lactate (Sigma-Aldrich, USA), 0.25 mM PMS, 0.025 mM DCPIP, and dH_2O up to 3 ml final volume. The reaction was started by the addition of L- or D-lactate and the reduction of DCPIP followed at 600 nm. Specific activities were calculated using an extinction coefficient at 600 nm for DCPIP of 21 mM^{-1} cm^{-1}.

NAD^+ dependent lactate dehydrogenase activity was determined at 37°C in a 1ml reaction mixture containing 10 mM Tris-HCl pH 7.5, 30 mM sodium pyruvate or D-lactate, 0.4 mM NADH or NAD^+, cell free extracts containing 0.3–0.5 mg total

protein, and dH_2O up to 1 ml final volume. The reaction was started by the addition of sodium pyruvate (Sigma-Aldrich, USA) or D-lactate (Sigma-Aldrich, USA) and the oxidation/reduction of NADH/NAD^+ followed at 340 nm. Specific activities were calculated using an extinction coefficient for NADH of 6.22 mM^{-1} cm^{-1}. Experiment was performed in duplicates at three separate occasions. Excel was used to calculate specific activity.

2.3.4 Assays for metabolites

2 ml samples of cell cultures grown aerobically (50 ml in 250 ml flask at 200 rpm) or microaerobically (200 ml in 250 ml flask at 100 rpm) were collected after 4, 6, 8 and 24 h for metabolite analysis. These samples were pelleted (5000 rpm for 5 min at 22°C, Beckman Avanti J20i XPi Centrifuge). The supernatant was then filter sterilized and used to assay metabolite concentrations. Quantitation of D-lactate and acetate concentrations in supernatant were performed using the D-lactic acid and acetic acid determination kits as described by the manufacturer (Megazyme, Ireland). Experiment was repeated at four separate occasions.

2.4 Methods specific to Chapter 4

2.4.1 Agarose Gel Electrophoresis

Electrophoresis was carried on 1% agarose gel prepared in 1xTBE (Tris-Borate-EDTA buffer. Prior to loading on the gel, loading dye was added to every sample. Electrophoresis was run at 120 V.

2.4.2 Oxygen consumption assays

Respiratory studies were performed using whole cells from wild type *N. gonorrhoeae* 1291 and the isogenic mutant strains, *ΔldhA, ΔldhD,* and *ΔlldD* by using a Clark-type oxygen electrode and accompanying software (Hansatech), based on the method of Markwell & Lascelles (1978). The reaction mixture contained 25 mM Phosphate buffer pH 7.5, 5 mM substrate (glycolate), plus 100 µl of the indicated membrane preparation. Reactions were carried out in a total volume of 2 ml at 25°C.

2.4.3 Enzyme assays

Prior to carrying out enzyme assays, cells were grown microaerobically (200 ml in 250 ml flask at 100 rpm) to mid exponential phase and harvested. The cell pellet was lysed with BugBuster (Protein Extraction Reagent, Novagen, Australia), debris removed by centrifugation at 14000 rpm, and cell free supernatant collected and used in the assays. The LDH activity of wild type (1291) and mutants defective in respiratory L-lactate dehydrogenases as well as LutABC (isogenic mutant strains *ΔlldD, ΔlldDΔlutABC* and *ΔlutABC*) was determined with L- or D-lactate, using cell free extracts containing ~0.5 mg total

protein. 2,6-dichlorophenolindophenol (DCPIP) linked assays were performed aerobically at 37°C in a 3 ml reaction mixture containing 20 mM potassium phosphate buffer pH 7.2, 2 mM $MgCl_2$, 30 mM sodium L- or D-lactate, 0.25 mM PMS, 0.025 mM DCPIP, and dH_2O up to 3 ml final volume. The reaction was started by the addition of L- or D-lactate (Sigma-Aldrich, USA) and the reduction of DCPIP followed at 600 nm. Specific activities were calculated using an extinction coefficient at 600 nm for DCPIP of 21 mM^{-1} cm^{-1}. Experiment was performed at four separate occasions in duplicates. Excel was used to calculate specific activity.

2.5 Methods specific to Chapter 5

2.5.1 Neutrophil killing assay

PMN isolation. Heparinized blood was drawn from healthy volunteers according to a protocol approved by the institutional review board at The University of Queensland. PMN were isolated by PolymorphPrep (AXIS-SHIELD) density gradient separation according to the manufacturer. Residual erythrocytes were removed by hypotonic lysis, and PMN were resuspended in Hanks balanced salt solution (Gibco) with Ca^{2+} and Mg^{2+} and supplemented with 0.15% dextrose and 1% human serum albumin (HBSS+).

2.5.2 Synchronized phagocytosis experiments

Glass coverslips coated with bovine tendon collagen were added to 24-well tissue culture plates and incubated in autologous normal human serum (NHS) for 30 min at 37°C prior to adherence of PMN. Approximately 2 x 10^6 PMN were added to each well of the 24-well tissue culture plate, centrifuged at 350 x *g*, and

allowed to adhere for 30 min at 37°C. Monolayers were washed with 0.9% NaCl to remove non-adherent PMN. Medium was replaced with HBSS+S, and cells were kept at 10°C. Bacteria were added to PMN at a multiplicity of infection (MOI) of 1:1 and centrifuged at 800 x *g,* 10°C, to allow the bacteria to bind to PMN but not be ingested. Supernatants were removed and replaced with HBSS+S warmed to 37°C to initiate phagocytosis.

2.5.2 Viable counts of intracellular bacteria

PMN were challenged using the synchronized phagocytosis protocol described above. Supernatants were removed and replaced with HBSS+S warmed to 37°C for 2 min to initiate phagocytosis. Monolayers were then washed three times with 0.9% NaCl to remove extracellular bacteria. PMN were placed in HBSS+S and incubated in a 37°C water bath for the indicated time points. This point in the assay was designated as zero time (T0). Monolayers were treated with 1% saponin for 10 min at 37°C and scraped to lyse the cells. Lysates were then serially diluted and plated to enumerate CFU. Data are expressed as CFU at respective time points relative to the CFU at *T0.* Experiment was performed in duplicates at three separate occasions. The percent association, invasion, or survival was determined as a function of the original inoculum and the number of colonies formed with subsequent plating of the cellular lysate.

2.5.3 *N. gonorrhoeae* Association, Invasion, Survival Assays

(performed by Dr Jennifer Edwards)

Primary cervical epithelial (pex) cells were procured from surgical cervical tissue and maintained as described previously (Edwards *et al.* 2000). Cervical tissue was obtained from pre-menopausal women undergoing hysterectomy at The Ohio State University Medical Center for medically-indicated reasons not related to our study and was provided by the Cooperative Human Tissue Network (The Research Institute at Nationwide Children's Hospital, Columbus, OH). In accordance with NIH guidelines, these tissues do not constitute human subjects. Quantitative association, invasion, and survival assays were performed as we have described previously (Edwards 2010). Briefly, pex cells were challenged with gonococci at a multiplicity of infection of 100 for 90 min. Gentamicin was then omitted from (association assays) or added to (invasion and intracellular survival assays) infected pex cell monolayers to kill extracellular cell-associated bacteria. Pex cell monolayers were subsequently lysed or they were subject to a second incubation in antibiotic-free medium before cell lysis (intracellular survival assays). Before the onset of pex cell challenge, gonococci and pex cells were cultured under standard laboratory conditions (*i. e.* 5% CO2) or, where indicated, under microaerobic conditions. Mitsubishi (Pouch-MicroAero) gas-generating pouch systems were used to generate microaerobic conditions, as they rapidly (under 30 min) generate the desired oxygen-limited (6% O_2; 5% to 8% CO_2) environment. For assays performed under microaerobic conditions; microaerobically-cultured gonococci were used to challenge microaerobically-incubated pex cell monolayers. After

the addition of gonococci, infected pex cells were returned to a microaerobic environment. Separate gas pouches were used for each assay time point and care was exercised throughout the assay to minimize exposure to air. In this regard, air was vacuum-evacuated from pouches before the 30 min incubation with gentamicin. For all assays, serial dilutions of the cell lysates were plated to enumerate viable colony-forming units. The percent association, invasion, or survival was determined as a function of the original inoculum and the number of colonies formed with subsequent plating of the cellular lysate. Each assay was performed in triplicate on at least three separate occasions. A Kruskal-Wallis analysis of variance was used to determine the statistical significance of the calculated percent association, invasion, or survival for each assay. A Student's *t*-test was used to determine the significance of the calculated invasion and survival indices.

Chapter 3

Phenotypic characterisation of *ldhA*, *lldD* and *ldhD* mutants provides a framework for the understanding of the physiology of lactate metabolism in *Neisseria gonorrhoeae*

3.1 Introduction

N. gonorrhoeae cause an estimated 106 million cases of gonorrhoea worldwide per year ((WHO) 2012). As an obligate human bacterial pathogen, it is highly evolved for colonization of human mucosal epithelial surfaces (Wiesner and Thompson 1980). Gonococci engage the epithelial surfaces encountered during sexual transmission, such as the male urethra, female cervix, the rectum and the pharynx (Wiesner and Thompson 1980). Whereas most urethral infections produce symptoms including dysuria and purulent discharge, infections of the cervix, rectum and pharynx are frequently asymptomatic, which complicates the diagnosis and treatment of gonorrhoea and contributes to its persistence (Edwards and Apicella 2004). The clinical hallmark of infection by the *N. gonorrhoeae* is a host innate immune-driven inflammatory response, characterized by a potent neutrophil influx. The subsequent tissue damage enables bacterial access to secondary anatomical sites, which promotes much of the morbidity and mortality associated with *N. gonorrhoeae* infections. These sites include the fallopian tubes, heart, skin and joints. At these sites, infection by *N. gonorrhoeae* has adverse outcomes, including pelvic inflammatory disease, dermatitis, endocarditis and arthritis. Infection by *N. gonorrhoeae* is poorly controlled by the adaptive immune system owing to high-frequency antigenic variation of three major surface antigens: lipo-oligosaccharide (LOS), opacity-associated (Opa) proteins and type IV pili (Virji 2009). These structures undergo phase and antigen variation. It means that in every population of *N. gonorrhoeae* there is a subpopulation with different surface antigens. This has made the vaccine development based on surface structures unsuccessful.

N. gonorrhoeae must resist the variety of oxidative and non-oxidative antimicrobial components produced by PMNs (Quinn and Gauss 2004, Borregaard 2010). To be able to resist PMNs *N. gonorrhoeae* has evolved different strategies. *N. gonorrrhoeae* prevents PMNs from performing their normal antimicrobial functions (phagocytosis, granule content release), or expresses defenses against oxidative and non-oxidative components produced by PMNs (Bjerknes, Guttormsen et al. 1995, Lorenzen, Gunther et al. 2000, Criss and Seifert 2008, Soderholm, Vielfort et al. 2011, Ball and Criss 2013). Electron microscopic analysis of exudates from males with experimental gonococcal infection revealed that a subset of *N. gonorrhoeae* inside PMNs appear intact. Also viable *N. gonorrhoeae* were found inside neutrophil phagosomes and were cultured from gonorrhoeal purulent exudates providing the initial evidence that gonococci may survive within PMN phagosomes (Ovcinnikov and Delektorskij 1971, Farzadegan and Roth 1975, Apicella, Ketterer et al. 1996, Criss, Katz et al. 2009, Johnson and Criss 2013). In the female model of infection, with estradiol-treated mice, there was a rapid appearance of PMNs in the genital tract (Jerse 1999). This was also observed with human CEACAM-expressing transgenic mouse. The recruitment of more PMNs to fight infection during early infection could be an effective innate immune strategy, but the persistent exposure of CEACAM3-expressing PMNs to Opa-expressing gonococci can promote a pathogenic response, which is associated with gonorrhoea or pelvic inflammatory disease (Sintsova, Sarantis et al. 2014). PMNs have high rate of glycolysis and one of the byproducts of glycolysis is lactate.

It has been demonstrated that *N. gonorrhoeae* can form biofilms on abiotic surfaces and over primary urethral and cervical epithelial cells (Greiner,

Edwards et al. 2005). Biofilms are structured communities of bacteria that exist within a self-produced extracellular matrix (Flemming and Wingender 2010). Principal component of the matrix of the gonococcal biofilm is DNA produced by the organism (Steichen, Shao et al. 2008). The membranous networks can also be observed throughout the biofilm that are presumed to arise from blebbing of the outer membrane (Greiner, Edwards et al. 2005, Steichen, Shao et al. 2008). There are concentration gradients of oxygen, nutrients, waste products and secreted bacterial signaling compounds that alter the microenvironment at different spatial locations within a biofilm (Flemming and Wingender 2010). Thus, organisms living in biofilms differ physiologically from bacteria growing in a free-swimming planktonic state. The gonococcal biofilms grow anaerobically or microaerobically. This was supported by study in which was found that genes upregulated in biofilm organisms were all required for anaerobic respiration. These genes are nitrite reductase (*aniA*), nitric oxide reductase (*norB*), and cytochrome c peroxidase (*ccp*) (Phillips, Steichen et al. 2012).

Lactate is present in the human body at millimolar concentrations and is a product of glycolysis. Lactate can be found in the muscles, in cerebrospinal fluid (CSF), in respiratory and urogenital secretions, blood and phagocytes (Morse and Bartenstein 1974, Morse and Hebeler 1978, Leighton, Kelly et al. 2001) In the context of the female genital tract lactate concentrations of about 6 mM (Smith, Yates et al. 2001) have been reported and in this case Lactic acid bacteria inhabiting this niche produce this molecule as an end product of metabolism.

Although it is a waste product of glucose catabolism lactate provides energy for growth when it is oxidised to pyruvate that is beneficial to the bacteria. *N.*

gonorrhoeae is an obligate human pathogen that can utilise lactate efficiently (Smith, Yates et al. 2001, Smith, Tang et al. 2007). Previous studies indicated that lactate stimulates overall metabolism of *N. gonorrhoeae*. This includes increased lipopolysaccharide (LPS) production, enhanced protein synthesis and larger pentose content (Smith, Yates et al. 2001). It also stimulates sialylation of *N. gonorrhoeae* LPS by host derived cytidine 5'-monophospho-*N*-acetyl neuraminic acid (CMP-NANA). This is catalyzed by gonococcal sialyltransferase that makes the bacteria resistant to complement-mediated killing by human serum (Britigan, Klapper et al. 1988, Parsons, Emond et al. 1996, Regan, Watts et al. 1999).

Also, studies with aerobically grown *N. meningitidis* where glucose, pyruvate or D/L-lactate were used as a single carbon source have shown accumulation of acetate (Leighton, Kelly et al. 2001). Activities of acetate kinase (ACK) and phospho-transacetylase (PTA) were also measured. Activity of PTA was higher than activity of ACK. The amount of acetate excreted was highest when glucose was used as a carbon source. Logical explanation would be that both lactate and pyruvate have to fulfil two functions, being used in gluconeogenesis and for energy production. This was also confirmed by lower rate of growth. *N. meningitidis* same as *N. gonorrhoeae*, avoids over reduction of quinone pool and produces ATP by alternative pathway, producing the acetate.

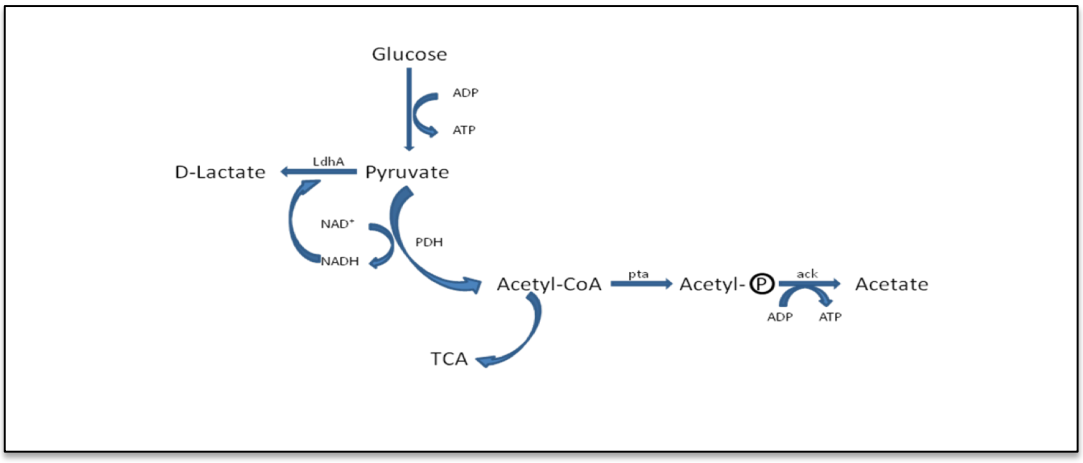


Figure 3.1 Simplified diagram of carbon metabolism in *N. gonorrhoeae* under aerobic condition (no lactate is produced) and microaerobic conditions (when both lactate and acetate are produced).

The accumulation of acetate is also present when *E. coli* is grown aerobically with glucose as a carbon source. There have been different suggestions explaining acetate accumulation. Possibility of imbalance between glucose metabolism and respiration (Erwin and Gotschlich 1996) has been suggested as well as the presence of excess NADH and condition in which influx of carbon into the cell exceeds demands for biosynthesis (Harvey, Post et al. 2002)

Inspection of the *N. gonorrhoeae* genome (FA1090) reveals that this bacterium possesses at least three lactate dehydrogenases. Two flavoproteins associated with the cytoplasmic membrane and a soluble enzyme found in the cytoplasm (Fischer, Martin et al. 1994). The membrane-bound lactate dehydrogenases oxidise L/D lactate isomers and feed electrons into the quinone pool of the respiratory chain. Soluble lactate dehydrogenase is an oxidoreductase using both D-lactate and pyruvate as substrates. Sequence comparison of *N. gonorrhoeae* genome led to the annotation of NGO0639 (*lldD*) which encodes the membrane bound L-lactate dehydrogenase , NGO0890 (*ldhD*) which encodes the membrane

bound D-lactate dehydrogenase and NGO1336 (*ldhA*) which encodes the soluble D-lactate dehydrogenase. These three genes have high sequence homology to enzymes from other bacteria such as *E. coli*.

Lactate permease (LctP) is the transporter protein that enables carriage of lactate into the gonococcal cell. To investigate the importance of lactate utilization during *N. gonorrhoeae* genital tract infection, mutant strain lacking *lctP* gene was generated and examined both *in vitro* and in murine model of infection. A mutant that lacks a functional copy of *lctP* was unable to take up exogenous lactate and did not grow in defined medium with lactate as the sole carbon source. It had also exhibited increased sensitivity to complement-mediated killing compared with the wild type strain (Exley, Wu et al. 2007). In the murine model of infection the *lctP* mutant was significantly attenuated in its ability to colonize and survive in the genital tract (Exley, Wu et al. 2007).

Studies have shown that phagocyte derived lactate, which accumulates as consequence of glucose catabolism, stimulates oxygen consumption (Britigan, Klapper et al. 1988). By competing for oxygen *N. gonorrhoeae* effectively decreases neutrophil formation of reactive oxygen species (Britigan, Klapper et al. 1988). This demonstrates the importance of gonococcal lactate dehydrogenase proteins for pathogenicity of this organism. However, some studies show that *N. gonorrhoeae* suppress the PMN oxidative burst by production of a factor requiring *de novo* protein synthesis, whose mechanism of action requires bacterial contact with PMNs but is independent of phagocytosis of *N. gonorrhoeae* (Criss and Seifert 2008).

This work is aimed to further investigate the role of these proteins and provide a framework for further studies of the contribution of lactate metabolism in gonococcal infections.

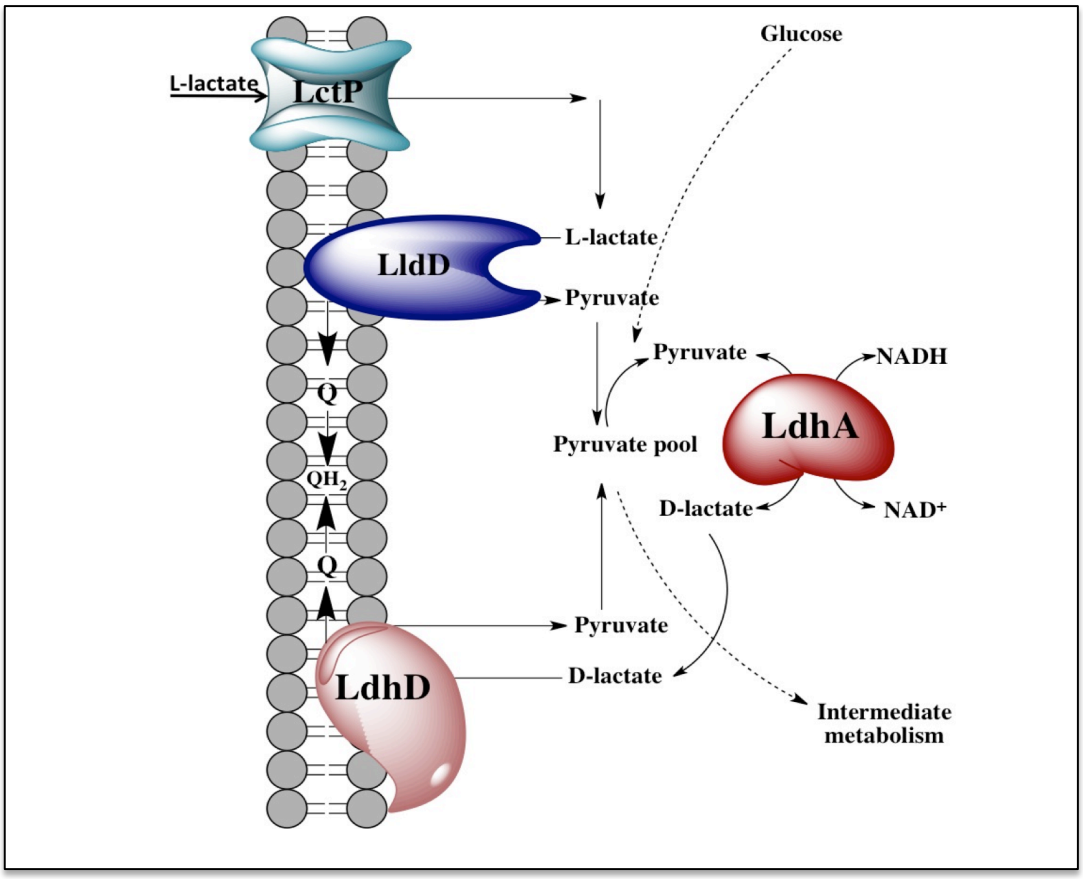


Figure 3.2 Model of lactate utilisation in *Neisseria gonorrhoeae* based on the concept of the existence of three lactate dehydrogenases. The model suggests that L-lactate is imported into the cell and oxidised to pyruvate by L-lactate dehydrogenase (LldD). This pyruvate also contributes to the pyruvate pool together with pyruvate from glycolysis. Pyruvate from pyruvate pool is then reduced by NADH dependent D-lactate dehydrogenase (LdhA) that leads to the accumulation of D-lactate. The D-lactate is then used by D-lactate dehydrogenase (LdhD) to push the electrons into the respiratory chain.

3.2 Results

3.2.1 Confirmation that *ldhA* encodes an NADH dependent D-lactate dehydrogenase

The bioinformatic analysis of the gonococcus 1291 genome sequence identified a gene (NGO1336), *ldhA*, which was annotated as a putative lactate dehydrogenase. To address the functional role of this lactate dehydrogenase a deletion mutant in which *ldhA* had been inactivated was grown in GW medium supplemented with glucose as the sole carbon source. The growth of this mutant strain was compared with that of mutants defective in each of the other two putative lactate dehydrogenases (encoded by NGO0639 and NGO0890) and wild-type strain 1291 in GW supplemented with 1% glucose as the single carbon source (Figure 3:3). All four strains where able to grow over the period of 8h.

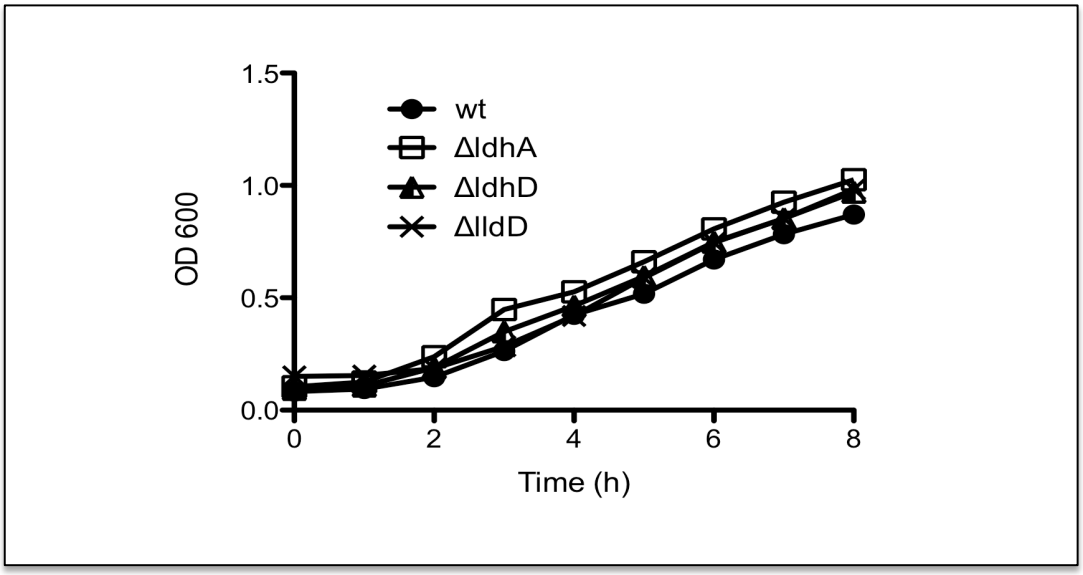


Figure 3.3 Growth of wt 1291, *ldhA* (NADH dependent D-lactate dehydrogenase) mutant, *ldhD* (D-lactate dehydrogenase) mutant and *lldD* (L-lactate dehydrogenase) mutant in GW supplemented with 1% glucose as the sole carbon source. The graph is representative of three independent experiments.

Initial experiments to investigate lactate dehydrogenases made use of cells grown on glucose. The model described above (Figure 3:2) suggested that D-lactate might accumulate in *N. gonorrhoeae* if it were not removed at a sufficiently high rate by the respiratory D-lactate dehydrogenase. Therefore, in order to determine how much D-lactate is produced by the *N. gonorrhoeae*, measurements of D-lactate from supernatant of cultures grown for 24 hrs on glucose as the only carbon source under microaerobic conditions was performed. wt1291 and mutant strains were examined for production of L-lactate. No L-lactate was detected in the medium after 24h of growth.

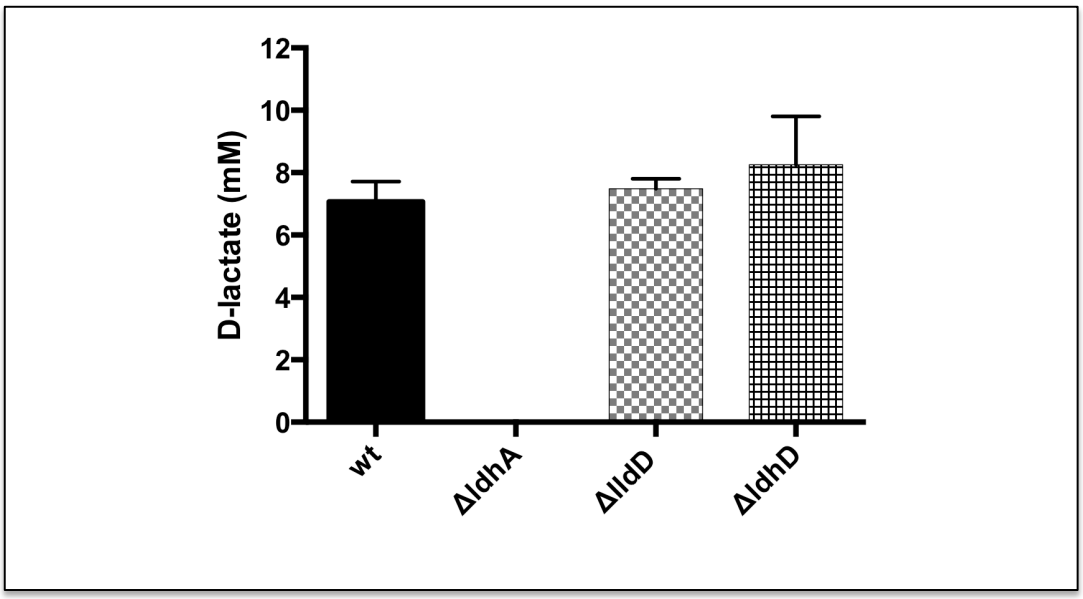


Figure 3.4 Amount of D-lactate accumulated in medium over a period of 24 h. wt 1291, soluble D-lactate dehydrogenase mutant (*ΔldhA*), respiratory D-lactate dehydrogenase mutant (*ΔldhD*) and L-lactate dehydrogenase mutant (*ΔlldD*) were grown microaerobically (200 mL GW in 250 mL flask, 100 rpm) in GW with glucose as the only carbon source. No accumulation of L-lactate was detected. The graph is representative of four independent experiments. Error bars are calculated as the standard error of the mean.

Figure 3.4 shows that approximately 6 mM D-lactate accumulated in the wild-type strain 1291 as well as mutants lacking both respiratory D-lactate and L-lactate dehydrogenases. However, no D-lactate formation was observed in the *ldhA* mutant. This is consistent with the model (Figure 3.2) where LdhA catalyzes the NADH-dependent reduction of pyruvate and formation of D-lactate.

To further address the question of LdhA acting as an oxidoreductase (not only reducing pyruvate to D-lactate but also oxidising D-lactate to pyruvate in an NAD^+-dependent reaction), pyridine nucleotide coupled enzyme assay was performed on soluble extracts from wt 1291 and *ldhA* mutant.

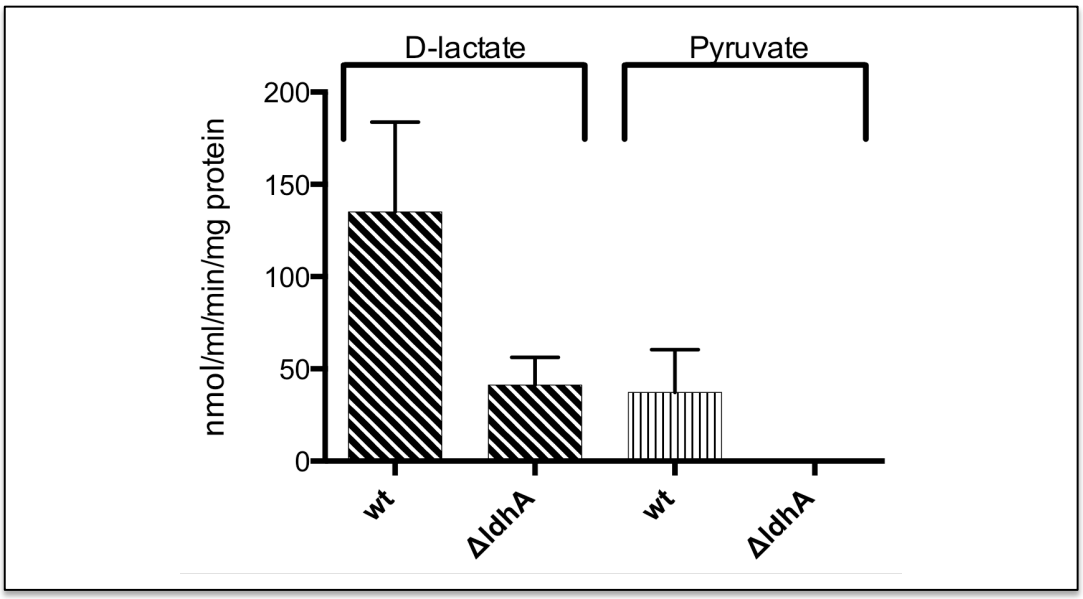


Figure 3.5 Lactate dehydrogenase activity in cell free extracts of wt 1291 and *ΔldhA* mutant. D-lactate and NAD^+ and pyruvate and NADH were used as substrates. Error bars are calculated as the standard error of the mean.

The activity of LdhA was measured using the two substrates: D-lactate and NAD^+, and pyruvate and NADH. Figure 3.5 displays the lactate dehydrogenase activity when D-lactate or pyruvate respectively, were used as substrates with soluble

cell extracts from wt 1291. Enzyme activity was measured to 135 mU for D-lactate as a substrate and 37 mU for pyruvate as a substrate. There was no detectable activity when soluble cell extract from *ldhA* deficient mutant was used in the NADH coupled reaction. Although LdhA was not functional, enzyme activity of up to 42 mU was detected in the cell extract from *ldhA* deficient mutant in NAD^+ coupled reaction. The activity recorded in the *ldhA* deficient mutant when D-lactate was used as a substrate could mean the presence of second soluble lactate-NAD^+ dehydrogenase acting only in one direction, oxidising D-lactate to pyruvate. This has not been investigated and further studies are necessary to confirm this.

3.2.2 D-lactate formation in *N. gonorrhoeae* under aerobic and microaerobic conditions

N. gonorrhoeae catabolises glucose via the Entner-Douderoff Pathway (Morse 1979). This pathway generates 1 ATP per mole of glucose converted to pyruvate. The logical conclusion would be that major generator of ATP generation in this bacterium is via membrane associated electron transport chain. D-lactate and acetate are the known products of gonococcal carbohydrate metabolism (Holten 1975, Morse 1979) but it was of interest to determine how the pattern of production of these metabolites was affected by aerobic and microaerobic conditions. To address this question, *N. gonorrhoeae* was grown aerobically and microaerobically with glucose as the only carbon source and the amount of acetate and D-lactate was measured.

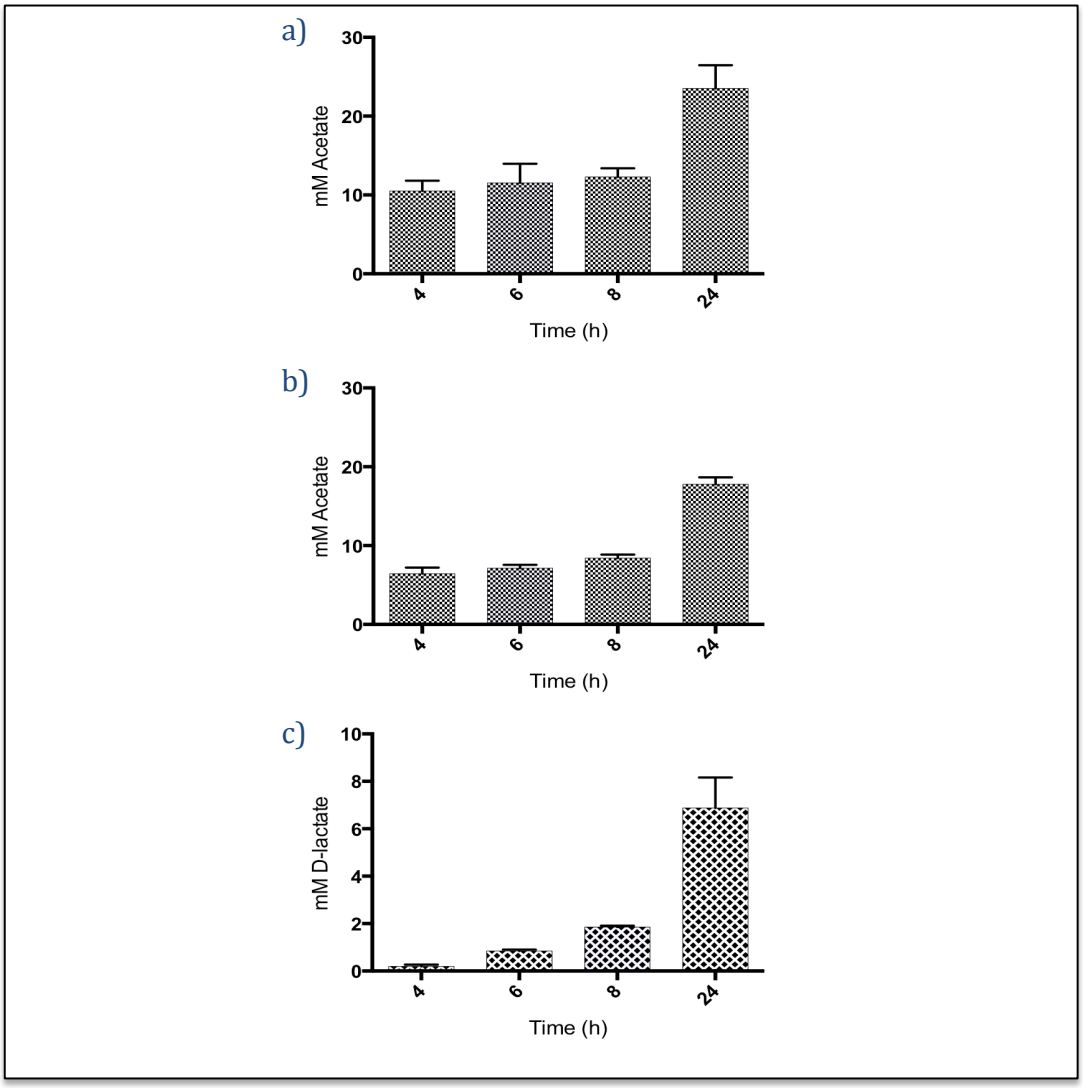


Figure 3.6 a) The amount of acetate produced under aerobic conditions over time. Time points measured for determination of acetate were 4, 6, 8, and 24 h. The experiment was repeated two times in independent experiments. b) The amount of acetate produced under microaerobic conditions over time. Time points measured for determination of acetate are 4, 6, 8, and 24 h. c) The amount of D-lactate produced under microaerobic conditions over time. Time points measured for determination of lactate are 4, 6, 8, and 24 h. The graphs are representative of four independent experiments**.** Error bars are calculated as the standard erorror of the mean.

Under aerobic conditions approximately 27 mM acetate was accumulated by 24 hours (Figure 3.6 a). No D-lactate was detected under these conditions. Under microaerobic conditions less acetate was accumulated by 24 hours (16 m)

(Figure 3.6 b). D-lactate accumulated to approximately 7 mM by 24 h under the same conditions (Figure 3.6 c). The accumulation of acetate under all conditions indicated that there was substantial amount of substrate level phosphorylation involving phosphotransacetylase/actate kinase. However, the shift towards D-lactate production under microaerobic conditions indicates that production of acetate was more restricted.

3.2.3 Characterisation of respiratory dehydrogenases

It has already been established that *Neisseria gonorrhoeae* posses two lactate dehydrogenase isoenzymes (Smyth, Friedman-Kien et al. 1976, Fischer, Martin et al. 1994). These are the respiratory membrane bound enzymes, LdhD and LldD. To determine the role and substrate preference of each enzyme, two sets of experiments were performed. First, the respiratory enzyme assay using cell free extract, and second, the oxygen consumption of the cell membranes from both wt 1291 and mutants lacking the lactate dehydrogenase isoenzymes

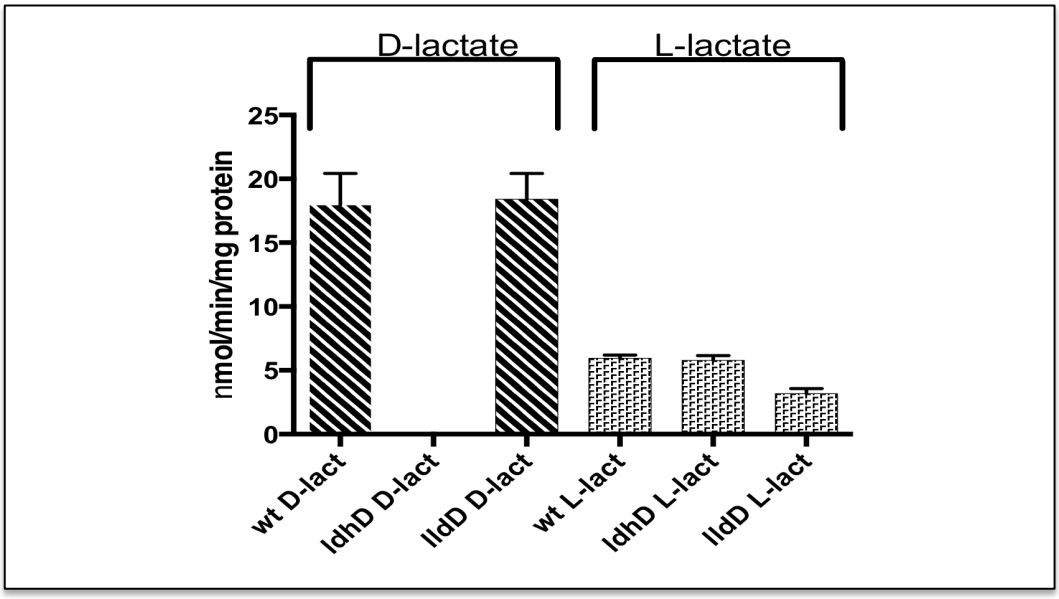


Figure 3.7 Respiratory enzyme assay using cell free extract from wt 1291, *ΔldhD* (D-lactate dehydrogenase) mutant and *ΔlldD* (L-lactate dehydrogenase) mutant. The graph is representative of five independent experiments. Error bars are calculated as the standard error of the mean.

Wt 1291 exhibited activity with both D- and L-lactate as a substrate though activity for D-lactate was three fold higher than for L-lactate (Figure 3.7). There was no detectable activity with D-lactate as the substrate in the *ldhD* (D-lactate dehydrogenase) mutant and it retained its activity with L-lactate as a substrate (Figure 3:7). This result confirms that LdhD it is specific for D-lactate. The *lldD* (L-lactate dehydrogenase) mutant demonstrated a different pattern to that of the *ldhD* mutant. It remained active with D-lactate as the substrate with activity similar to the wt 1291. However, when L-lactate was used as a substrate, the activity was half of the wild type activity. To further confirm this data experiment measuring respiration in membranes prepared from wt 1291 and the two mutants in Clark-type oxygen electrode was applied. The result of the

oxygen consumption experiment (Figure 3:8) confirms the results achieved with enzyme assay for respiratory dehydrogenases. There is no oxygen consumption by the *ldhD* mutant when D-lactate is used as the substrate. In contrast to that, there is still oxygen consumption in the *lldD* mutant when L-lactate is used as a substrate.

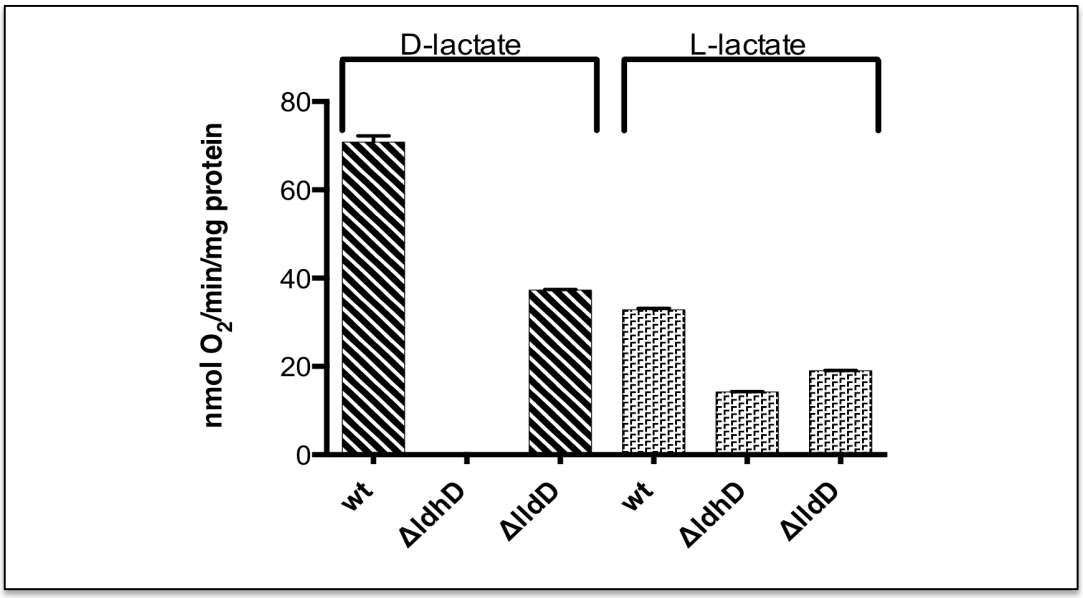


Figure 3.8 Oxygen consumption by wt 1291, respiratory D-lactate dehydrogenase mutant *(ΔldhD*) and L-lactate dehydrogenase mutant (*ΔlldD*). *lldD* mutant exhibit respiration with L-lactate as the substrate. The graph is representative three separate experiments performed in duplicates**.** Error bars are calculated as the standard error of the mean.

3.3 Discussion

Previous studies suggested that lactate metabolism plays an important role in the virulence of *N. gonorrhoeae* (Exley, Wu et al. 2007). However, biochemical analysis of lactate dehydrogenases has not kept pace with the developments in genomics. Thus, for further understanding in this area it was important to characterise the biochemical properties of lactate dehydrogenase enzymes responsible for lactate metabolism in *N. gonorrhoeae* and relate these properties to specific genes. Three decades ago it was thought that *N. gonorrhoeae* possessed only two lactate dehydrogenases, one soluble NAD^+-dependent and one respiratory membrane-bound enzyme (Smyth, Friedman-Kien et al. 1976). A few years later Fisher and colleagues made an observation that *N. gonorrhoeae* possesses two respiratory Ldh enzymes, one specific for L- and the other for D-lactate (Fischer, Martin et al. 1994). In this study it was demonstrated that *N. gonorrhoeae* possesses two respiratory lactate dehydrogenases and possibly a third one also specific for L-lactate. It was also demonstrated that a third lactate dehydrogenase LdhA is a cytoplasmic enzyme that operating under physiological conditions as a NADH:pyruvate oxidoreductase generating D-lactate as a product. This leads to a model (Figure 3:2) in which L-lactate is imported into the cell by LctP (lactate permease) and is oxidised to pyruvate by the respiratory L-lactate dehydrogenase. The pyruvate is reduced to D-lactate by LdhA (pyridine nucleotide-dependent D-lactate dehydrogenase). The D-lactate can than be oxidised to pyruvate. In this way, *N. gonorrhoeae* maintains the pyruvate pool generated both from glucose and lactate. In the absence of L-lactate, when *N. gonorrhoeae* is grown only on glucose, LldD would be inactive as it is specific

only for L-lactate.

By using a spectrophotometric assay for LdhD and LldD activity, it was found that the *lldD* mutant had significantly lower activity than the parental 1291 strain with L-lactate as a substrate, while activity with *ldhD* mutant was completely abolished when D-lactate was used as a substrate. This confirms that LdhD is the respiratory D-lactate dehydrogenase. The residual activity found in *lldD* mutant suggests that there is possibly another L-lactate dehydrogenase. The same result was found in *N. meningitidis* (Gotschlich 1996).

The accumulation of only acetate and no D-lactate under aerobic conditions indicates that there is a considerable amount of substrate level phosphorylation involving phosphotransacetylase/acetate kinase that consumes acetyl-CoA and generates ATP. It appears that under conditions of glucose excess it is more efficient for gonococcus to grow by producing the acetate and not use the TCA cycle to completely oxidise carbon. Under these conditions the respiratory chain serves a role as an oxidiser of NADH and this would also generate some ATP.

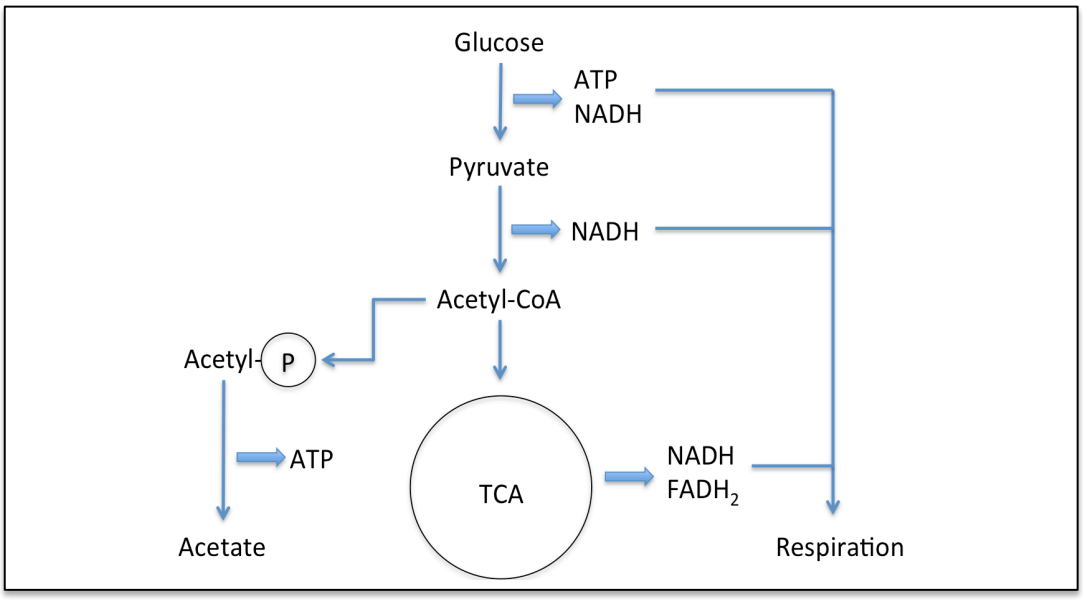


Figure 3.9 Model of aerobic glucose metabolism in *N. gonorrhoeae*

Under microaerobic conditions, the pattern of carbon end product shifted with reduced amount acetate and appearance of D-lactate (Figure 3:10) presumably because the respiratory chain has a more limited capacity to oxidise NADH under these conditions. In *N. gonorrhoeae,* glucose is metabolised by the Entner-Doudoroff pathway, which yields to only one ATP produced per molecule glucose. This is inferior to the electron transport phosphorylation in terms of ATP produced per molecule of glucose. NADH produced in glucose catabolism and TCA cycle is used in electron transport phosphorylation, thus being recycled to generate NAD^+.

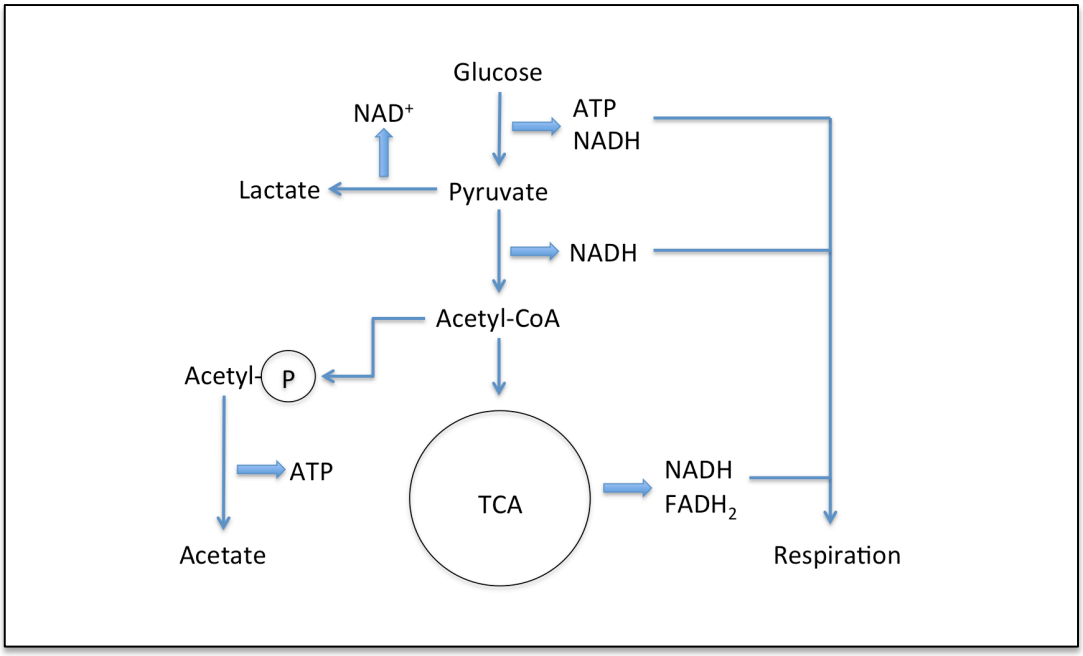


Figure 3.10 Model of microaerobic respiration in *N. gonorrhoeae*

Respiratory lactate dehydrogenases are flavoproteins that reduce ubiquinone. Unlike Nuo (NADH: ubiquinone oxidoreductase) they do not contain Fe-S clusters. It is known that *nuo* gene expression is up-regulated in the presence of iron (Ducey, Carson et al. 2005). Thus, iron availability may have an important influence on the pattern of carbon metabolism in pathogenic *Neisseria*. In *N. meningitidis* one small RNA was identified, named NrrF (for neisserial regulatory RNA responsive to iron [Fe]), which was demonstrated to be both iron responsive and Fur regulated and which has a well-conserved orthologue in *N. gonorrhoeae* (Mellin, Goswami et al. 2007). *sdh* operon, encoding succinate dehydrogenase, is demonstrated to be under NrrF regulation. Approximately 30% of the operons regulated by iron are under the direct control of the (Fur). Fur also indirectly controls gene expression by affecting the transcription of three secondary regulators. Fur, in the presence of ferrous iron, binds as a dimer to DNA regulatory sequences (Fur boxes), which typically results in the

repression of transcription of many iron-repressible genes (Coy and Neilands 1991). Iron-repressible promoters in the gonococcus, including *fetA*, *hmbR*, *fbpABC*, *hpuAB*, *lbpAB*, and *tbpAB*, have this consensus Fur box (Genco and Desai 1996, Lewis, Gray et al. 1997). Genes encoding proteins involved in protein synthesis, energy metabolism, and transcription were preferentially expressed under high-iron conditions, suggesting an overall increase of cellular metabolism (Ducey, Carson et al. 2005). Thus, it might be expected that under conditions of iron-restriction there would be limited flux through the TCA cycle. This is not case with lactate *ldh* genes which leads to the hypothesis that they probably operate under iron-restricted conditions. Although the respiratory lactate dehydrogenases are inferior to NADH dehydrogenase (Nuo) in terms of energy conservation they have the advantage of their simplicity of composition and lack of iron requirement. As lactate was not accumulated during aerobic growth one may speculate that lactate dehydrogenase expression is under the control of transcriptionl regulator which, upregulates expression of proteins involved in anaerobiosis. One such regulator is fumarate and nitrate reductase regulator protein (FNR). FNR regulates expression of the major anaerobically induced outer membrane protein in *N. gonorrhoeae* AniA (Householder, Belli et al. 1999, Lissenden 2000). *S. aureus* is capable of metabolically adapting to nitrosative stress by expressing an NO-inducible L-lactate dehydrogenase. Lactate production allows *S. aureus* to maintain redox homeostasis during nitrosative stress and is essential for its virulence (Richardson, Libby et al. 2008). Real time PCR in which expression of microaerobically grown *N. gonorrhoeae* with or without NO_2^- was compared, showed that *ldhD* and *lldD* expression was up-regulated, while expression of *ldhA* was approximately the same over a period of

4, 6, 8 and 24 h with addition of NO_2^- (Appendix Fig. 1). With that we can include NO_2^- as an additional inducer of expression of D- and L-lactate dehydrogenase proteins. Indeed, accumulation of D-lactate in medium was higher for cells, which were cultured with addition of 4 mM NO_2^- (Appendix Figure 2) showing enhanced lactate metabolism in these cells. In *E. coli* genes coding *lldD*, *ldhD* and *lctP* are situated in one operon and are under the control of LldR (Aguilera, Campos et al. 2008). LldR is a GntR type of promotor. This family, named after the repressor of the *Bacillus subtilis* gluconate GntR operon, includes about 270 members, which are distributed among the most diverse bacterial groups and regulate various biological processes (Haydon and Guest 1991, Rigali, Derouaux et al. 2002). This regulator is absent in *N. gonorrhoeae*. Just upstream of *lctP* gene, there is a GntR type of regulator but it does not show any sequence similarity with other known lactate dehydrogenase regulators. In the future, it would be interesting to gain more knowledge about regulation of lactate dehydrogenases in *N. gonorrhoeae.*

Chapter 4

Does *N. gonorrhoeae* possess additional respiratory system for L-lactate utilisation Characterisation of a possible new hydroxyl-carboxylic acid dehydrogenase

4.1 Introduction

Neisseria gonorrhoeae is an obligate human pathogen. As a result, this organism is adapted to evade killing by the human host. It causes a purulent discharge in the urethra and/or cervix containing polymorphonuclear leukocytes (PMNs), cell debris, and serum components (Edwards and Apicella 2004).

Gonococci utilize a number of resistance mechanisms, including antimicrobial inactivation, target modification and strategies that reduce antimicrobial concentration, such as reduced permeability of the cell envelope mediated through alteration of porin proteins and active export of multiple antimicrobial compounds from the cell by efflux pumps.

The importance of gonococcal Opa surface adhesins and type IV pili in mediating interactions with host cells has been a long-appreciated characteristic of *N. gonorrhoeae* biology (Swanson, Sparks et al. 1975). These structures undergo phase and antigenic variation. The frequency of phase and antigenic variation results in a high potential for surface polymorphism in any given population.

Inflammation during gonorrhoeal infection is characterized by the influx of PMNs to the site of infection (Wiesner and Thompson 1980). One feature of neutrophils is their production of reactive oxygen species (ROS), or the oxidative burst. Neutrophil NADPH oxidase catalyzes the initial requisite step of ROS production by formation of superoxide through the transfer of an electron from NADPH to oxygen (Quinn and Gauss 2004). Despite the presence of neutrophils at sites of gonococci infection, viable *N. gonorrhoeae* bacteria are found inside neutrophil phagosomes and can be cultured from gonorrhoeal purulent exudates (Wiesner and Thompson 1980, Johnson and Criss 2013). The mechanisms

underlying the inability of neutrophils to clear gonococci infection are incompletely understood. Although *N. gonorrhoeae* expresses many antioxidant gene products, implying a role for neutrophil-derived ROS during human infection, resistance of *N. gonorrhoeae* to neutrophil killing is independent of the neutrophil oxidative burst (Frangipane and Rest 1992, Seib, Simons et al. 2005, Seib, Wu et al. 2006, Criss, Katz et al. 2009, Wu, Soler-Garcia et al. 2009).

Lactate is a major product of anaerobic metabolism, but it also serves as a carbon and energy source for anaerobic and aerobic microorganisms.

One of the first indications that lactate metabolism might contribute to the pathogenesis of gonococcal infection was the demonstration that lactate from human neutrophils stimulated oxygen consumption by gonococci, which in turn could impair oxygen-dependent bactericidal mechanisms (Britigan, Klapper et al. 1988).

N. gonorrhoeae express an α-2,3-sialyltransferase (Lst) that can scavenge sialic acid from the host and use it to modify lipooligosaccharide (LOS). Sialylation of gonococcal LOS converts serum-sensitive strains to serum resistance, decreases antibody binding, and resists killing by neutrophils and antimicrobial peptides (Smith, Parsons et al. 1995, Gill, McQuillen et al. 1996). Mutant *N. gonorrhoeae* that lack Lst (cannot sialylate LOS) are attenuated in a mouse model (Wu and Jerse 2006). The donor molecule for sialic acid is cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA). Sialylation of gonococcal LOS converts serum-sensitive strains to serum resistant strains. This phenomenon has been termed unstable serum resistance and is used to explain its dependence on

exogenously supplied CMP-NANA. It has also been shown that lactate from blood cell extracts enhances LPS sialylation of the gonococcus as it emerges from lag phase during growth in a medium containing glucose. This results from a stimulation of metabolism, leading to a more rapid emergence from lag phase, a 20% increase in the rate of growth with enhanced LPS, and protein production (McGee and Rest 1996, Parsons NJ 1996 , Gao, Parsons et al. 1998).

This suggests that, in the human genital tract, where glucose is present in millimolar concentrations, lactate metabolism could enhance the successful colonization of the *N. gonorrhoeae*.

It has been established that *N. gonorrhoeae* possess three lactate dehydrogenases; two respiratory, inner membrane bound and one cytoplasmic (Erwin A. L. 1993, Fischer, Martin et al. 1994, Erwin and Gotschlich 1996). Transporter protein, lactate permease (LctP), is responsible for transport of lactate into the gonococcal cell. The *lctP* mutant demonstrated reduced early growth and increased sensitivity to complement-mediated killing compared with the wild-type strain when grown in defined media containing physiological concentrations of lactate. Also, the enhanced susceptibility to complement-mediated killing was associated with a reduction in lipopolysaccharide sialylation of the *lctP* mutant (Exley, Wu et al. 2007).

The studies described in Chapter 3 showed that there was residual L-lactate dehydrogenase activity in membranes from L-lactate dehydrogenase (*lldD*) mutant when L-lactate was used as a substrate. This Chapter is aimed to examine if there is an additional L-lactate dehydrogenase in *N. gonorrhoeae.*

4.2 Results

4.2.1 Growth characteristics of an *lldD* mutant suggests the presence of a second L-lactate dehydrogenase

To compare the growth of wt1291 and mutants lacking the three lactate dehydrogenases, all four strains were grown in chemically defined medium (CDM) with L-lactate as a sole carbon source. As expected, both the *ΔldhA* and *ΔldhD* mutants had a very similar growth profile to the wt (Figure 4.1). Surprisingly, the L-lactate dehydrogenase (*ΔlldD*) deletion mutant was also able to grow at a similar level to wt1291. This result was not expected as the deletion of *lldD* should have rendered the bacterium incapable of utilising L-lactate for energy, hence resulting in the inability to survive on L-lactate alone. Therefore, the next step was to analyse the genome of *N. gonorrhoeae* in detail to identify other potential proteins that could perform a similar role as LldD.

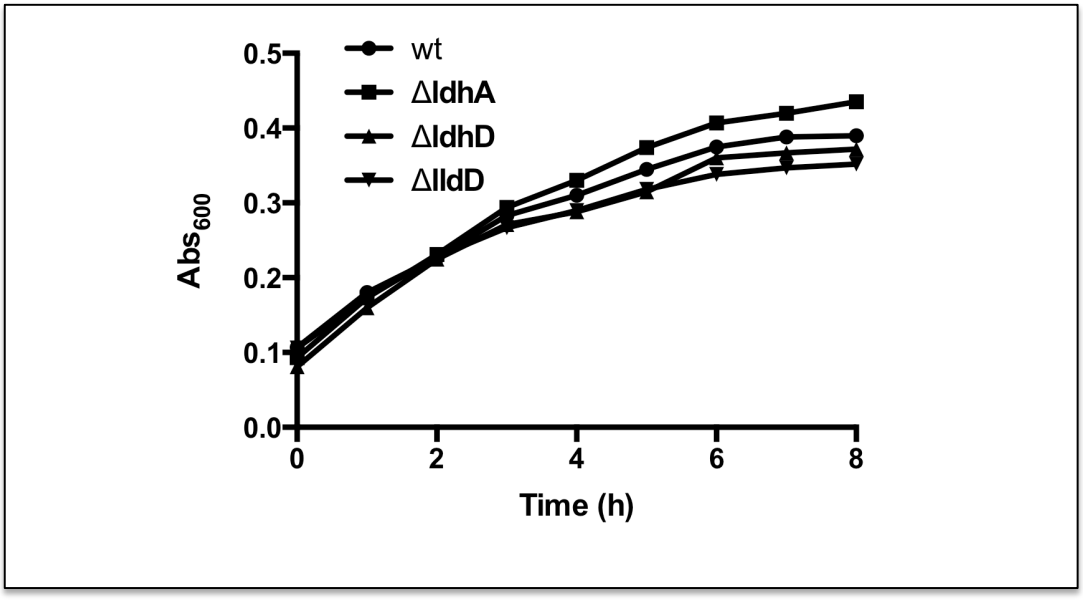


Figure 4.1 Growth of wt1291, *DldhA*, *DldhD* and *DlldD* strains in CDM supplemented with 6 mM L-lactate as a single carbon source.

4.2.2 Bioinformatic identification and characterisation of *lutABC* genes

B. subtilis possesses a different system for lactate utilisation. L-lactate is oxidised by LutABC. This system is also found in *C. jejuni*. BLAST search of the *N. gonorrhoea* genome revealed the existence of a putative *lutABC* operon (NGO0904, NGO0905, NGO0906). Based on sequence comparison and residual activity with L-lactate as a substrate in the *lldD* mutant, it was hypothesised that *N. gonorrhoeae* could have two respiratory systems for lactate utilisation.

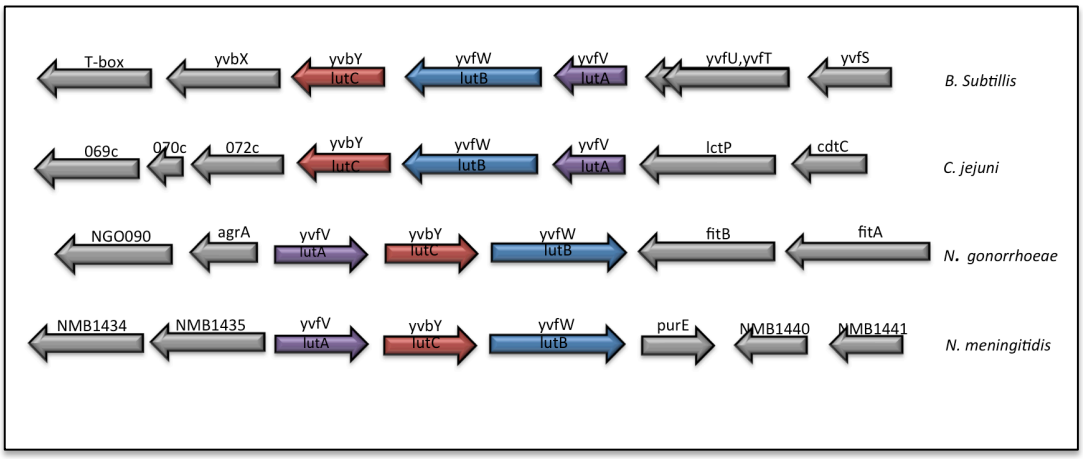


Figure 4.2 Organisation of *lutABC* in genomes of *B. subtilis*, *C. jejuni*, *N. gonorrhoeae* and *N. meningitidis*

B. subtilis(168): Uptream *lutABC*: *yvbX* epimerase,
Downstream *lutABC*: *yvfU* two-component response regulator, *yvfT* two-component sensor histidine kinase, *yvfS* ABC transporter permease

C .jejuni(NCTC 11168): Upstream *lutABC*: *072c* transport protein-pseudo-one frameshift, *070c* unknown function, *069c* unknown function, Downstream *lutABC*: *cdtC* functional classification - Pathogenicity

N. gonorrhoeae: Upstream *lutABC*: NGO0902 putative phospholipase D-family protein, *argA* aminoglycoside resistance efflux transporter protein, Downstream *lutABC*, *fitB* trafficking protein B, *fitA* trafficking protein A.

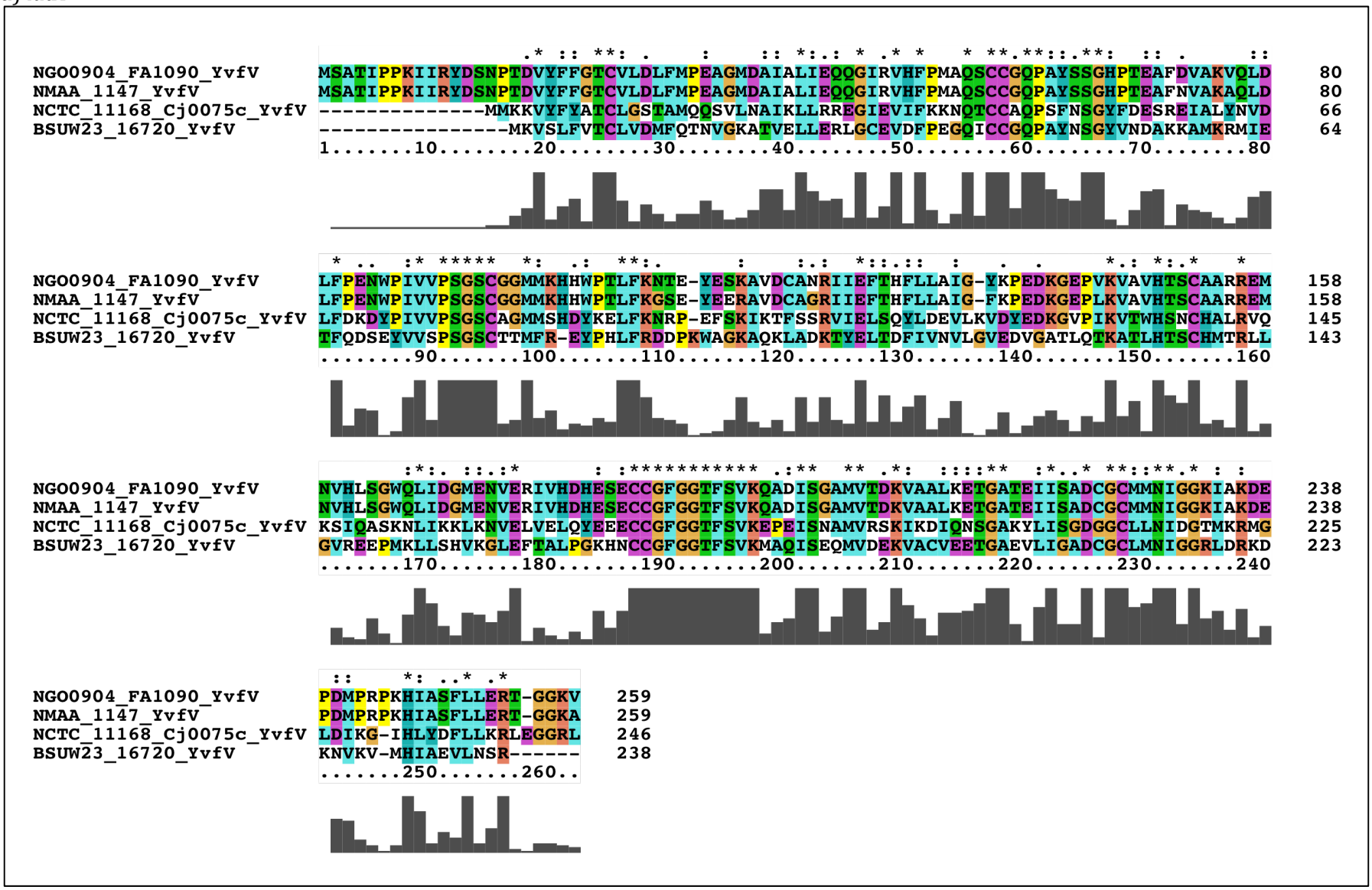
N. meningitidis: Upstream *lutABC*: NMB1435 drug resistance translocase, NMB1434 phopholipase D-family protein, Downstream *lutABC*: *purE* phosphoribosylaminoimidazole carboxylase catalytic subunit, nmb1440 hypotetical protein, *nmb1441* O-methyltransferase.

It was interesting to examine the organisation of *lutABC* genes in the genomes of the different bacterial strains. Figure 4.2 shows organisation of *lutABC* in genomes of *B. subtilis*, *C. jejuni*, *N. gonorrhoeae* and *N. meningitidis*. There is no similarity between genes surrounding *lutABC* in genomes of these bacterial strains. In *N. gonorrhoeae* upstream of *lutABC* are found genes encoding putative phospholipase D-family and aminoglycoside resistance efflux transporter proteins. Downstream of *lutABC* are genes encoding trafficking protein B and trafficking protein A. In *B. subtilis lutABC* is under dual regulation by a GntR like transcription repressor LutR and a regulator for biofilm formation SinR (Mantovani, Cassatella et al. 2011). LutR represses transcription of *lutABC* when there is no lactate present, and the operon is derepressed in the presence of lactate. At present there is no information for regulation of *lutABC* in *C. jejuni*. A GntR transcription regulator is found in the *N. gonorrhoeae* genome but it is not known whether it regulates transcription of lactate dehydrogenases as well as *lutABC*. Microarray data indicated that expression of *lutABC* in *N. gonorrhoeae* under high iron conditions (Dyer 2010). Thus, *lutABC* may be also under transcriptional regulation of ferric uptake regulator (Fur) protein.

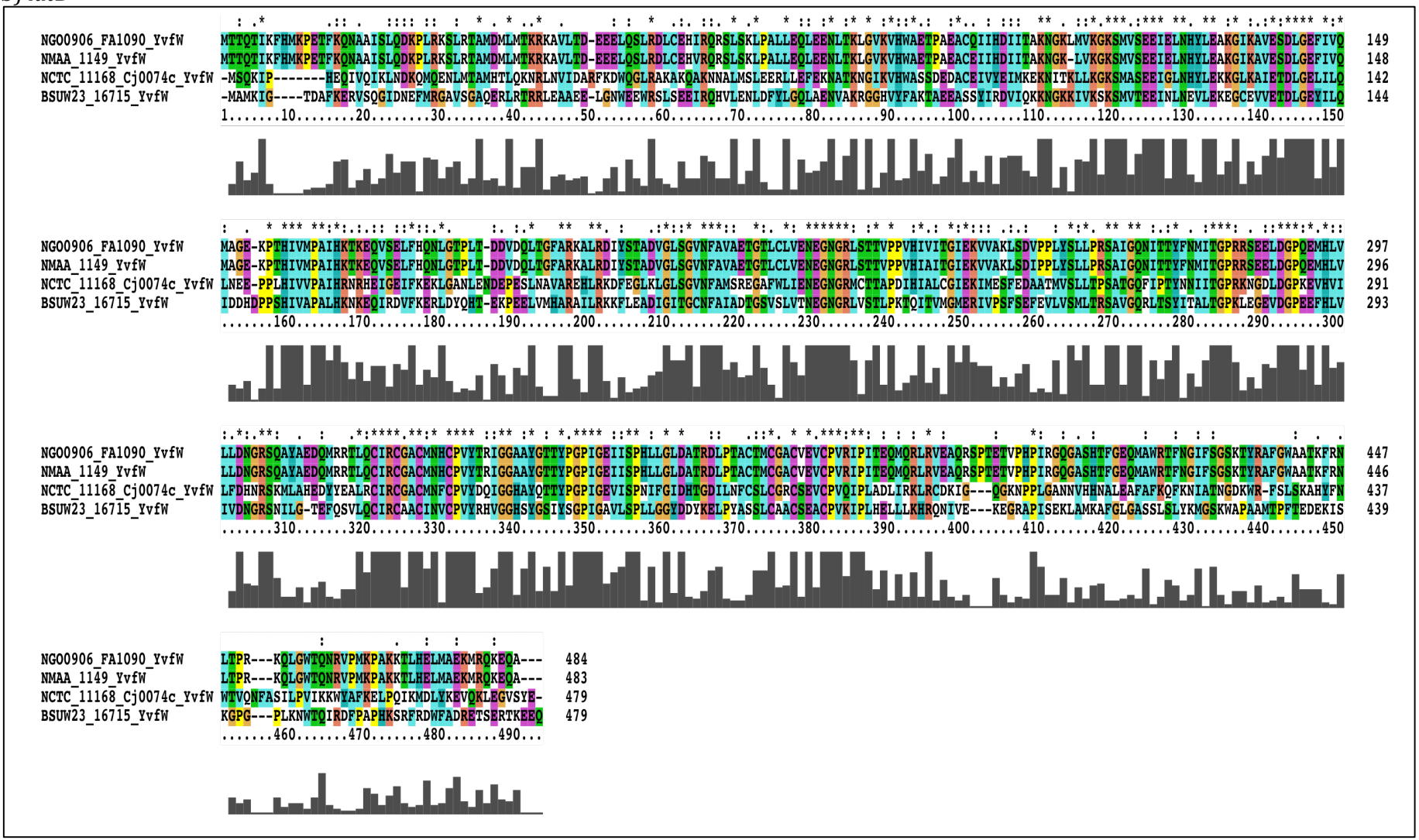
4.2.3 Analysis of the putative LutABC proteins

ClustalW analysis showed that amino acid identity between *B. subtilis* and *N. gonorrhoeae* LutABC was 31%, 38 % and 36%, respectively.

a) *lutA*


NGO0904_FA1090_YvfV
NMAA_1147_YvfV
NCTC_11168_Cj0075c_YvfV
BSUW23_16720_YvfV
MSATIPPKIIRYDSNPTDVYFFGTCVLDLFMPEAGMDAIALIEQQGIRVHFPMAQSCCGQPAYSSGHPTEAFDVAKVQLD 80
MSATIPPKIIRYDSNPTDVYFFGTCVLDLFMPEAGMDAIALIEQQGIRVHFPMAQSCCGQPAYSSGHPTEAFNVAKAQLD 80
----------MMKKVYFYATCLGSTAMQQSVLNAIKLLRREGIEVIFKKNQTCCAQPSFNSGYFDESREIALYNVD 66
----------MKVSLFVTCLVDMFQTNVGKATVELLERLGCEVDFPEGQICCGQPAYNSGYVNDAKKAMKRMIE 64
1.....10.....20.....30.....40.....50.....60.....70.....80
LFPENWPIVVPSGSCGGMMKHHWPTLFKNTE-YESKAVDCANRIIEFTHFLLAIG-YKPEDKGEPVKVAVHTSCAARREM 158
LFPENWPIVVPSGSCGGMMKHHWPTLFKGSE-YEERAVDCAGRIIEFTHFLLAIG-FKPEDKGEPLKVAVHTSCAARREM 158
LFDKDYPIVVPSGSCAGMMSHDYKELFKNRP-EFSKIKTFSSRVIELSQYLDEVLKVDYEDKGVPIKVTWHSNCHALRVQ 145
TFQDSEYVVSPSGSCTTMFR-EYPHLFRDDPKWAGKAQKLADKTYELTDFIVNVLGVEDVGATLQTKATLHTSCHMTRLL 143
.....90.....100.....110.....120.....130.....140.....150.....160
NVHLSGWQLIDGMENVERIVHDHESECCGFGGTFSVKQADISGAMVTDKVAALKETGATEIISADCGCMMNIGGKIAKDE 238
NVHLSGWQLIDGMENVERIVHDHESECCGFGGTFSVKQADISGAMVTDKVAALKETGATEIISADCGCMMNIGGKIAKDE 238
KSIQASKNLIKKLKNVELVELQYEEECCGFGGTFSVKEPEISNAMVRSKIKDIQNSGAKYLISGDGGCLLNIDGTMKRMG 225
GVREEPMKLLSHVKGLEFTALPGKHNCCGFGGTFSVKMAQISEQMVDEKVACVEETGAEVLIGADCGCLMNIGGRLDRKD 223
.....170.....180.....190.....200.....210.....220.....230.....240
PDMPRPKHIASFLLERT-GGKV 259
PDMPRPKHIASFLLERT-GGKA 259
LDIKG-IHLYDFLLKRLEGGRL 246
KNVKV-MHIAEVLNSR------ 238
.....250.....260..

b) *lutB*


NGO0906_FA1090_YvfW
NMAA_1149_YvfW
NCTC_11168_Cj0074c_YvfW
BSUW23_16715_YvfW

c) *lutC*

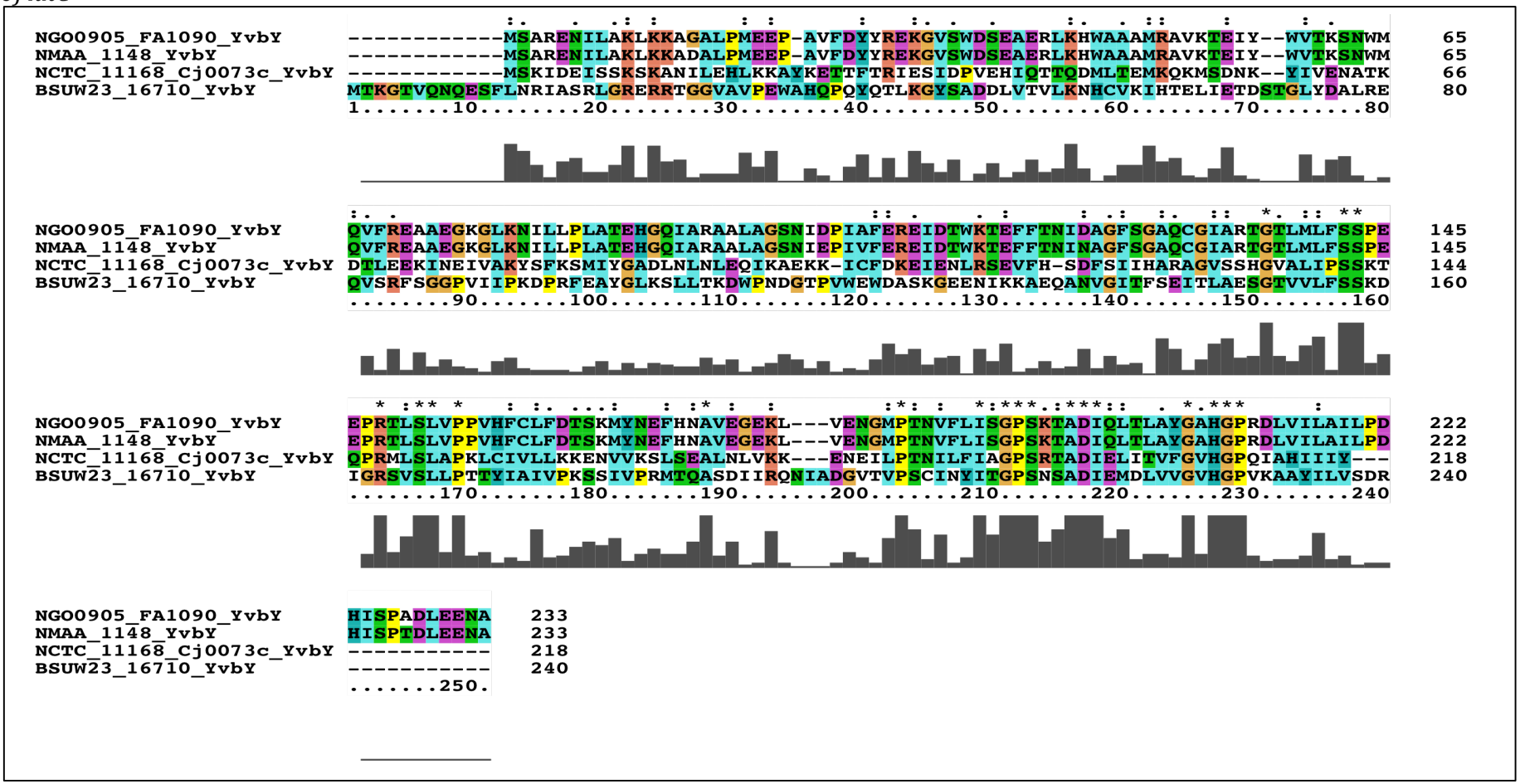


Figure 4.3 ClustalW of LutA, LutC and LutB amino acid sequences. Comparison of four different bacterial strains, *N. gonorrhoea* FA1090, *N. meningitidis* WUE 2594, *C. jejuni* NCTC 11168 and *B. subtilis* BSUW23 16720.

Further analysis of the LutA homolog NGO0904 indicated that it does not possess any transmembrane regions, which was confirmed with PredictProtein server. NGO0904 is thus cytoplasmic protein. This protein belongs to YgkE protein family from *E. coli* and other organisms. In *B. subtilis* it is formally named YvfV or LutA. It has two conserved cysteine residues containing Fe-S clusters. It is involved in electron transfer and contains the active site for lactate oxidation, sending the electrons further to LutB, LutC and finally to the quinone pool (Chai Y 2009 Apr).

NGO0905 is also found among *Neisseria* species as well as other bacterial species. It is annotated as an YkgG protein in *E. coli* and some other bacteria. InterPro Scan confirms BLAST search (Figure 4.4). NGO0905 contains a domain found in 5-formyltetrahydrofolate cyclo-ligase, also known as 5,10-methenyltetrahydrofolate synthetase. It is a single-domain alpha/beta protein. This domain is also found in a number of other proteins, eg LutB/YkgF and LutC/YkgG. Search with PredictProtein for transmembrane domains gave result of one possible transmembrane helix. Thus, one may speculate that this subunit could be loosely attached to the membrane and likely make it possible for electrons to reach the quinone pool. At the other hand, as data is based on bioinformatics analysis more studies at the protein level must be performed to confirm this model.

NGO0906 is annotated as an iron-sulphur protein. A search with BLAST and InterPro Scan revealed the presence of four Fe-S clusters. It is also annotated as L-lactate dehydrogenase among some bacterial species. This protein does not possess any transmembrane domains. The predicted organisation of subunits in LutABC is shown in Figure 4.5. LutA is a protein with catalytic site, oxidising L-

lactate to pyruvate. Electrons released from oxidation are transferred from LutA to LutB and finally to LutC which then sends them to the respiratory chain.

Figure 4.4 a) InterPro Scan result NGO0904 It shows cysteine rich domains. The protein belongs to YkgE family of proteins. This tool searches in database for family and domain of proteins. b) InterPro Scan result NGO0906. Domain found in YkgG/YkgF proteins. c) InterPro Scan result NGO0905 confirms presence of iron-sulphur clusters**.**

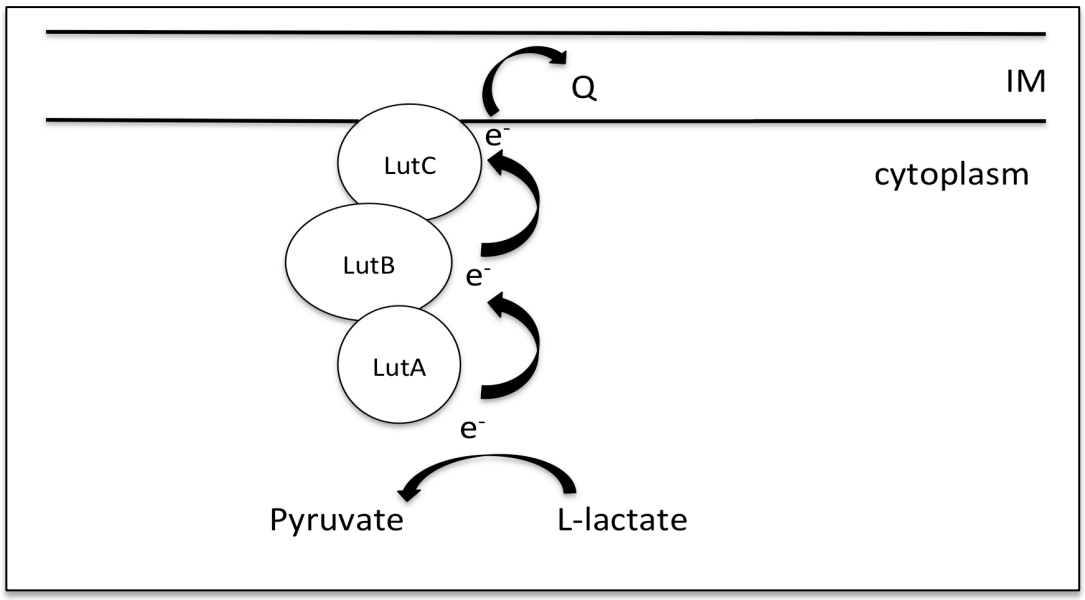


Figure 4.5 Predicted organisation of Lut subunits in the LutABC protein and suggested function.

4.2.4 Construction of a *lutABC* mutant

In order to construct a *lutABC* mutant strain, splice overlap PCR described in the Methods section was used. First, the 1000 bp upstream and downstream regions of *lutABC* were amplified with primers lutA F and lutA R, and, lutB F and LutB R, respectively (Figure 4.6). The amplified PCR fragments from the primers described are shown in Figure 4.7. The fragments and the chloramphenicol gene were then merged using splice overlap PCR. The positive splice overlap PCR fragment (Figure 4.8) was cloned in the cloning vector pGEM T-Easy. This construct was linearised and transformed into either wt1291 or *ΔlldD* mutant background generating the *ΔlutABC or ΔlldDΔlutABC* mutant strain respectively. Screening of mutant strains was done with lutA screen F, Cm screen R and Cm screen F, lutB screen R. These primers should create fragments of 1851 and 1704 bp if a positive mutant was constructed. Figure 4:9 demonstrates that insertion deletions strains were correct. This was further confirmed by sequencing.

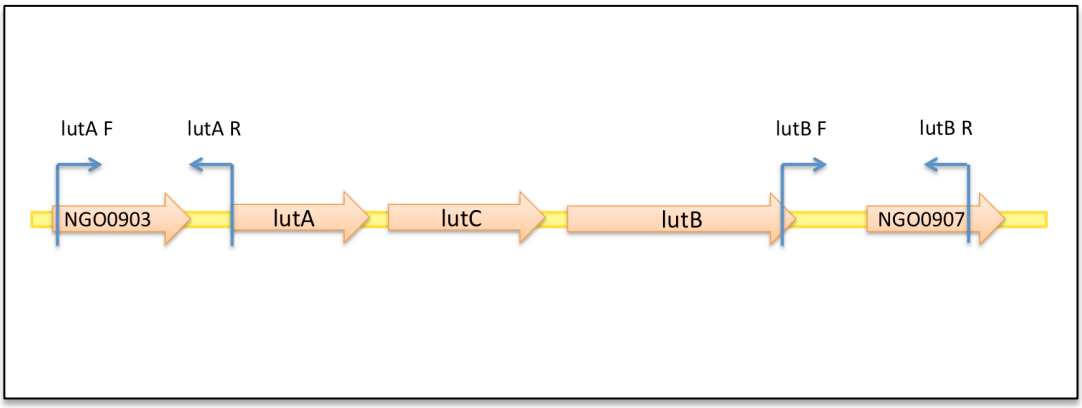


Figure 4.6 A schemetic figure showing the location of primers used to amplify 1000 bp upstream and downstream fragments of *lutABC* for generation of the deletion mutant. Primers used to amplify upstream fragment were lutA F and lutA R. For amplifying the downstream fragment, primers lutB F and lutB R were used. Gene NGO0904 is formally named *lutA*, gene NGO0905 *lutC* and gene NGO0906 *lutB*.

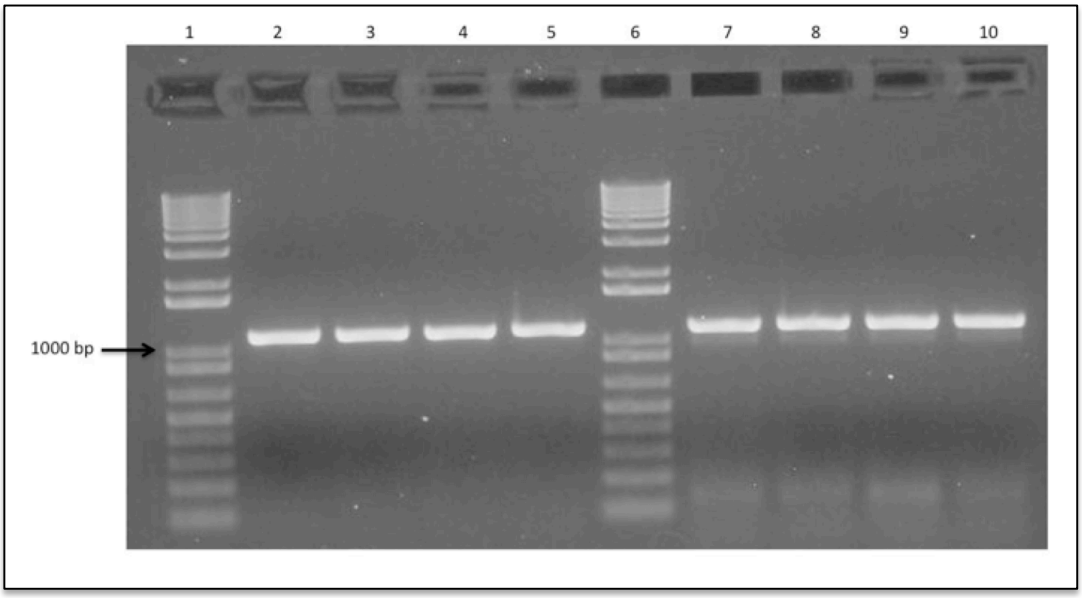


Figure 4.7 The 1% agarose gel image showing the positive PCR products for the *lutABC* upstream fragment amplified with primers lutA F and lutB R (lanes 2-5) and *lutABC* downstream fragment amplified with primers lutB F and lutB R (lanes 7-10). Lane 1 and 6 is the DNA ladder.

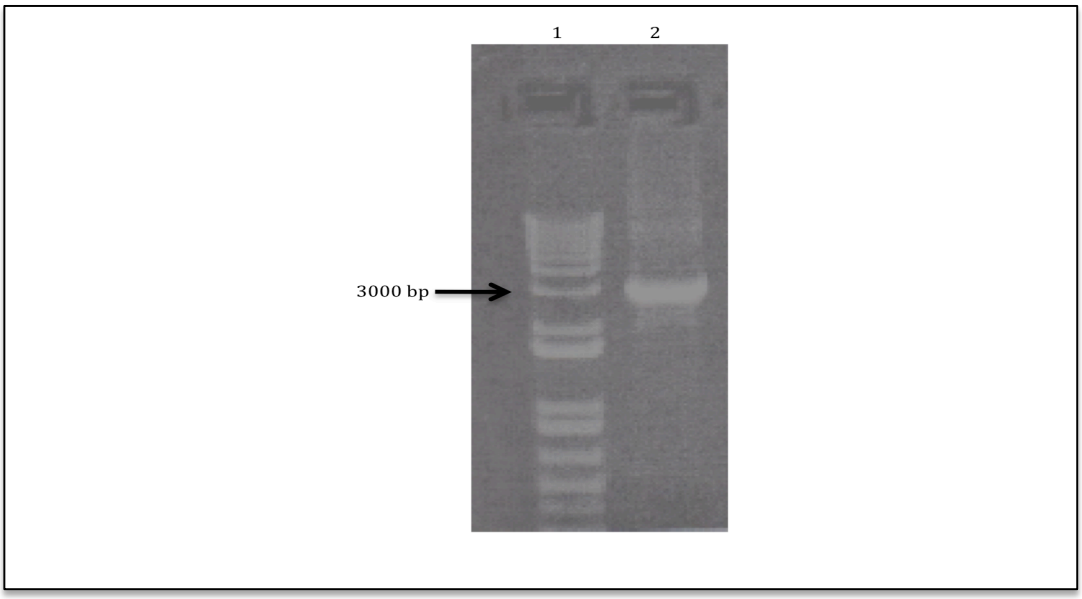


Figure 4.8 The 1% agaraose gel image showing the positive splice overlap PCR product.

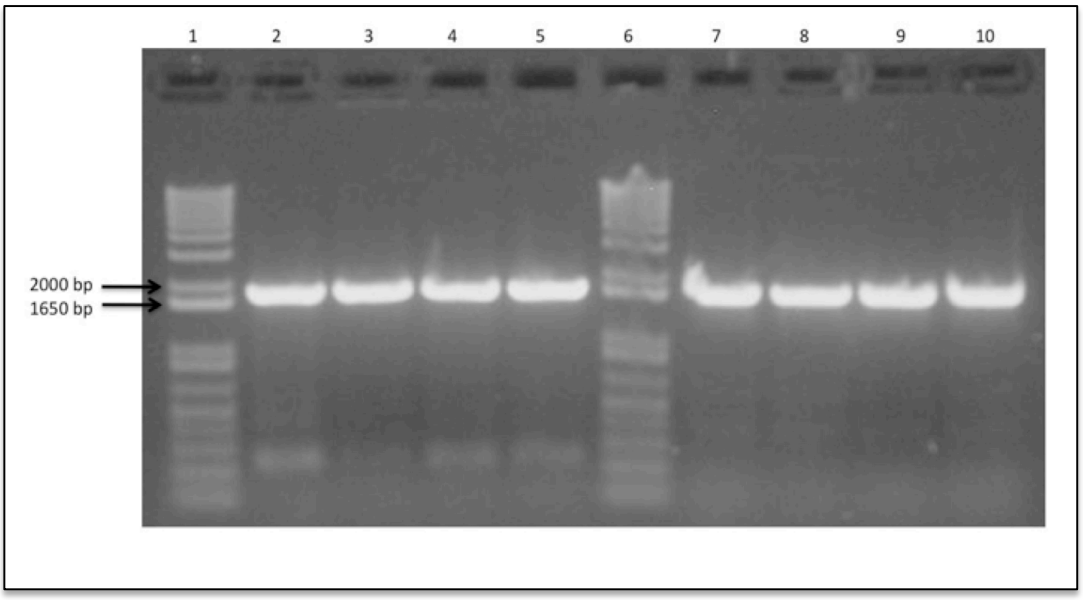


Figure 4.9 Screening of *ΔlldDΔlutABC* mutant with primers binding into Cm cassette and outside the *lutABC*. Expected size upstream fragment ~1800bp and downstream fragment ~1700bp. Lanes 2-5 are upstream fragments and lanes 7-10 are downstream fragments of four different colonies screened. Lane 1 and 6 is the DNA ladder. Sequencing of these fragments showed insertion of Cm cassette in *lutABC.*

4.2.5 Characterisation of a *lutABC* mutant

To address the question about the involvement of LutABC in lactate metabolism in *N. gonorrhoeae*, a double mutant lacking both *lldD* and *lutABC* was generated. The double mutant was examined for activity with L-lactate as a substrate in a respiratory enzyme assay in which DCPIP was used as an electron acceptor (Figure 4.10). In this experiment D-lactate was used as a control substrate. As seen previously (Figure 3.7), when L-lactate is used as a substrate, *ΔlldD* mutant exhibited activity. This mutant was compared to the double *ΔlldDΔlutABC* mutant. There was no difference in activity between these two mutants. This result implied that LutABC is not involved in lactate metabolism.

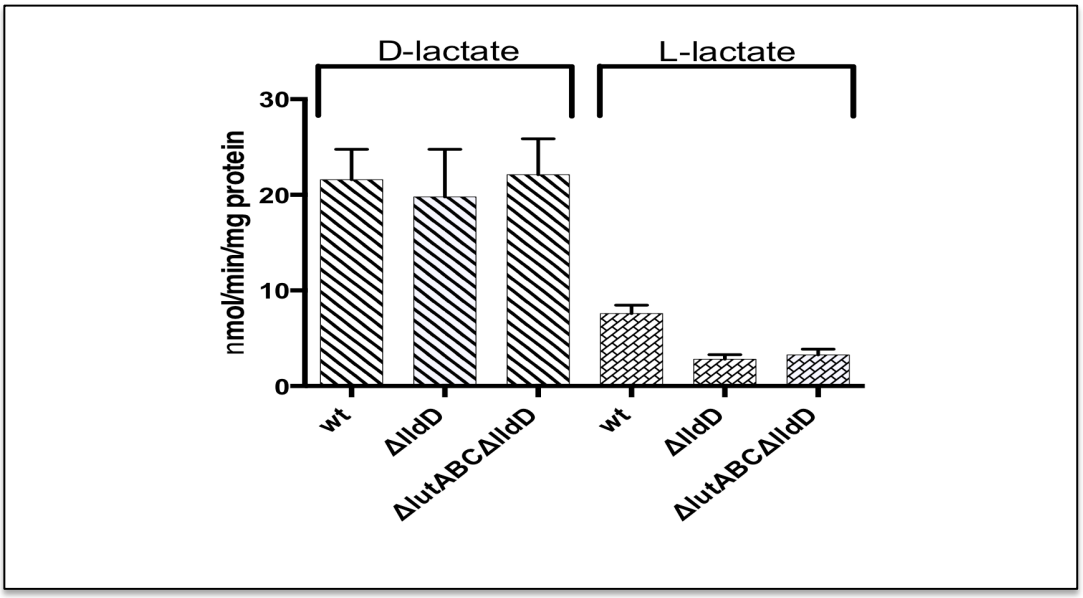


Figure 4.10 Lactate dehydrogenase assay with DCPIP as an electron acceptor. The assay was performed using L-lactate as the substrate with cell free extracts from wt1291, L-lactate dehydrogenase mutant (*ΔlldD*) and double *ΔlldDΔlutABC* mutants. Graph is representative of four independent experiments. Error bars are calculated as the standard error of the mean.

4.2.6 Glycolate oxidation in *N. gonorrhoeae* – Is LutA a glycolate dehydrogenase?

BLAST analysis also revealed that LutA also had sequence similarity to glycolate oxidase. ClustalW showed that identity between GlcF and LutA is 19% (Figure 4.11). As glycolate is present in urogenital tract (Merz and So 2000), LutA would be a candidate for exhibiting the role of a glycolate oxidase. There was no similarity between LutC and LutB subunits and other subunits of glycolate oxidase in *E. coli.*

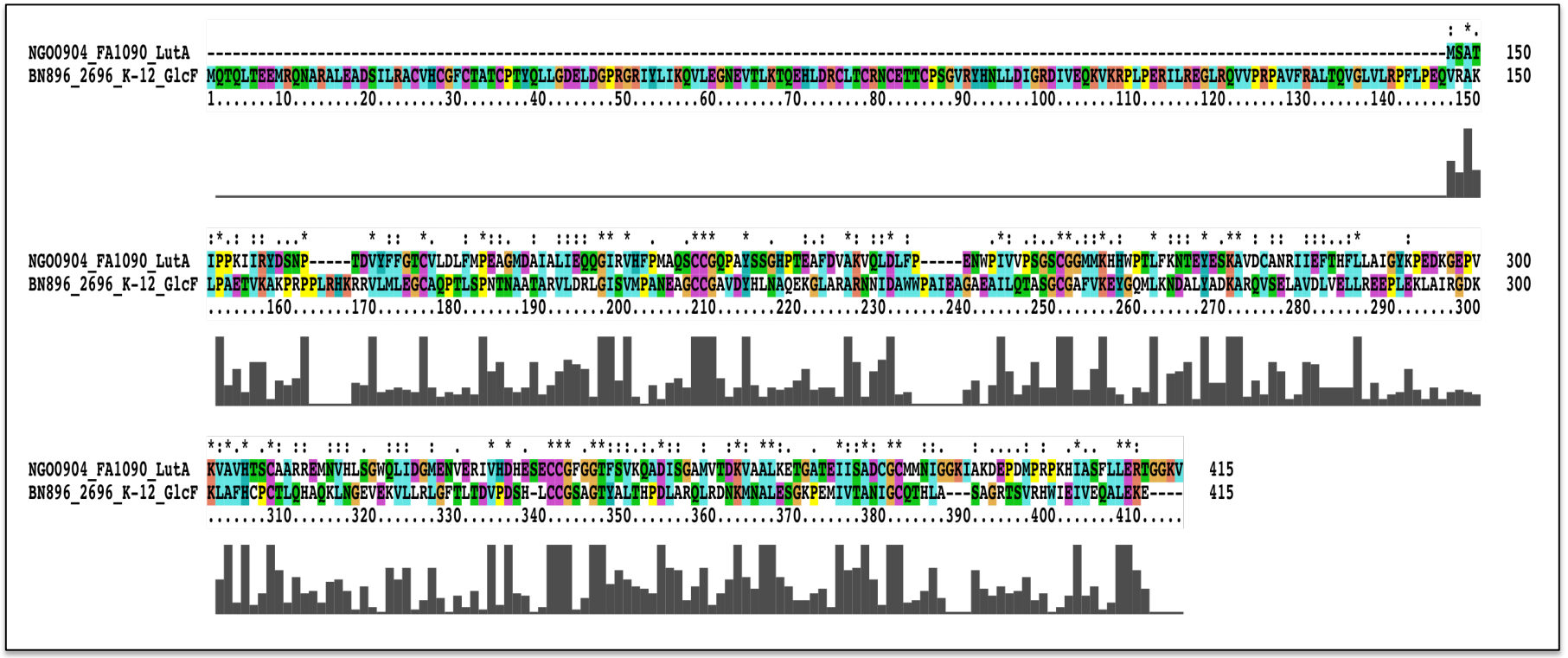


Figure 4.11 BLAST analysis of LutA from *N. gonorrhoeae* FA 1090. It shows 19% identity to GlcF subunit of glycolate oxidase found in *E. coli* K12.

To determine if LutABC is involved in glycolate oxidation, respiration by whole cells was measured using glycolate as a substrate with Clark-type oxygen electrode. Though respiration was low it was measurable. Induction of LutABC with glycolate during the growth did not change the rate of respiration. When wt1291 was compared to *ΔlutABC* mutant, there was no significant difference in the rate of respiration (Figure 4.12). Induction of LutABC with glycolate and excess of iron did not have any effect on the activity.

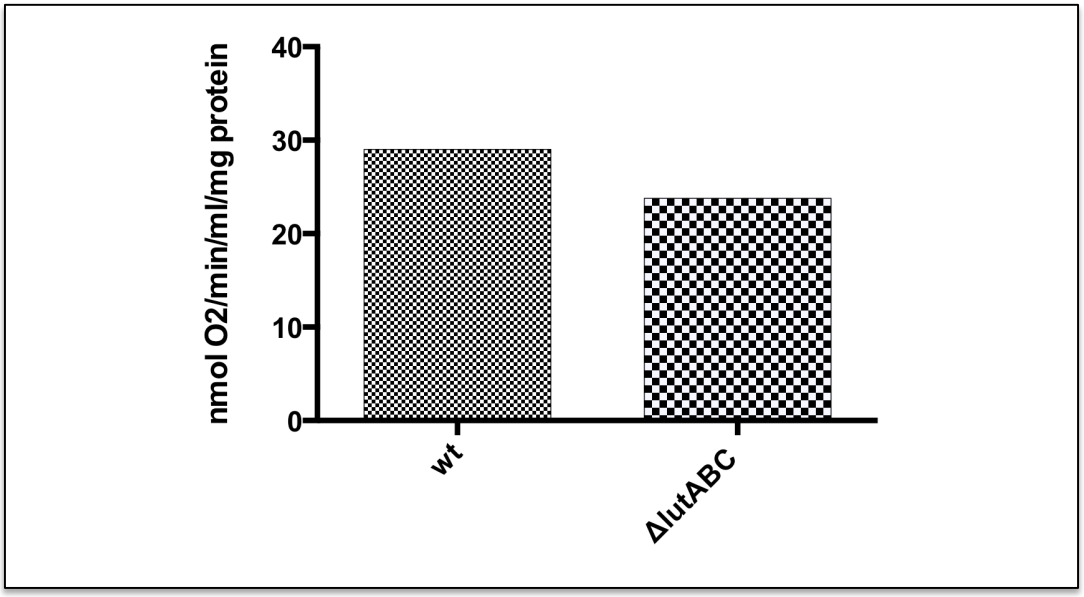


Figure 4.12 Respiration by whole cells with glycolate as a substrate. Respiration was measured in Clark-type oxygen electrode. Wt1291 respired with rate of 29 and *ΔlutABC* mutant with rate of 23.8 nmolO_2/min/mg protein.

4.3 Discussion

Lactate is a key source of energy and plays an important role in pathogenicity of *N. gonorrhoea* (Britigan, Klapper et al. 1988, Exley, Wu et al. 2007). Previous studies have established existence of two respiratory lactate dehydrogenase proteins (Fischer, Martin et al. 1994) but their organisation was not established. Analysis of mutants led to the model of lactate utilisation (Chapter 3). On the journey towards confirming the model it was discovered that there is not only one L-lactate dehydrogenase but also possibly a second one, yet uncharacterised. This is not uncommon among bacterial species. *Campylobacter jejuni* possesses two respiratory systems for utilisation of lactate (Thomas, Shepherd et al. 2011). Growth studies, in which both wt1291 and mutants lacking lactate dehydrogenases were grown with L-lactate as a single carbon source, has confirmed existence of the second L-lactate dehydrogenase in *N. gonorrhoeae*. It was expected that a mutant lacking *lldD* would not be able to grow in CDM with L-lactate as a single carbon source. By being able to grow in this medium (Figure 4:1), it was confirmed that second LlldD protein is equally important for growth.

Closer scrutiny of the *N. gonorrhoeae* (FA 1090) genome revealed the existence of *lutABC* genes that code for L-lactate dehydrogenase both in *Bacillus subtilis*, *Campylobacter jejuni* and *Shewanella onidensis.* BLAST comparison between *B. subtilis* 16710 and *N. gonorrhoeae* FA 1090 amino acid sequence of LutABC, showed that identity between LutA was 36%, LutB 38 % and LutC 31%. These proteins are present in many bacterial species. Even though they are usually annotated as conserved proteins, in some bacteria the function has been determined. This function is L-lactate oxidation and they have been annotated as

L-lactate dehydrogenases (Chai, Kolter et al. 2009, Pinchuk, Rodionov et al. 2009, Thomas, Shepherd et al. 2011). LutC contains four Fe-S clusters and has one transmembrane helix, meaning that this protein is could be attached to the membrane sending the electrons into the respiratory chain (Chai, Kolter et al. 2009). There was no available information of LutB possessing Fe-S clusters. This protein is the anchor between LutA and LutC. LutA binds two cysteine rich domains, which means that it probably contains Fe-S clusters. This is the first subunit in three subunit protein embracing the active site (Chai, Kolter et al. 2009). This led to the hypothesis that LutABC might be involved in L-lactate utilisation in *N. gonorrhoeae*. Therefore, mutants lacking *lutABC* and mutant lacking both *lldD* and *lutABC* were constructed. These mutants were examined for activity with L-lactate as a substrate with enzyme assay for respiratory dehydrogenases. Figure 4.10 demonstrates that there was no significant difference in activity between single *ΔlldD* and double *ΔlldDΔlutABC* mutant. *ΔlutABC* mutant exhibited activity similar to the wt. These results led to the conclusion that LutABC is not involved in lactate utilisation in *N. gonorrhoeae*. This would not mean that LutABC is not involved in some other metabolism in *N. gonorrhoeae,* similar to the lactate utilisation.

Further BLAST analysis revealed that LutA (FA 1090) shows similarity to GlcF subunit of glycolate oxidase from *E. coli* K12. Identity between these two proteins was 19%. Glycolate oxidase in *E. coli* is a three-subunit enzyme. Expression of this enzyme is induced by glycolate (Unemoto and Hayashi 1993). Glycolate permease is a transporter protein responsible for transport of glycolate in *E. coli*. This transporter protein shows high similarity to lactate

permease (Ducey 2005). Glycolate permease is not present in *N. gonorrhoeae* genome which leads to conclusion that maybe lactate permease has a dual role, both as a lactate and as a glycolate transporter. Glycolate is present in the kidneys as a by-product of collagen degradation and would therefore also be present in the urogenital tract (Merz and So 2000). This would then provide another energy source that could be utilised by *N. gonorrhoeae*.

LutA contains two cysteine rich domains. They are highly conserved and probably coordinating Fe-S clusters. These cysteine rich domains are normally found in archaeal and bacterial (heterodisulphide reductase) Hdr-like proteins. The family includes a subunit from heterodisulphide reductase and a subunit from glycolate oxidase and glycerol-3-phosphate dehydrogenase (Marchler-Bauer, Lu et al. 2011). To approach the question about the involvement of LutABC in glycolate oxidation, experiments were performed in which whole cells were examined for respiration with glycolate (Figure 4.12). In this experiment wt1291 was compared to a single *lutABC* mutant. Even though respiration had a slow rate it was detectable. There was no difference in respiration between wt1291 and *ΔlutABC* and so it was concluded that LutABC is not involved in oxidation of glycolate. Thus function of LutABC in *N. gonorrhoeae* is still not clear.

Due to residual activity measured with *ΔlldD* mutant when L-lactate was used as a substrate it was assumed that LdhD could be responsible for this activity. A study has shown that D-lactate dehydrogenase (LdhD) from *N. meningitidis* was purified and substrate specificity was examined for this enzyme. In that study, the D-lactate dehydrogenase showed high affinity for D-lactate, as expected, but

also demonstrated some activity with L-lactate (Criss, Katz et al. 2009). Further studies in which D-lactate dehydrogenase is inactivated together with L-lactate dehydrogenase would give more insight in residual activity with L-lactate mutant strain and L-lactate as a substrate. Alternatively, purification and characterisation of D-lactate dehydrogenase would also provide more information.

Chapter 5

Lactate as a carbon and energy source during gonococcal-host cell interactions

5.1 Introduction

N. gonorrhoeae causes around 106 million cases of gonorrhoea worldwide annually ((WHO) 2012). Symptomatic infection is characterized by an inflammatory response and a purulent discharge composed almost entirely of gonococci and PMNs. Gonococci can ascend to the upper genital tract, leading to serious diseases, such as epididymitis in men and cervicitis, endometriosis, and pelvic inflammatory disease in women (Densen, MacKeen et al. 1982, Bolan 1999). In rare cases, gonococci can disseminate to the blood stream from the initial site of infection, causing disseminated gonococcal infection (DGI) and the associated complications of arthritis and endocarditis ((WHO) 2012, CDC 2013). *N. gonorrhoeae* can also infect the eyes of newborns as they pass through the birth canal, resulting in ocular gonorrhoea, which is a leading cause of infectious blindness in the developing world (Workowski and Berman 2010)

As *N. gonorrhoeae* is an obligate human pathogen there have been difficulties in developing an animal model of infection. The human model for gonococcal infection is most relevant for studying early events in gonococcal urethritis but there are limitations to this model due to ethical restrictions. This leads to the inability to fully study the effect of innate immune responses to gonococcal infection *in vivo*. To some extent the experimental urethral infection of male volunteers has been used to address certain questions concerning *N. gonorrhoeae* pathogenesis (Cohen, Cannon et al. 1994). Antigenic variation of *N. gonorrhoeae* pilin, lipooligosaccharide (LOS), and opacity (Opa) protein *in vivo* may play a role in evasion of the specific immune response as well as provide functionally different phenotypes (Swanson, Robbins et al. 1987, Schneider,

Griffiss et al. 1991, Seifert, Wright et al. 1994, Jerse, Cohen et al. 1994). Indeed, a high percentage of *N. gonorrhoeae* expressing Opa proteins were isolated from volunteers who were inoculated with an Opa-negative population of *N. gonorrhoeae* (Schneider, Cross et al. 1995, Schmidt, Deal et al. 2000). This observation suggests that Opa protein expression confers a selective advantage in the male urethra. *N. gonorrhoeae* pathogenesis studies cannot be performed with female volunteers due to the risk of serious complications. That means that the group that suffers the greatest consequences from *N. gonorrhoeae* infection is nearly excluded from experimental research. Recently, female mice have been used as an animal model of human infection. The experimental murine infection cannot fully mimic human disease due to the absence in mice of several human-specific factors that are utilized by *N. gonorrhoeae* during infection (Jerse, Wu et al. 2011). However, the genital tract of female mice shares many other features with that of humans, including similarities in oxygen tension, cervical pH, commensal flora, hormonally driven changes in mucus, and certain histological characteristics (Corbeil, Chatterjee et al. 1985).

Manifestation of disease differs between males and females. In females, infection is frequently asymptomatic, while in males infection causes an inflammatory reaction resulting in acute urethritis in which epithelial cells produce pro-inflamatory cytokines (Ramsey, Schneider et al. 1995, Harvey, Post et al. 2002). These cytokines recruit professional phagocytes to the site of infection. Phagocytes, such as neutrophils, are the first line of defence in the human body. Neutrophil influx, as well as shedding of damaged urethral epithelia produces urethral discharge, which is characteristic for infection in men.

Neutrophils posses cytoplasmic granules that contain antimicrobial peptides, enzymes and reactive molecules (Burg N. 2001). They take up microorganisms by phagocytosis and use this arsenal of defence to clear them. They also use extracellular traps to kill microorganisms that have not been phagocytised (Mantovani, Cassatella et al. 2011). *N. gonorrhoeae* has developed different strategies to avoid phagocytosis and also resist killing in the neutrophils. It can vary antigens on the surface by phase variation during the replication thus preventing phagocytosis to some extent (Virji 2009). However, a proportion of bacterial cells still get phagocytised. *N. gonorrhoeae* has evolved strategies to avoid the actions of various antimicrobial agents that the neutrophils possess (Seib, Tseng et al. 2004, Simons, Nauseef et al. 2005). It also prolongs the life of neutrophils (Binnicker, Williams et al. 2003), as their necrosis would trigger the reaction of macrophages. By prolonging the lifespan of neutrophils, and avoiding the antimicrobial effect at the same time, they are able to survive and replicate within them. This would provide *N. gonorrhoeae* an advantage in the course of disease, as neutrophils migrate through the epithelia allowing the bacteria to spread to the surrounding tissues.

Neutrophils exhibit a very high rate of glycolysis, which results in production of lactate (Borregaard and Herlin 1982). Lactate is also present in urogenital tract in millimolar concentrations. *N. gonorrhoeae* can efficiently use only few carbon sources among which is lactate. Millimolar concentrations of lactate enhance the growth of *N. gonorrhoeae* when added to media containing glucose (Smith, Yates et al. 2001). It has been demonstrated that neutrophil derived lactate enhances the oxygen consumption by *N. gonorrhoeae* (Britigan, Klapper et al. 1988).

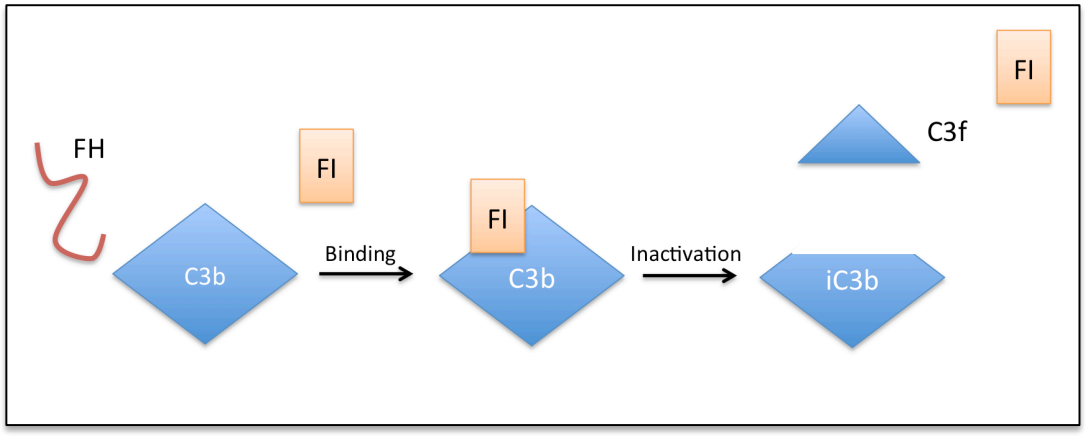


Figure 5.1 Regulation of complement activation via cofactor activity. Cofactor activity is a critical mechanism for controlling complement activation. A regulator in plasma (such as factor H) or on the cell surface (such as membrane cofactor protein) binds to deposited C3b. Following this interaction, the plasma serine protease factor I inactivates C3b by limited proteolytic cleavage that releases a small proteolytic fragment (C3f). The target bound iC3b cannot participate further in the feedback loop.

Infection in women often does not trigger an inflammatory reaction. There are a number of possible explanations for that one of which could be that female tract exhibits anomalous complement (C') regulatory characteristics. With its multiple pathways and numerous components, regulators, and receptors, the C' is a major player in innate immunity and instructs the adaptive immune response. Designed to handle bacterial as well as viral infections, especially to block their invasion into the bloodstream, the complement system is also a key participant in the immune and inflammatory response at sites of tissue injury and debris deposition. If the C' was fully functional in female's reproductive tract, seminal fluid would be recognised as antigens, which would influence reproduction (Vanderpuye, Labarrere et al. 1992).

N. gonorrhoeae initiates infection in females by binding to the complement receptor 3 (CR3). Studies have shown that the presence of this receptor on ecto- and endocervix, on endometrium and Fallopian tube tissue (Edwards, Brown et

al. 2001). These receptors are absent in tissues and cells from female urethra and vagina. This confirms role of CR3 in the infection as gonococcal infection occurs at the sites where CR3 is present. *N. gonorrhoeae* possesses complement C' inactivation product, iC3b which is the ligand for CR3. Binding of *N. gonorrhoeae* to CR3 elicits membrane ruffling and phagocytosis. This again occurs independently from proinflammatory response in immune cells (Caron and Hall 1998).

Primary cervical epithelial cells (pex cells) possess serine/threonine kinase (Akt) (Edwards and Apicella 2006) that is involved regulation of cell cycle as well as a variety of cellular responses including increased glucose uptake. The multifunctional properties of this enzyme make it an attractive target for microorganisms, including *N. gonorrhoeae*. By controlling the function of this enzyme, *N. gonorrhoeae* induces transcription of glucose transporter proteins as well as lactate dehydrogenases. Flow of glucose into the cervical epithelial (pex) cells means higher rate of glycolysis. In this way *N. gonorrhoeae* secures energy source important for survival. This Chapter is aimed to further explore importance of lactate in gonococcal-host cell interactions. By introducing the mutants strains defective in lactate dehydrogenases, already described in Chapters 3 and 4, to neutrophils and pex cells it will give better understanding of possible significance of lactate during the infection.

Mutants strain lacking lactate permease (*lctP*) has already been generated in strain F62 and examined in murine model of infection (Exley, Wu et al. 2007). By constructing this mutant in strain 1291, possible strain differences will be examined. It is worth mentioning that *N. gonorrhoeae* is an obligate human pathogen. Ability for survival and replication of this strain in neutrophils will

also provide insight of importance of lactate during the course of the infection in the human host.

5.2 Results

5.2.1 Construction and characterisation of a Lactate permease mutant (*lctP*)

A mutant lacking lactate transporter protein has already been constructed in strain F62 (Exley, Wu et al. 2007). This mutant was not able to grow in CDM with L–lactate as a single carbon source. It also showed less ability to colonise and survive in murine model of infection. *N. meningitidis* like *N. gonorrhoeae* can use only few carbon sources effectively and among them is lactate. Studies have shown that *N. meningitidis* LctP was required for nasopharyngeal colonisation (Exley, Goodwin et al. 2005).

Sequence comparison of the *N. gonorrhoeae* strain 1291 genome revealed existence of lactate permease transporter (NGO1449). To examine if LctP is responsible for lactate influx in this strain, mutant lacking LctP transporter was generated. In order to construct an *lctP* mutant strain, the splice overlap PCR technique described in the Methods section was used in which upstream (1089 bp) and downstream fragments (1051 bb) were amplified with primers lctP-KO F1, lctP-Ko R1 and lctP-KO F2, lctP-KO R2 (Figure 5.2). These fragments contained part of the *lctP*. For this amplification tag primers with overhangs for Kanamycin (Km) were used. Both fragments and Km were merged using PCR and the subsequent fragment (Figure 5.2) was cloned in the cloning vector pUC19. This construct was linearized and transformed into wt1291 generating the *ΔlctP* mutant strain. Screening of mutant strains was done with lctP screen-F2,

kanScreen-F and kanScreen screen- R, lctP screen-R2. These primers should create fragments of 1522 and 1401 bp if a positive mutant was constructed. Figure 5.3 demonstrates that insertion deletions strains were correct. This was further confirmed by sequencing.

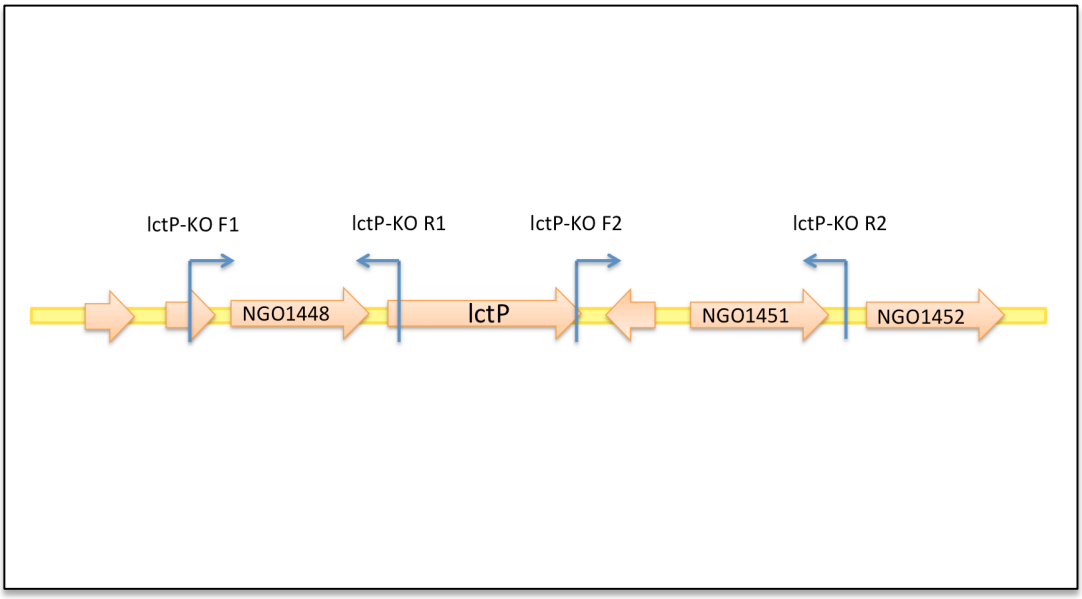


Figure 5.2 Primers used to amplify upstream and downstream fragments for knock out of *lctP*. Primers used to amplify upstream fragment were lctP-KO F1 and lctP-Ko R1. For amplifying the downstream fragment, primers lctP-KO F2 and lctP-KO R2 were used.

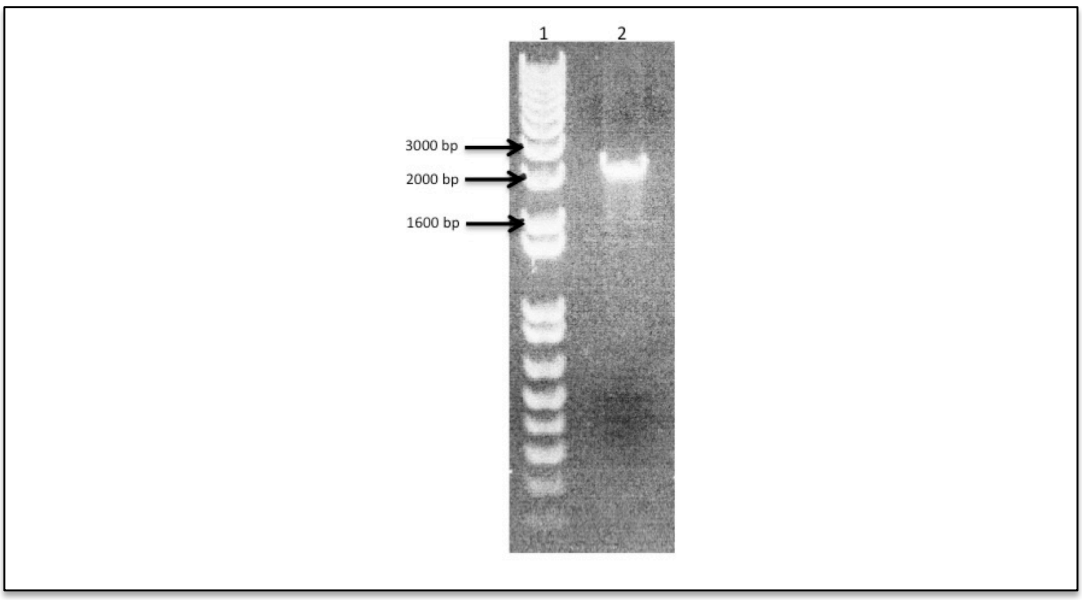


Figure 5.3 The 1% agarose gel image showing the positive splice overlap PCR product.

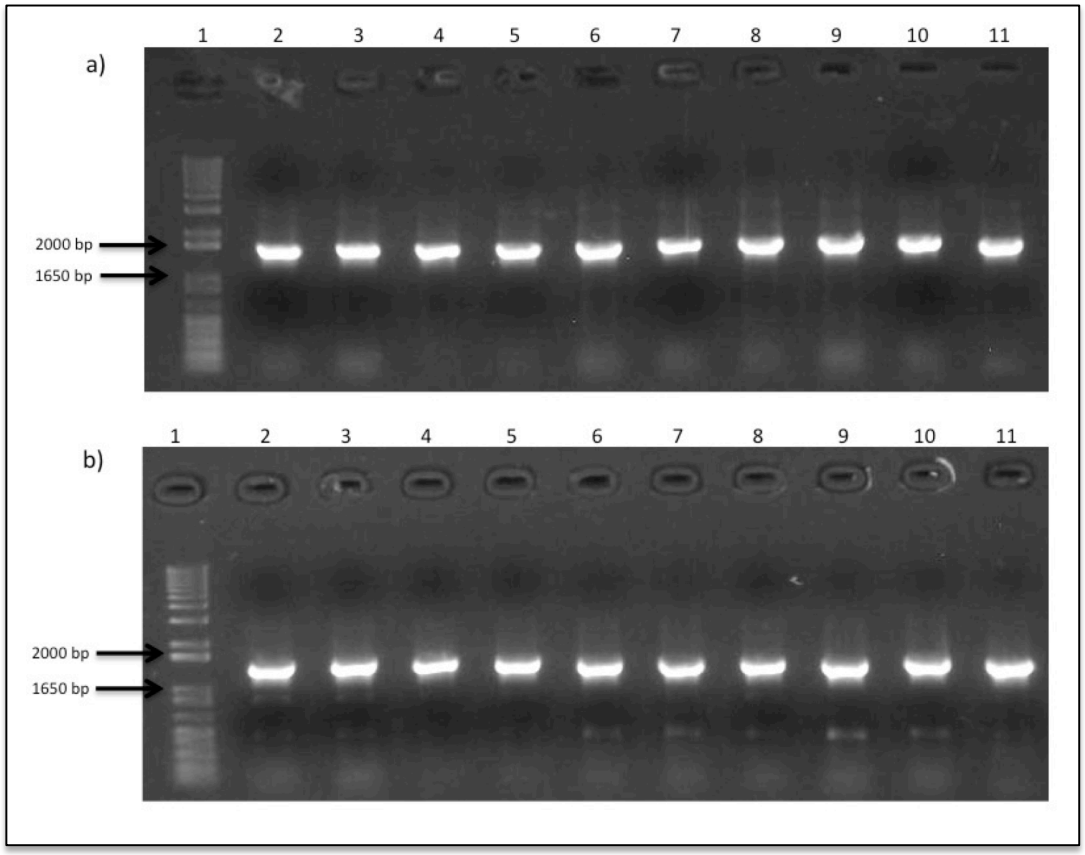


Figure 5.4 Screening of *ΔlctP* mutant with primers binding into Km cassette and outside the *lctP*. Expected size upstream fragment ~1522bp and downstream fragment ~1401bp. a) Lanes 2-11 are upstream fragments and b) lanes 2-11 are downstream fragments of ten different colonies screened. Lane 1 a) and 1 b) is the DNA ladder. Sequencing of these fragments showed insertion of Km cassette in *lctP.*

To compare growth of wt1291 and mutant lacking *lctP*, two strains were grown in chemically defined medium (CDM) with or without L-lactate as a single carbon source. Neither strain could grow without carbon sources. When L-lactate was used as a single carbon source, only wt1291 could grow. The data show that deletion mutation in *lctP* abolishes the ability of *N. gonorrhoea* to grow on L-lactate as a single carbon source (Figure 5.5).

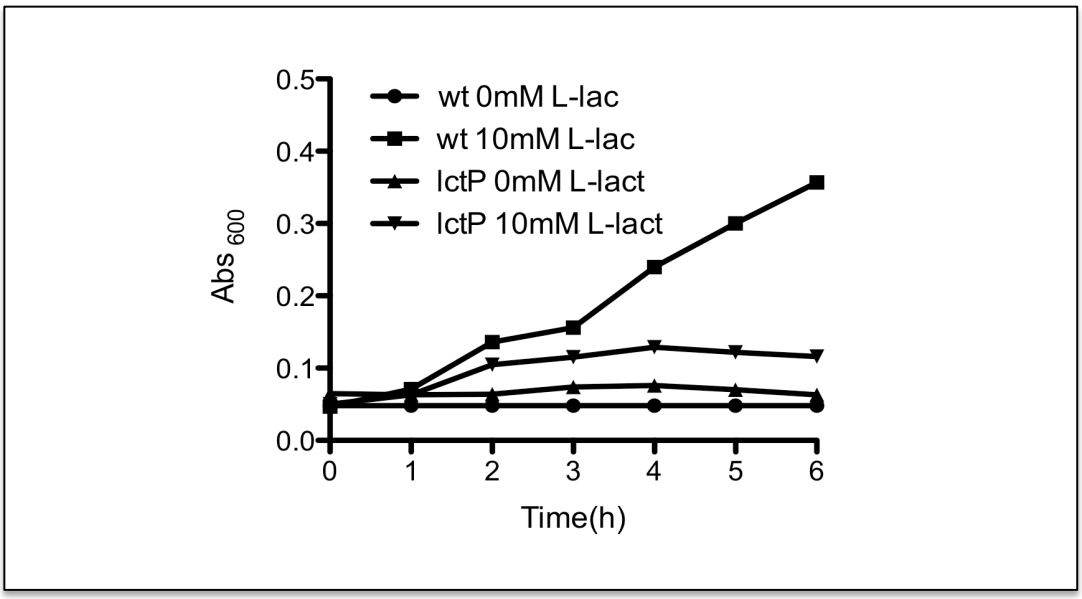


Figure 5.5 Growth of wt1291, *ΔlctP* strains in CDM supplemented with 0 or 10 mM L-lactate as a single carbon source.

5.2.2 Importance of lactate for *N. gonorrhoeae* survival in neutrophils

The importance of lactate for survival of GC was tested using mutants lacking lactate dehydrogenases or lactate permease. Neutrophils extracted from the blood where infected with wt1291 and mutant strains lacking lactate dehydrogenases as well as lactate permease with MOI 1:1. Figure 5.6 demonstrates that after initial killing, 30 min post infection, wt1291 is able to survive and replicate within neutrophils. This is consistent with previous studies (Simons, Nauseef et al. 2005). Mutant strains lacking either lactate dehydrogenases or lactate permease were almost cleared 6h post infection. From this observation it is concluded that respiratory lactate dehydrogenases and lactate permease are required for survival of *N. gonorrhoeae* within neutrophils.

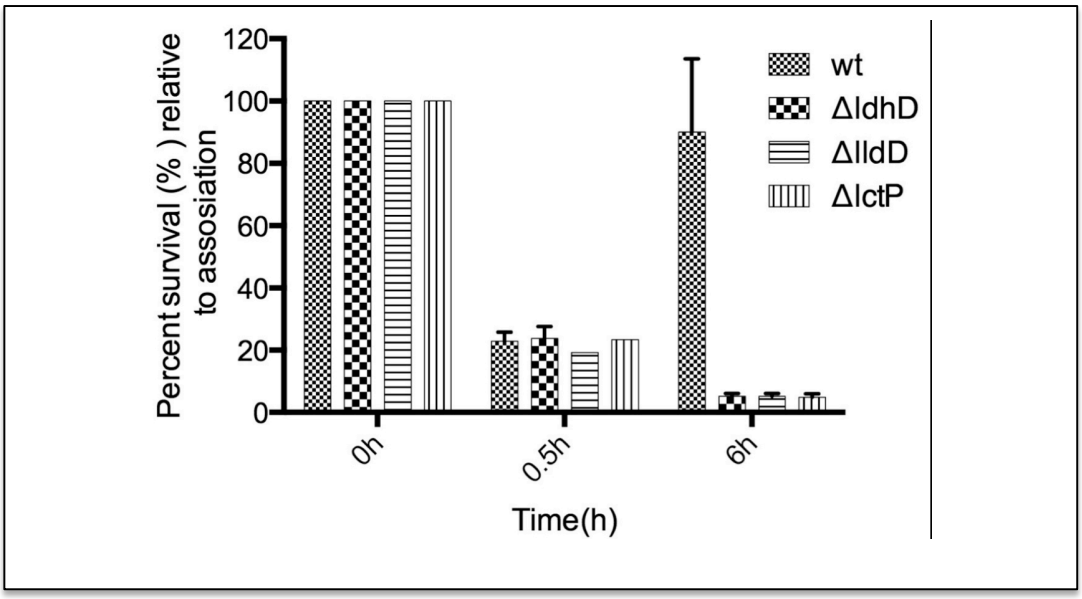


Figure 5.6 Neutrophil killing assay. Neutrophils were infected with wt1291, *ΔldhD*, *ΔlldD* and *ΔlctP* with MOI 1:1. The number of bacteria associated with neutrophils at Time 0h was taken to represent 100%. Survival was determined relative to association. Each assay was performed in duplicates on at least three separate occasions.

5.2.3 Are lactate dehydrogenases important for infection of primary cervical epithelial (pex) cells?

Primary human cervical epithelial (pex) cells are derived from cervical epithelia and represent good model for study of gonorrhoeal infection in women (Edwards, Shao et al. 2000). The relationship between lactate production and the survival of *N. gonorrhoeae* in pex cells has not been previously studied.

Dr Jennifer Edwards performed the infection and survival assay with pex cells from The Center for Microbial Pathogenesis, Nationwide Hospital Children's Research Institute and The Ohio State University, Columbus. Pex cells were infected with wt1291 and mutants lacking all three lactate dehydrogenase with MOI 100:1. Association of *N. gonorrhoeae* to pex cells is determined relatively to

initial inoculum. Around 30% of initial inoculum associated to pex cells. Figure 5:6 demonstrates that wt1291 is able to survive and replicate 3h post infection, after initial killing. However, *ΔldhA* mutant shows approximately the same rate of survival as the wt1291. Mutants lacking respiratory dehydrogenases, *ΔldhD* and *ΔlldD*, were almost all cleared 3h post infection. This demonstrates possible importance of respiratory dehydrogenases in the course of infection.

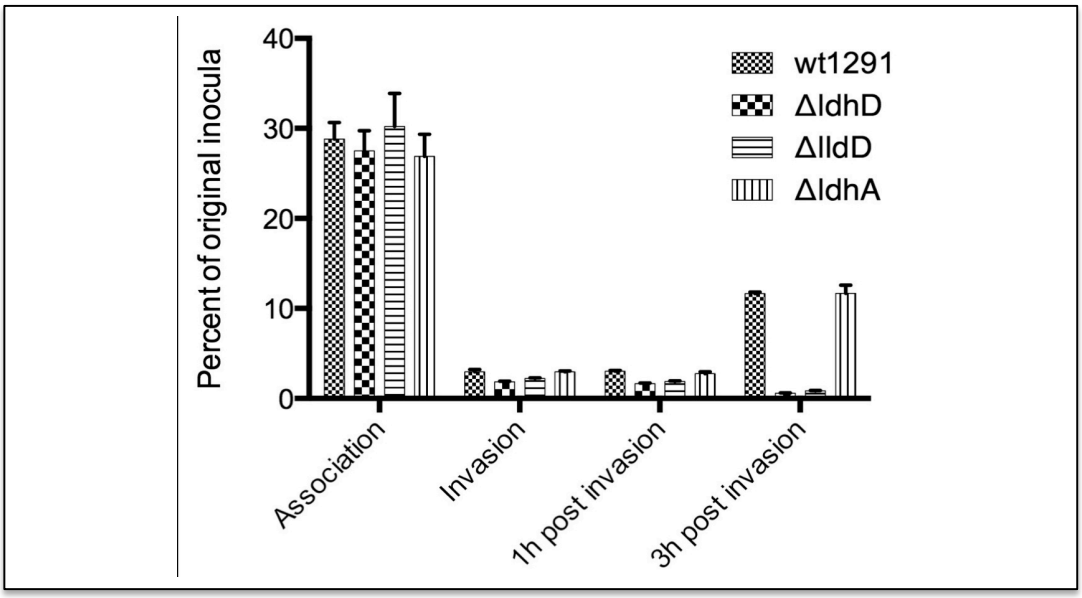


Figure 5.7 Invasion and survival assay of *N. gonorrhoeae* in pex cells under hypoxic conditions. Pex cells were infected with wt1291, *ΔldhD*, *ΔlldD* and *ΔldhA* strains. Survival is determined relatively to initial inoculum. Each assay was performed in triplicate on at least three separate occasions.

When pex cells were grown aerobically there was no difference between wt1291 and mutant srains (Figure 5.8).

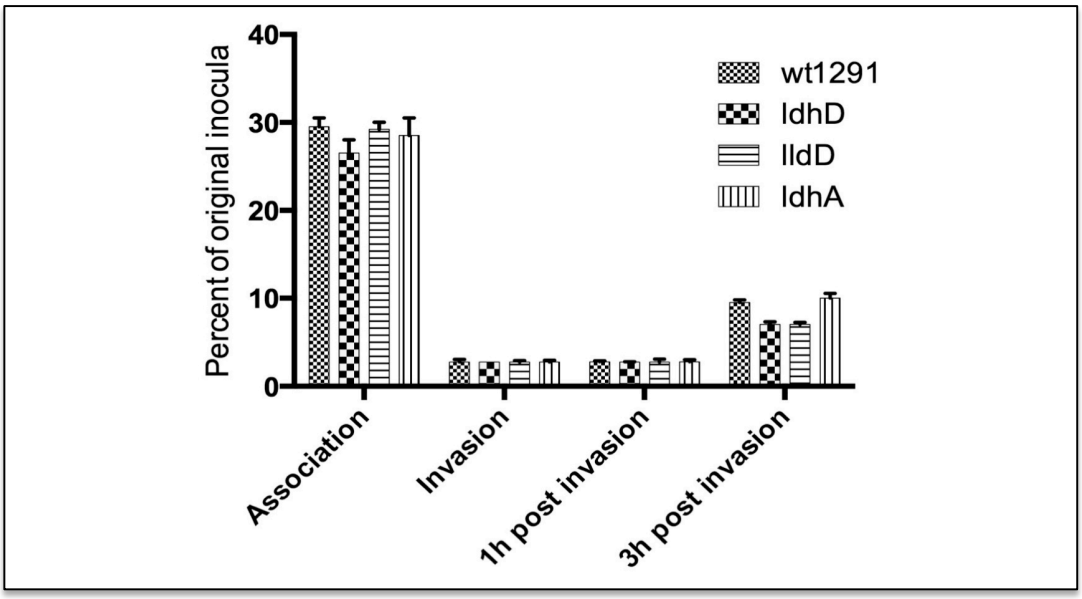


Figure 5.8 Invasion and survival assay of *N. gonorrhoeae* in pex cells under normoxic conditions. Pex cells were infected with wt1291, *DldhD*, *DlldD* and *DldhA* strains. Survival is determined relatively to initial inoculum. Each assay was performed in triplicate on at least three separate occasions.

5.3 Discussion

During the course of infection bacteria must acquire nutrients for growth and replication. *N. gonorrhoeae* can use only lactate, glucose and pyruvate effectively (Smith, Tang et al. 2007). Lactate is present in the urogenital tract, normal habitat of *N. gonorrhoeae*. Studies *in vitro* have shown that millimollar concentrations of lactate enhance the metabolism of *N. gonorrhoea* when added to cultures containing glucose (Sun, Bakshi et al. 2000, Smith, Yates et al. 2001). *N. gonorrhoeae* possesses three lactate dehydrogenases responsible for lactate utilisation and lactate permease responsible for lactate influx into the cell.

Lactate permease (LctP) is a transporter protein with fourteen transmembrane domains. A mutant lacking *lctP* in *Neisseria meningitidis* was attenuated in the infant rat model of bacteremia (Sun, Bakshi et al. 2000). There is a high sequence similarity between gonococcal and meningococcal *lctP*. Importance of the lactate for the survival of *N. gonorrhoeae* was also investigated *in vivo* in murine model of infection. In this study mice were infected with *lctP* mutant strain and parental strain F62. The *lctP* mutant was impared in its ability to colonise and survive in comparison with the parental strain (Exley, Wu et al. 2007). Millimolar concentrations of lactate enhance the metabolism of the gonococcus when added to cultures containing glucose. This is most obviously seen as a rapid emergence from lag phase, an effect that may be important during the initial stages of *N. gonorrhoeae* colonization at mucosal surfaces (Gao, Parsons et al. 1998). The inability to utilize lactate as an effective carbon source could affect replication in the genital tract mucosa. *N. gonorrhoeae* is able to resist complement-mediated killing through LPS sialylation. The LPS sialylation is enhanced by lactate. The *lctP*

mutant's LPS were less sialylated, leading to impaired resistance to complement-mediated killing by normal human serum (NHS) (Exley, Wu et al. 2007). This is comparable to findings in *N. meningitidis.*

Colonization ability of *N. meningitidis lctP* mutant was also tested. The *lctP* mutant was attenuated in its ability to colonize nasopharyngeal tissue. It has also been shown that the *N. meningitidis lctP* mutant is susceptible to complement-mediated killing, a finding consistent with the effect of adding lactate to the *N. gonorrhoeae* (Parsons NJ 1996 , Smith, Yates et al. 2001).

A mutant lacking *lctP* was generated and characterised by its ability to grow in chemically defined medium (CDM) with L-lactate as a single carbon source. By not being able to utilise lactate, *lctP* mutant strain did not exhibit growth in CDM with L-lactate as a single carbon source, compared to the wt1291. This confirmed one more time that LctP is the transporter protein, solely responsible for influx of lactate into the cell.

As nature of infection differs in males and females, by causing the inflammatory reaction with recruitment of neutrophils to the site of infection in males and being frequently asymptomatic in females, the question of possible lactate involvement in the infection was raised.

N. gonorrhoeae is resistant to oxidative and non-oxidative antimicrobials produced by neutrophils, thus it can survive and replicate within the same. Neutrophils also have high rate of glycolysis with lactate as a by-product. Older studies concentrate on relationship between lactate and oxidative burst, claiming that neutrophil derived lactate stimulates *N. gonorrhoeae* respiration (Britigan, Klapper et al. 1988). This enhanced respiration would render *N.*

gonorrhoeae resistant to neutrophil killing by off the oxidative burst. More recent studies demonstrate that *N. gonorrhoeae* survives neutrophil derived oxidative burst even when enzymes that protect this bacterium against it *in vitro* are not functional (Simons, Nauseef et al. 2005). The exact mechanism is not recognised yet.

By performing the neutrophil killing assay with mutants lacking respiratory dehydrogenases as well as lactate permease, it is demonstrated that lactate is indeed important in the course of infection (Figure 5.6). The mutant lacking respiratory L-lactate dehydrogenase (*ΔlldD*) has reduced ability to use L-lactate in respiration and also reduced production of pyruvate. L-lactate, as an important carbon source cannot be used even though it is available and this results in attenuated ability of survival within neutrophils. *In vitro* studies in Chapter 3 and 4 suggested the existence of a second L-lactate dehydrogenase in *N. gonorrhoeae*. One could conclude that this second L-lactate dehydrogenase would take over the function of the mutated one and that *lldD* mutant would survive. We have to have in mind that *in vitro* studies often have another outcome than experiments closer to those *in vivo*. Also, the second L-dehydrogenase can operate under the different oxygen tensions (Appendix Figure 3). Thus, if the second LldD is active under conditions more close to aerobic conditions, although this is speculative, pyruvate would be produced. Consequently this pyruvate would be used to synthetise acetate and electrons released from L-lactate oxidation would be used in aerobic respiration.

The D-lactate dehydrogenase mutant (*ΔldhD*) showed the same attenuated ability to survive within neutrophils as L-lactate dehydrogenase mutant (*ΔlldD*).

Indeed, available L-lactate is oxidised by L-lactate dehydrogenase but at the same time D-lactate is produced by NADH dependent D-lactate dehydrogenase. This enzyme creates a D-lactate pool, which cannot be released due to inactive LdhD. Excretion and oxidation of D-lactate work in synergy to release the D-lactate pool, as the D-lactate is poisonous in high concentrations. If not released it causes cell necrosis (Htyte, White et al. 2011). The Lactate permease mutant (*ΔlctP*) was also at a disadvantage compared to wt1291. This mutant was not able to import lactate produced by neutrophils, which left it without one important available carbon source.

N. gonorrhoeae attaches and invades cervical epithelial cells in complement receptor 3' (CR3') dependent mechanism (Edwards, Brown et al. 2001). This mechanism is independent from immune response and partially explains nature of infection in women, which is frequently asymptomatic. *N. gonorrhoeae* can survive and replicate within cervical epithelial cells. Mammalian cells, among them cervical epithelial cells, possess Akt serine/threonine kinase. Akt has the ability to regulate cell cycle, gene transcription, uptake of nutrients and metabolism. By being multifunctional enzyme involved in cell metabolism, it is often targeted by microorganisms that subvert its function to their own favour. *N. gonorrhoeae* possesses phospholipase D (PLD), which competes with Akt's natural ligand. Studies have shown that *pld* mutant had lower ability to survive within primary cervical epithelial (pex) cells (Edwards and Apicella 2006). By activating Akt with PLD, *N. gonorrhoeae* induces expression of glucose transporters as well as expression of lactate dehydrogenases. This means higher influx of glucose into the cervical cells, which is followed by higher rate of glycolysis and of course more lactate. In this way *N. gonorrhoeae* provides itself

energy source required for survival and replication.

To further confirm importance of the lactate, *ex vivo* experiment was performed with pex cells. Figure 5.7 shows that after initial killing, wt1291 as well as NAD^+ dependent D-lactate dehydrogenase mutant (*ΔldhA*) are able to survive and replicate 3h post infection, while respiratory lactate dehydrogenase mutants were almost all cleared. Even though extracellular lactate is present and could be imported into the cell, ability for utilisation of this important carbon source is decreased. The *ldhA* mutant is able to use L-lactate effectively by having LldD fully functional. Cells are able to respire and pyruvate pool is created. This pyruvate is then used in intermediary metabolism, which allows this mutant to survive and replicate.

The *N. gonorrhoeae* enters a protective environment early in disease where they survive and replicate. Experimental infection of men demonstrates that the cytokines and chemokines are prevalent within the urethral lumen with progressive gonococcal disease (Ramsey, Schneider et al. 1995). Release of cytokines and chemokines from the urethral epithelium may, therefore, potentially initiate the inflammatory response associated with gonococcal urethritis by triggering PMN influx. Microscopic examination of urethral exudates from men documented to have culture-proven gonorrhoea indicates that gonococci are found within PMNs and urethral epithelial cells (Apicella, Ketterer et al. 1996). As mentioned above and showed (Figure 5.6) *N. gonorrhoeae* is able to survive and replicate within neutrophils. I have shown that fully working lactate metabolic pathway is necessary for proliferation of *N. gonorrhoeae* during neutrophil phagocytosis. This could be important during the infection in males. With

prevalence of asymptomatic gonococcal disease in women, there is no influx of neutrophils. Also, the presence or absence of sialic acid on the gonococcal surface does not influence the ability of this bacterium to bind and inactivate C3b or to invade primary cervical epithelial cells. As lactate enhances sialylation of LPS, this would mean that lactate is not crucial for attachment and internalization but crucial for survival of *N. gonorrhoeae* within cervical epithelia. This can be seen in Figure 5.7 and 5.8.

Lactate is important energy source. It renders *N. gonorrhoeae* resistant to complement mediated killing by human sera and it is important, as shown in this study, for survival of *N. gonorrhoeae* in neutrophils and pex cells. This study opens the way for better understanding of the role of lactate metabolism during the course of infection in both women and men.

Chapter 6

Conclusion and Future Directions

Even though the importance of lactate as a carbon source and its importance in virulence had been studied little was known about organisation of lactate dehydrogenases, their characteristics and possible involvement in pathogenicity of *N. gonorrhoeae*. This work was aimed to give some answers about vital interplay between *N. gonorrhoeae* and lactate, to determine the organisation of lactate dehydrogenases involved in lactate utilisation and provide directions for future antimicrobial strategies.

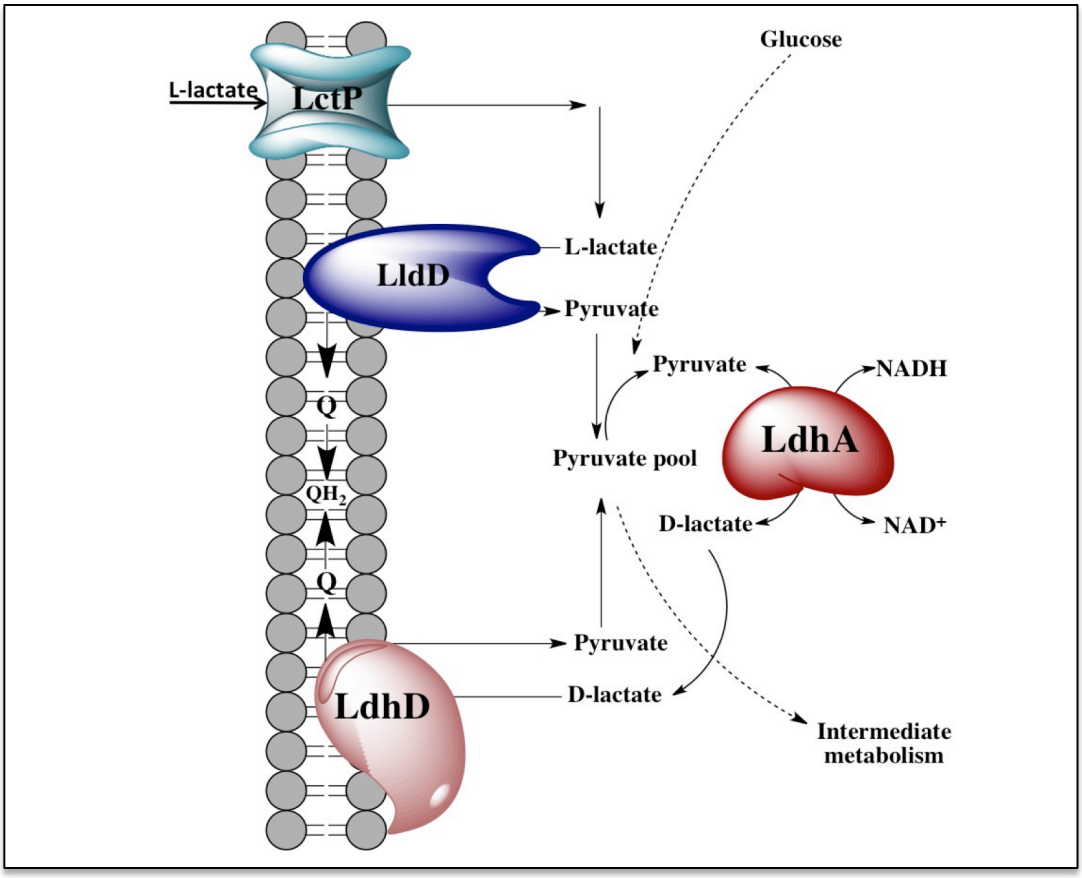


Figure 3.2 Model of lactate utilisation in *Neisseria gonorrhoeae* based on the concept of the existence of three lactate dehydrogenases.

To address the question about organisation of lactate dehydrogenases, a model of lactate utilisation was created (Figure 3.2). The model was generated by

comparison between genomes of *E. coli* and *N. gonorrhoeae*. In this model L-lactate, which is present only in the host, is imported into the cell by lactate permease (LctP). L-lactate is then oxidised to pyruvate by L-lactate dehydrogenase (LldD). Pyruvate from pyruvate pool, created both from L-lactate oxidation and glycolysis, is reduced to D-lactate by NADH dependent D-lactate dehydrogenase (ldhA). This D-lactate is oxidised to pyruvate by respiratory D-lactate dehydrogenase (LdhD). To confirm the role of respiratory lactate dehydrogenases, set of experiments were performed, including the enzyme assay for respiratory dehydrogenases and respiration by membranes derived from wt1291 and mutants lacking these enzymes (Figure 3.7 and 3.8). As expected, *ldhD* mutant did not show any activity with D-lactate as a substrate. Activity with L-lactate was simular to the wt1291, confirming the role of this protein as respiratory D-lactate dehydrogenase. The situation was slightly different with *lldD* mutant strain. Residual activity with this mutant and L-lactate as a substrate was measured (Figure 3.7 and 3.8). Despite this residual activity it was confirmed that LldD is respiratory L-lactate dehydrogenase. The same result was reported in *N. meningitidis* (Erwin A. L. 1993, Gotschlich 1996). Gotschlich and colleagues speculated about second L-lactate dehydrogenase present in *N. meningitidis*, thus it is a common phenomenon among pathogenic *Neisseria* species.

Accumulation of metabolic end products was also of interest. Under aerobic conditions only acetate accumulated while under conditions of restricted oxygen accumulation of D-lactate was observed (Figure 3.5). Aerobically, there is a considerable amount of substrate level phosphorylation involving phosphotransacetylase/acetate kinase that consumes acetyl-CoA and generates

ATP. This is alternative pathway by which *N. gonorrhoeae* produces ATP and at the same time avoids over reduction of quinone pool. The same result was observed in *N. meningitidis* when it was grown aerobically on glucose, pyruvate or D/L-lactate (Leighton, Kelly et al. 2001). Acetate accumulates also when *E. coli* is grown aerobically with glucose as a single carbon source. This was explained as possibility of imbalance between glucose metabolism and respiration (Erwin and Gotschlich 1996). Other suggestion was the presence of excess NADH and condition in which influx of carbon into the cell exceeds demands for biosynthesis (Harvey, Post et al. 2002).

Appearance of D-lactate followed by lower concentration of acetate under microaerobic conditions implicates shift in metabolism. Due to restricted oxygen, respiratory chain probably exhibits lowered ability to oxidise NADH. Lack of oxygen and alternative respiration pathway by *N. gonorrhoeae* would lead to over accumulation of NADH produced by glycolysis and TCA cycle. Production of lactate provides alternative pathway to recycle NADH.

The role of LdhA as an oxido-reductase has been determined. *In vitro*, this enzyme exhibits preferences for D-lactate as a substrate. The situation in the cell grown microaerobically is different. Amount of pyruvate produced during the glycolysis and oxidation of L-lactate as well as amount of NADH formed under microaerobic conditions is enough to pull the reaction towards NADH oxidation.

The role and organisation of lactate dehydrogenases were answered but there was still unanswered question about residual activity detected with *lldD* mutant and L-lactate as a substrate. The natural approach to it was search for the second L-lactate dehydrogenase. *B. subtilis* does not possess any homolog to *Neisserial*

LldD. L-lactate, in this bacterium, is oxidised by LutABC. This is a three subunit Fe-S containing protein. LutABC is found also in *C. jejuni*, which surprisingly possesses two L-lactate-dependent respiratory enzyme systems. The first protein involved in L-lactate utilisation is an iron-sulfur and flavin containing (Dld-II) protein. The second is LutABC. Orthologs of *lutABC* are also found in *Shewanella onidensis*, *Neisseria meningitidis* and *Escherichia coli* (Pinchuk, Rodionov et al. 2009). BLAST analysis of *N. gonorrhoeae* genome revealed existence of putative *lutABC* operon. Bioinformatic characterisation of LutA, LutB and LutC subunits revealed possible function and organisation of each subunit in the LutABC complex. LutA contains iron-sulfur clusters and active site. This subunit would be responsible for lactate oxidation. There was no information about LutB containing iron-sulfur clusters, thus it could be either flavoprotein or ferredoxin. This subunit is anchor between LutA and LutC. LutC is loosely attached to membrane with one transmembrane helix sending the electrons to the respiratory chain (Figure 4.5). To further examine if LutABC is involved in lactate utilisation in *N. gonorrhoeae*, a mutant lacking this operon was constructed both in wt1291 and *lldD* mutant strain. These mutants did not show any difference to wt1291 or *lldD* mutant strain when examined with enzyme assay for respiratory dehydrogenases (Figure 4.10). Thus, it was concluded that LutABC was not a respiratory L-lactate dehydrogenase. However, further bioinformatics analysis revealed similarity between LutA and GlcF subunit of glycolate oxidase from *E. coli*. When respiration by whole cells was measured with glycolate as a substrate there was no significant difference between wt1291 and *lutABC* mutant strain. This led to conclusion that LutA is not a glycolate oxidase. As D-lactate dehydrogenase from *N. meningitidis* was purified and

characterised showing the preferences for D-lactate but to some extent for L-lactate also, it would be possible that the same would apply to *N. gonorrhoeae* D-lactate dehydrogenase. Detailed study of this protein is necessary to confirm this.

Being the obligate human pathogen *N. gonorrhoeae* does not provide any good animal model for studies *in vivo*. Exley and colleagues in a few studies used murine model of infection (Jerse 1999, Exley, Wu et al. 2007, Jerse, Wu et al. 2011, Sintsova, Wong et al. 2015). Male human volunteers, in whom outbreak of disease is followed by inflammatory response, are used in some studies until now (Schneider, Cross, Kuschner et al. 1995, Hobbs, Sparling, Cohen et al. 2011). Female volunteers are not used because of the nature of this disease in women, which is frequently asymptomatic. To examine if lactate dehydrogenases are possibly important during the course of infection, neutrophils and pex cells were infected both with wt1291 and mutant strains defective in lactate dehydrogenase proteins as well as *lctP* mutant strain. Neutrophils and pex cells represent good models for study of *N. gonorrhoeae* as they are derived from fresh blood and cervical epithelia.

Neutrophils have high rate of glycolysis, thus lactate is ever present in these professional phagocytes. Previous studies have shown that *N. gonorrhoeae* is able to survive and replicate within them (Seib, Simons et al. 2005, Simons, Nauseef et al. 2005, Ball and Criss 2013, Johnson and Criss 2013). Arsenals of defence used by neutrophils, such as oxidative and non-oxidative antimicrobials are not effective against *N. gonorrhoeae* (Seib, Wu et al. 2006, Criss, Katz et al. 2009, Wu, Soler-Garcia et al. 2009). So, is lactate something that contributes to resistance of this bacterium? Result presented in Figure 5.5 clearly demonstrates

that *N. gonorrhoeae* is vulnerable when not able to use lactate. Mutants lacking respiratory dehydrogenases as well as *lctP* mutant were almost all cleared 6h post infection. Wt1291 survived and replicated which is consistent with previous studies. The same was observed when pex cells were infected with the difference for *ldhA* mutant strain. This mutant strain showed the same trend as wt1291. The lactate metabolism is not disrupted by elimination of LdhA. Lactate is still imported into the cell and oxidised to produce pyruvate, which is used in intermediary metabolism. Cervical epithelial cells possess serine/threonine kinase (Akt). Akt is involved in many cellular mechanisms. *N. gonorrhoeae* possesses phospholipase D (PLD), which competes with Akt's natural ligand (Edwards and Apicella 2006). By activating Akt with PLD, *N. gonorrhoeae* induces expression of glucose transporters as well as expression of lactate dehydrogenases. Higher influx of glucose means higher rate of glycolysis and of course more produced lactate.

This work has confirmed the role and organisation of lactate dehydrogenases in *N. gonorrhoeae*. It has shown under what conditions lactate is produced in this bacterium. It has clearly demonstrated importance of respiratory dehydrogenases as well as LctP in conditions closer to those *in vivo*. It has shown that possible ability of LdhD to oxidise L-lactate is not of importance in experiments closer to those. This work provides foundation for deeper understanding of importance of lactate for *N. gonorrhoeae*.

The metabolism and virulence cannot be considered as distinct functions within the bacterial cell. A novel class of antimicrobial compounds and vaccines could be designed that block lactate metabolic pathway and render the bacterium

more sensitive to innate immune killing. In future mutants lacking both respiratory and NADH dependent lactate dehydrogenases could be constructed as well as mutant lacking only both respiratory dehydrogenases. These mutants as well as single mutants could be examined in mouse model of infection. This study would give more insight in female model of infection. Also, if possible, *in vivo* studies of male model of infection could be conducted. Even though expression of lactate dehydrogenases is not up-regulated in biofilm, it could be interesting to gain knowledge if they are important for survival of *N. gonorrhoeae* within the same setting.

Apendix

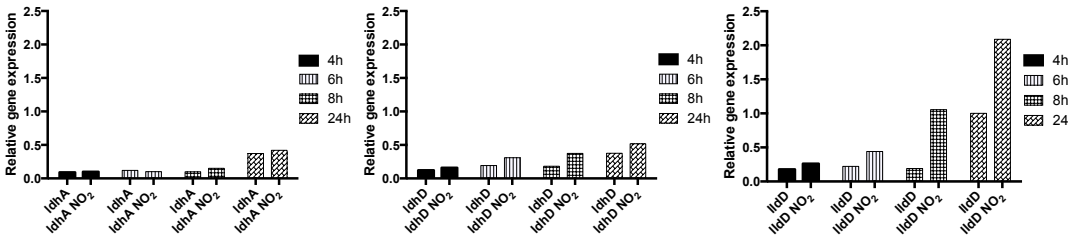


Figure 1. Relative gene expression of *ldhA*, *ldhD* and *lldD* genes. *N. gonorrhoeae* was grown without and with 4mM NO_2. Time points are 4, 6, 8, 24h.

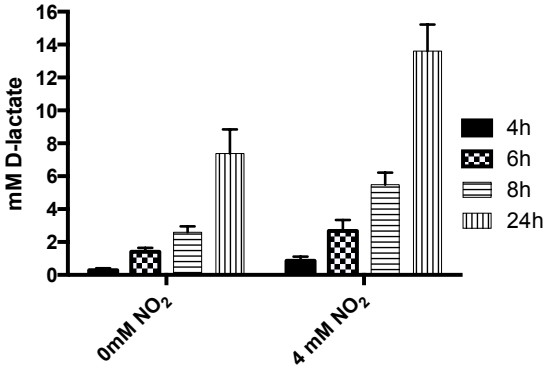


Figure 2. The amount of D-lactate produced under microaerobic conditions without and with 4mM NO_2 over time. Time points measured for determination of lactate are 4, 6, 8, and 24 h. The graphs are representative of three indipendent experiments**.** Error bars are calculated as: Standard deviation/√N where N is number of repllicates.

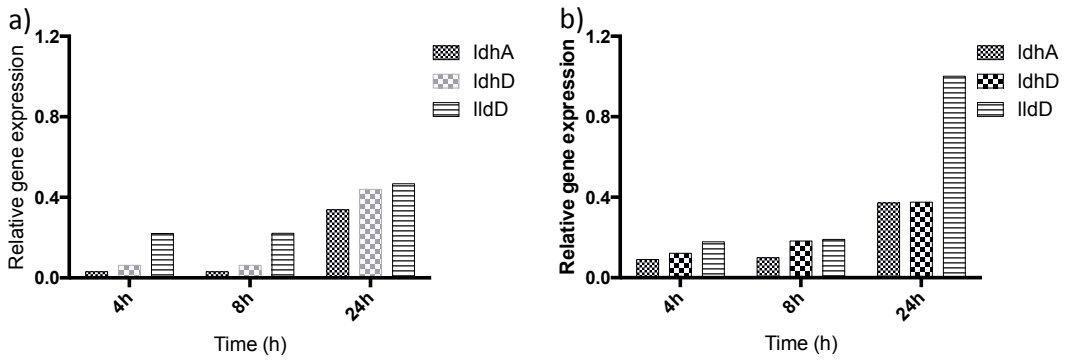


Figure 3. Relative gene expression of *ldhA*, *ldhD* and *lldD* genes under the a) high and b) the low oxygen conditions

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