Streptococcus dysgalactiae : INTERACTIONS WITH HOST CELLS AND CHARACTERIZATION OF GapC

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ABSTRACT

To determine if *Streptococcus dysgalactiae* GapC plays a role in the pathogenesis of *S. dysgalactiae*, an attempt was made to inactivate *gapC* gene expression by insertion of a Ω Km^r cassette, resulting in a single-crossover recombination event. However, attempts to obtain a double-crossover recombination were not successful. The failure to obtain double-crossover mutants together with the result of this study showing that there is only a single copy of *gapC* in *S. dysgalactiae*, and the impact of iodoacetate, a specific GAPDH inhibitor on viability of bacteria, suggests that *gapC* is an essential gene required for basic metabolism.

The role of GapC in the *S. dysgalactiae* adherence/penetration on/into MAC-T cells (a bovine mammary epithelial derived cell line) was investigated with a GapC specific polyclonal antibody. The addition of a molar excess antibody (10 μg/mL) did not directly inhibit bacterial adherence/penetration on/into MAC-T cells. This suggested that GapC, presented on the bacterial surface, probably does not directly participate in adherence/penetration during the pathogenesis.

The effect of *S. dysgalactiae* on MAC-T cell gene expression was also investigated using bovine microarrays. The data was analyzed by B-statistic and three genes that showed the highest possibility of differential expression were (i) succinate dehydrogenase complex, subunit A, flavoprotein (accession number BF046015), (ii) myosin, light polypeptide kinase (accession number AW465934) and (iii) an open reading frame (ORF) (accession number AW463818), respectively. None of the selected genes showed differential expression between infected and non-infected MAC-T cells when tested by Northern blot analysis. It is possible that the conditions used did

not mimic the pathogenesis of mastitis *in vivo* since some of the genes we had expected to detect (i.e. inflammation-associated genes) were not on the list of 25 genes with the highest possibility to show differential expression. It should also be noted that the data analysis performed might not be accurate due to the limited number of microarray slides used.

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Ω Km^r	omega kanamycin resistance
α_2-M	alpha 2-macroglobulin
α_2-M-T	alpha 2-macroglobulin-trypsin
β2M	beta-2-microglobulin
°C	degree Celsius
µg	microgram(s)
µL	microliter(s)
(n) xg	(n) time(s) of gravity
A_n	absorbance at wavelenght n nanometer
aadUTP	aminoallyl-2'-deoxyuridine 5'-triphosphate
ACTB	beta actin
ADHE	alcohol dehydrogenase
ADP	adenosine diphosphate
AK	acetate kinase
Ala	alanine
Ap^r	ampicillin resistance
ATCC	American Type Culture Collection
BG	background
C3b	complement factor 3b

C. bovis	*Corynebacterium bovis*
CAB	chlorophyll a/b binding protein
cDNA	complementary DNA
cfu	colony forming unit(s)
cm	centimeter(s)
Cy	cyanine
dATP	2'-deoxyadenosine 5'-triphosphate
dco	double-crossover
dCTP	2'-deoxycytidine 5'-triphosphate
ddH_2O	double distilled water
DE	differential expressed
dGTP	2'-deoxyguanosine 5'-triphosphate
DIG	digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
DTT	dithiothreitol
EC	enzyme commission
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e.g.	(Latin *exempli gratia*) for example

erm	gene encoding erythromycin resistant protein
Erm^r	erythromycin resistance
Erm^s	erythromycin sensitivity
EST	expressed sequence tag(s)
et al.	(Latin *et alii*) and others
Fig	figure(s)
FA	formaldehyde-agarose
G	green intensity
g	gram(s)
gap, gapdh	gene encoding glyceraldehyde-3-phosphate dehydrogenase
gapC::Ω Km^r	*gapC* interrupted with Ω Km^r cassette
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	group A streptococci
Gb	background green intensity
Gf	foreground green intensity
HPRT	hypoxanthine phosphoribosyl transferase
IAA	iodoacetate
i.e.	(Latin *id est*) that is
IgA	immunoglobulin A
IgG	immunoglobulin G

IL	interleukin
IMI	intramammary infection
ipc	frequency of integrations per cell
IPTG	isopropyl-beta-D-thiogalactopyranoside
IQR	interquartile range of the background
kb	kilobase pair(s)
kDa	kilodalton(s)
Km^r	kanamycin resistance
L. bulgaricus	*Lactobacillus bulgaricus*
LB	Luria-Bertani
LDH	lactate dehydrogenase
Lys	lysine
MAC-T	bovine mammary epithelial cell line
mg	milligram(s)
mL	milliliter(s)
M	molar(s)
mM	millimolar(s)
moi	multiplicity of infection
mol	mole(s)
mRNA	messenger ribonucleic acid
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide

	(reduced form)
ng	nanogram(s)
NHS	N-hydroxysuccinimide
nm	nanometer(s)
NMR	nuclear magnetic resonance
OCI	Ontario Cancer Institute
ORF	open reading frame(s)
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PEP-PTS	phosphoenolpyruvate-dependent phosphotransferase system
PFL	pyruvate formate lyase
pg	picogram(s)
PGK	3-phosphoglycerate kinase
PI	propidium iodide
PK	pyruvate kinase
Plr	plasmin(ogen)-binding protein/receptor
plr	gene encoding plasmin(ogen)-binding protein/receptor
PMN	polymorphonuclear cell(s)
R	red intensity
Rb	background red intensity

RBS	soy rubisco small chain
$RepA^{ts}$	a temperature sensitive RepA
$repA^{ts}$	gene encoding temperature sensitive RepA
Rf	foreground red intensity
RNA	ribonucleic acid
rpm	revolution(s) per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
S. dysgalactiae	*Streptococcus dysgalactiae*
S. equisimilis	*Streptococcus equisimilis*
S. typhimurium	*Salmonella typhimurium*
S. uberis	*Streptococcus uberis*
sco	single-crossover
SDH	streptococcal surface dehydrogenase
SDS	sodium dodecyl sulfate
SFPF	serum-free and protein-free
subsp	subspecies
SV	simian virus
THYE	Todd-Hewitt medium supplemented with 0.5% yeast extract

TIGR	The Institute for Genomic Research
TMS	tris minimal succinate medium
TPI	triosephosphate isomerase
tpi	gene encoding triosephosphate isomerase
Ts	thermosensitive
U	unit(s)
US	United States of America
V	volt(s)
VIDO	Veterinary Infectious Disease Organization
vol	volume
v/v	volume by volume

1.0 INTRODUCTION

Mastitis results in severe economic losses to the world's dairy industry and one of the contributors to these losses is *Streptococcus dysgalactiae*. Unfortunately, little is known regarding the factors which affect virulence of this bacterium in the bovine mammary gland (Leigh et al., 1998). The *gapC* gene from *S. dysgalactiae*, characterized at the Veterinary Infectious Disease Organization (VIDO), University of Saskatchewan, has been shown to possess high homology to the *plr* gene of group A streptococci (GAS). The plasmin binding protein (Plr) or streptococcal surface dehydrogenase (SDH) of GAS was demonstrated to have multiple binding activity to fibronectin, lysozyme, myosin and actin (Pancholi and Fischetti, 1992). Due to its ability to bind fibronectin, the Plr was proposed to play a role in adherence and colonization of the pharyngeal epithelium (Cunningham, 2000). It was also found that whole streptococci and/or purified GAS SDH could activate tyrosine kinase and protein kinase C in human pharyngeal cells (Pancholi and Fischetti, 1997a) which may be important in the infectious process (Cunningham, 2000).

To determine if GapC plays a role in *S. dysgalactiae* virulence, an attempt was made to disrupt the *gapC* gene by insertional inactivation. In addition, the effect of the GapC-specific polyclonal antibody on bacterial adherence/penetration on/into MAC-T cells was studied. Microarray technology was used to investigate gene expression by

MAC-T cells following infection with *S. dysgalactiae* and this was a pilot study for investigating host response to GapC.

In this dissertation, a literature review precedes a chapter of hypothesis and objectives and three chapters which describe the three main studies in manuscript form. Therefore, each chapter contains a short introduction, materials and methods, results, a discussion, and a conclusion. The three chapters are followed by a general discussion which interprets the results of the entire research project, the references for the thesis and the appendix which is presented in the last chapter of the dissertation.

2.0 LITERATURE REVIEW

2.1 Bovine Mastitis

2.1.1 Epidemiology

Mastitis is the pathological response of the mammary gland to microbial infection (McDonald, 1984). It is one of the most widespread and costly diseases affecting dairy herds (Broadbent et al., 1989), costing over $2 billion US per year or at least $180 US per cow per year (McDonald, 1984). Nearly 50% of cows suffer at least one outbreak of clinical mastitis per lactation (Broadbent et al., 1989).

Udder infection may develop when the cow is lactating or dry. Infection rates are highest in the early dry period although these infections often do not persist or develop into clinical mastitis until the next lactation. Clinical mastitis is most common at calving and in the first weeks of lactation. The incidence of clinical disease and new infection increases with lactation number. The high incidence of mastitis around calving is largely a consequence of a high infection rate in the dry period and a periparturient suppression of host defence (Bramley, 1992).

2.1.2 Pathogenesis

The teat duct is the usual port of entry for microbial invasion and, conversely, the major barrier to infection. It has a heavily keratinized surface and the keratin lining

is crucial to the maintenance of its barrier function. Removal of keratin by mechanical means increases susceptibility to bacterial colonization and invasion. Certain defects in machine milking operation can adversely affect the defensive properties of the teat duct, increasing mastitis incidence. Colonization or infection of the streak teat canal, particularly adjacent to the teat orifice, is common with pathogens such as *Staphylococcus aureus* or *Streptococcus agalactiae*. Such colonization may persist for long periods in the absence of intramammary infection (IMI) but are largely prevented or eliminated by postmilking teat disinfection. Other organisms are often present within the teat duct including *Corynebacterium bovis* and coagulase-negative staphylococci and there are reports of the isolation of anaerobic bacteria as well. The environmental organisms, such as coliforms and *Streptococcus uberis,* rarely colonize the teat duct and this difference is important in the pathogenesis of infection (Bramley, 1992).

Both systemic and local signs of mastitis may be present. Usually, only one type of streptococcal and staphylococcal infection will produce systemic signs of disease. Systemic signs include anorexia, depression, toxemia, elevated body temperature, and recumbency. Early in the disease, the body temperature can be as high as 42°C; later the temperature decreases to about 40°C. Local signs of mastitis in each infected gland include swelling, redness, heat, tenderness, increased firmness, and subcutaneous edema, which may extend ventrally along the mammary vein. Changes in the appearance of the gland secretions start with the appearance of fine flakes and clots. Larger clots and flakes appear later, and the secretions take on a watery appearance. In most cases of mastitis, laboratory testing of milk samples is required to determine the etiologic agent (McDonald, 1984).

2.1.3 Mastitis organisms

Mastitis organisms have been categorized as contagious or environmental pathogens based on their distinct characteristics of distribution and interaction with the teat and teat duct. Contagious pathogens live and multiply on and in the cow's mammary gland and are spread from animal to animal primarily during milking. Environmental pathogens are those whose primary reservoir is the environment where cows live rather than infected mammary glands (Smith and Hogan, 1993). Contagious pathogens include *S. aureus*, *S. agalactiae*, *Mycoplasma* species and *C. bovis*, while environmental pathogens such as *S. uberis* are a heterogenous group of bacteria genera, species and strains (Calvinho et al., 1998).

For *S. dysgalactiae*, it has characteristics of both a contagious and an environmental pathogen. It was isolated from infected mammary glands and teat injuries and is transmitted primarily during milking (Bramley and Dodd, 1984). However, detection of several potential extramammary reservoirs, such as cattle tonsils, mouth and vagina (Cruz Colque et al., 1993), and occurrence of *S. dysgalactiae* IMI during the nonlactating period in herds with no previous history of *S. dysgalactiae* IMI (Bramley and Dodd, 1984) suggests that *S. dysgalactiae* also behaves as an environmental pathogen.

2.1.4 Control of mastitis

Control of mastitis involves hygienic practices such as teat dipping and infusion of antibiotic drugs into the udder. Gilmore et al. (1986) estimated 33 million antibiotic treatments are given each year in the United States. However, postmilking teat

disinfection and antibiotic dry cow therapy have been less effective against environmental streptococci and *Escherichia coli* (Smith et al., 1985). Therefore, environmental mastitis has become a major problem in many well-managed dairy farms that have successfully controlled contagious pathogens. In these herds, *Streptococcus dysgalactiae* accounts for a significant number of both subclinical and clinical IMI (Oliver and Mitchell, 1984; Todhunter et al.,1995). In addition to the required practices of good herd management and hygiene, alternative control measures should include manipulation of host defense mechanisms. This approach requires knowledge of host and pathogen factors involved in the development and establishment of IMI as well as defense mechanisms within the udder and ways to enhance these mechanisms (Broadbent et al., 1989).

2 . 2 *Streptococcus dysgalactiae*

2.2.1 *S. dysgalactiae*

Streptococcus dysgalactiae belongs to the Lancefield serological group C based on their dominant carbohydrate cell wall antigens (Garvie et al., 1983). This bacterium is able to cause mastitis on its own and is one of a number of pathogens involved in the aetiology of "summer" mastitis. Strains showing the highest infectivity are those that can adhere to mammary gland epithelial cells the best. *Streptococcus dysgalactiae* can bind several host cell proteins at different levels. For example, it binds high levels of fibronectin, low levels of collagen and moderate amounts of fibrinogen. Protein G is present in the cell walls of some group C streptococci strains. Non-specific binding of

immunoglobulin by this organism may protect it from the defence mechanism of the gland. In addition, some strains produce a fibrinolysin specific for bovine fibrin. Group C streptococci produce hyaluronidase and deoxynuclease (Kenny et al., 1992).

Streptococcus dysgalactiae is a gram-positive coccus with oval cells present in short- to medium-length chains. The cell wall peptidoglycan contains a L-Lys-L-Ala linkage (Schleifer and Kandler, 1972). Growth in glucose nutrient broth is poor and the culture has a pH of 4.7 to 4.9. On blood agar plates, colonies are surrounded by a wide zone of alpha hemolysis (greening). The optimum temperature for growth is 37°C. DNA-DNA hybridization shows that *S. dysgalactiae* is a distinct cluster with a low relationship to *S. agalactiae*, *Streptococcus acidominus*, *S. uberis* or *Streptococcus bovis* (Garvie and Bramley, 1979a; Garvie and Bramley, 1979b).

Streptococcus dysgalactiae is involved in the multietiological clinical entity referred to as summer mastitis which affects dry cows and heifers during summer months primarily in Northern Europe and Japan (Madsen et al., 1990). The organism also has been isolated from the common cattle fly *Hydrotaea irritans*, which appears to play a significant role in establishment and maintenance of bacterial contamination of teats of healthy cattle (Bramley et al., 1985 ; Madsen et al., 1991). *Streptococcus dysgalactiae* is considered to be the first bacterial species to colonize the bovine teat, and apparently provides a favorable environment for colonization by *Actinomyces pyogenes* and anaerobic bacteria such as *Peptostrepococcus indocans* and *Fusobacterium necrophorum* (Madsen et al., 1991).

Outbreaks of clinical mastitis caused by *S. dysgalactiae* frequently follow a breakdown in herd hygiene practices or increases in teat lesions. The incidence of teat

lesions can increase rapidly following failures in pulsation, excessive milking vacuum or adverse housing or climatic conditions. In such outbreaks the machine should be thoroughly examined by an expert and the teats closely inspected for damage. The inclusion of high levels of a suitable emollient in the teat dip can promote rapid healing of lesions and prevent colonization (Bramley, 1992).

Clinical *S. dysgalactiae* mastitis can be acute with anorexia and pyrexia in addition to the local signs. However, response to therapy is usually rapid and elimination rates with penicillins are high. *Streptococcus dysgalactiae* is also encountered in mixed culture with other organisms, notably *Aeromonas pyogenes* and *Pasteurella indolicus* in summer mastitis. The bacteria can be isolated from bovine tonsils and the bovine genital tract and these sources, allied with the ability of the organism to infect and colonize lesions, may be important in the pathogenesis of dry-period infections (Bramley, 1992).

2.2.2 Virulence factors and their roles in infection

The ability to initiate growth *in vivo* and stably infect a host requires the expression of virulence factors by the invading pathogen capable of neutralizing mechanisms of the host's defense. These factors include structural components, toxins, and enzymes that serve to overcome the defensive measures of the host. *Streptococcus dysgalactiae* isolated from bovine IMI produces a number of cell-associated as well as extracellular potential virulence factors (Calvinho et al., 1998).

2.2.2.1 α_2-macroglobulin (α_2-M) - immunoglobulin G (IgG) - and immunoglobulin A (IgA) - binding protein Mig

IgG and α_2-M receptor activities have been identified in a surface-expressed protein, Mig. The IgG receptor activity expressed by Mig belongs to the type III IgG-binding receptor family (Jonsson and Müller, 1994). Although the role of the IgG receptor of Mig in *S. dysgalactiae* virulence is unclear, the IgG receptor of GAS strains has been found to be involved in virulence in a mouse skin infection model (Raeder and Boyle, 1993). The α_2-M receptor of Mig can bind to the α_2-macroglobulin-trypsin (α_2-M-T) complex which is a complex form (fast form) of the universal protease inhibitor, α_2-M (Müller and Blobel, 1985) and also can specifically bind bovine serum immunoglobulin A (IgA) (Song et al., 2002). Pretreatment of *S. dysgalactiae* with α_2-M-T led to a concentration-dependent inhibition in phagocytosis of this bacterium by bovine neutrophils (Valentin-Weigand et al., 1990). In addition, a *mig* mutant strain, constructed by allele replacement mutagenesis of *S. dysgalactiae*, was significantly less resistant to phagocytosis and killing by bovine neutrophils than the wild-type strain when bacteria were preincubated with bovine serum, suggesting that the Mig protein of *S. dysgalactiae* plays a role in virulence of the bacteria by binding to the plasma protein α_2-M or IgG and thus preventing phagocytosis by bovine neutrophils (Song et al., 2001).

2.2.2.2 Fibrinogen-and IgG-binding M-like proteins

Fibrinogen can inhibit C3b-fixation and subsequent phagocytosis by PMN. Phagocytosis killing was inhibited by fibrinogen β- and γ-chains nearly to the same extent as by the intact fibrinogen molecule (Traore et al., 1991). The fibrinogen-binding protein of *S. dysgalactiae* has been considered to represent a pathogenic factor similar to group A streptococcal M protein which can bind to fibrinogen or complement factor

H as mechanisms to evade phagocytosis (Kehoe, 1994). The genes encoding for these proteins may be members of a regulon similar to the described *mga* regulon in *S. pyogenes*, which encodes several virulence factors in this species (Vasi et al., 2000).

2.2.2.3 Fibronectin-binding protein

Fibronectin is a high-molecular-weight glycoprotein found in soluble form in plasma and body fluids and in an insoluble form in connective tissue and basement membranes. A ligand binding site, termed Au, in a fibronectin-binding receptor of *S. dysgalactiae* was demonstrated. A monoclonal antibody raised against the Au binding site only recognized the epitope following binding of Au to fibronectin suggesting that these amino acid residues adopt a specific conformation on binding to fibronectin. Conformational changes in the Au sequence induced by binding to fibronectin may favor bacterial adherence to host tissue (Speziale et al., 1996), and thus, may play an important role in adherence to mammary tissue (Calvinho et al.,1998).

2.2.2.4 Vitronectin-binding protein

Vitronectin is a multifunctional plasma protein that plays a role in complement-dependent lysis, the coagulation system and cellular adherence (Hayman et al., 1983; Preissner et al., 1985). In adherence experiments, prior binding of bovine vitronectin to *S. dysgalactiae* enhanced streptococcal adherence to bovine epithelial cells. The enhancing effects by bovine vitronectin were abolished when the respective binding sites on the streptococci were digested by trypsin. Thus, bovine vitronectin could be an important mediator of adherence of *S. dysgalactiae* to bovine epithelial cells (Filippsen, et al., 1990).

2.2.2.5 Streptokinase

Streptokinase is produced by a number of streptococcal species, including *S. dysgalactiae* and forms a strong complex with plasminogen causing its activation to plasmin which hydrolyses fibrin as well as connective tissue proteins. It has been hypothesized that plasmin may enhance the ability of streptococci to spread in host tissues and promote the growth of cocci by making peptides and amino acids available for bacterial growth during the early stages of infection (Leigh, 1993; Leigh, 1994).

2 . 3 Glyceraldehyde-3-Phosphate Dehydrogenase

2.3.1 GAPDH

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an NAD+-dependent enzyme that catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate and, therefore, is a key enzyme in the glycolytic pathway. This enzyme has a molecular weight of about 145 kDa, and each molecule is invariably composed of four identical subunits. In most cases except the yeast glyceraldehyde-3-phosphate dehydrogenase (Byers and Koshland, 1975), the holoenzyme contains one firmly-bound NAD+ molecule per subunit. The kinetics of coenzyme binding, as well as that of the acylation of the unique cysteine residue involved in this binding, show different types of co-operativity depending on the source of the enzyme (Lee et al., 1982). Mass spectrometric analysis of the purified SDH from *S. pyogenes* revealed a monomer of 35.8 kDa. Molecular sieve chromatography and SDS-gel electrophoresis suggest that the native conformation of the protein is likely to be a tetramer of 156 kDa

(Pancholi and Fischetti, 1992). The *gapC* genes were found to encode a 336-amino-acid polypeptide (approximately 35.9 kDa) in *Streptococcus equisimilis* (Gase et al., 1996) and for a 334-amino-acid polypeptide (approximately 35.7 kDa) in *S. dysgalactiae* (Potter et al., 2001).

In eukaryotes, glycolytic enzymes, of which GAPDH is a member, are found to be associated with membranes and subcellular cytoskeletal structures (Arnold and Pette, 1968). Proteins with a similar enzymatic activity have been shown to be membrane bound (Caswell and Corbett, 1985; Allen et al., 1987; Tsai et al., 1982; Kliman and Stock, 1980) and involved in a variety of functions independent from its catalytic property (Caswell and Corbett, 1985; Huitorel and Pantaloni, 1985; Kawamoto and Caswell, 1986; Perucho et al., 1977; Meyer-Siegler et al., 1991). In prokaryotes, recent studies have confirmed the presence of GAPDH on the surface of group A streptococci (Broder et al., 1991; Pancholi and Fischettii, 1992), on *S. equisimilis* (Gase et al., 1996), on *S. uberis* and *S. agalactiae* (Fontaine et al., 2002), and on *S. aureus* (Modun and Williams, 1999).

2.3.2 GAPDH and pathogenesis

GAPDH on the surface of group A streptococci or surface glyceraldehyde-3-phosphate dehydrogenase (SDH) has been identified as a plasmin(ogen)-binding receptor, Plr (Broder et al., 1991; Broeseker et al.,1988; Lottenberg et al., 1987; Lottenberg et al., 1992). In group B (*S. agalactiae*) and C (*S. equisimilis*) or ungroupable (*S. uberis*) streptococci, surface GAPDH has been identified as GapC (Gase et al., 1996; Fontaine et al., 2002). In group A streptococci, lysine-binding sites in

the region of the plasmin(ogen) molecule interact with the streptococcal Plr (Broder et al., 1989; Broder et al., 1991). Surface-bound plasmin would activate extracellular matrix metalloproteases or collagenases (Lottenberg et al.,1994), enhancing bacterial invasion or movement through normal tissue barriers (Boyle and Lottenberg, 1997).

SDH from *S. pyogenes* was demonstrated to bind fibronectin, lysozyme, and the cytoskeletal proteins myosin and actin (Pancholi and Fischetti, 1992). Due to its binding of fibronectin, the dehydrogenase was proposed to play a role in adherence and colonization of the pharyngeal epithelium (Cunningham, 2000). The surface dehydrogenase was also discovered to be an ADP-ribosylating enzyme, an activity which was enhanced in the presence of nitric oxide (Pancholi and Fischetti, 1993). Since ADP-ribosylation is an important component of intracellular signaling events, ADP-ribosylating activity of SDH suggests that SDH may play a role in mediating cell-to-cell communication between group A streptococci and pharyngeal cells (Pancholi and Fischetti, 1997a). It was also found that whole streptococci or streptococcal surface dehydrogenase activated tyrosine kinase and protein kinase C in human pharyngeal cells (Pancholi and Fischetti, 1997b). The signaling of host pharyngeal cells may be important in the infectious process. Other studies have shown that under conditions of iron starvation, Plr is released from the surface into the growth medium (Eichenbaum et al., 1996). The significance of this mechanism in pathogenesis is yet to be determined, but it could play a role in bacterium-host cell communication (Cunningham, 2000).

3.0 HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

GapC has a role in the adherence/penetration of *S. dysgalactiae* on/into host cells.

3.2 Objectives

The objectives of this study were to determine the role, if any, of GapC in the pathogenesis of *S. dysgalactiae* infection and to determine the host gene expression profile in response to *S. dysgalactiae* infection. The specific aims of this study were to: (1) construct a *gapC* mutant strain of *S. dysgalactiae,* (2) study the effect of GapC on *S. dysgalactiae* adherence/penetration *in vitro*, and (3) study the effect of *S. dysgalactiae* on MAC-T cell gene expression.

4.0 INACTIVATION OF *S. dysgalactiae* GapC

4.1 Introduction

Allele replacement by homologous recombination is a common strategy used to generate site-specific insertions into the chromosome of a target organism. It relies on the presence of one or more DNA fragments with homology to the chromosome which can undergo a single- or double-crossover recombination event.

Single-crossover (sco) recombination, in which a conditionally replicating vector is commonly used, results in 2 copies of the appropriate gene on the host chromosome. The conditional plasmid vectors usually contain a *repA*^{ts} gene which results in temperature-sensitive plasmid replication. Therefore, as long as a permissive temperature is maintained (i.e usually 30°C or lower) where RepA^{ts} can function, the plasmid can replicate and stable transformants can be isolated. When exposed to non-permissive temperatures (i.e 37°C), plasmid replication is inhibited unless it integrates into the chromosome. (Yim and Rubens, 1998).

Allele exchange mutagenesis or double-crossover (dco) recombination is an extension of sco recombination. Usually, an antibiotic-resistance gene is cloned into the gene of interest, and following a sco recombination event as described above, excision of the integrated plasmid is promoted by a second recombination event induced by growth at 30°C or lower without the presence of antibiotics in the growth medium. The

recombinants would be phenotypically resistant to antibiotic corresponding to the antibiotic-resistant gene inserted into the middle of gene of interest but sensitive to an antibiotic corresponding to the antibiotic-resistant gene on the plasmid vector backbone. This event results in high-frequency excision of the replicon, giving rise to a chromosome containing either the wild type or mutant gene. If recombination occurs on the same site that was used during plasmid integration, the strain reverts to wild type and the original plasmid is excised in its entirety. However, if the second recombination occurs on the site opposite to the original recombination event, an allele exchange occurs (Yim and Rubens, 1998; Biswas et al., 1993) resulting in a mutant phenotype.

To determine if *S. dysgalactiae* GapC plays a role in the pathogenesis of *S. dysgalactiae*, an attempt was made to inactivate the *gapC* gene by insertion of an Ω Km^r (omega kanamycin) cassette. A Streptococcal integration plasmid, pG^+host9, was used in this allele exchange mutagenesis. The pG^+host replicon is a replication thermosensitive (Ts) derivative of pWV01 (Otto et al., 1982). In *Lactococcus lactis*, pG^+host replicates at 28°C but is lost above 37°C (Maguin et al., 1996). This plasmid can replicate in both *E.coli* and streptococci. Additionally, it provides an erythromycin resistance gene, *erm,* that can be expressed in both *E.coli* and streptococci. Therefore, sco recombinants would be Km^r and Erm^r whereas dco recombinants would be Km^r and Erm^s. In addition, since one function of GapC is as a GAPDH enzyme, iodoacetate, a specific GAPDH inhibitor (Webb, 1966; Campbell-Burk et al., 1987), was studied for its effect on bacterial GapC.

4.2 Materials and Methods

4.2.1 Bacteria, cultivation conditions and plasmids

The *E. coli* strains used were TG1(dev) {*sup*E Δ(*hsd*M-*mcr*B)5 *thi*Δ(*lac-pro*AB) F'[*tra*Δ36*pro*A^+B^+ *lac*$I^qZ\Delta M15$] *rec*A *aph*A *rep*A}, kindly provided by Dr. E. Maguin, Institut National de la Recherche Agronomique (INRA), France. EC768 (dam^- dcm^-), and BL21(DE3) [*hsd*S *gal*(λ*cIts*857 *ind*1 Sam7 *nin*5 *lac*uv5-T7gene*1*)] from our laboratory collection. *E. coli* cells were grown either in liquid or solid (1.5% agar) Luria-Bertani (LB) medium (Difco Laboratories). *Streptococcus dysgalactiae* was obtained from the American Type Culture Collection (ATCC number 43078). *Streptococcus dysgalactiae* was grown in Todd-Hewitt broth or on Todd-Hewitt agar (1.5%) (Difco Laboratories) supplemented with 0.5% yeast extract (THYE). Liquid cultures of *S. dysgalactiae* and solid cultures of *S. dysgalactiae* and *E. coli* were incubated with 5% CO_2 without agitation. Liquid cultures of *E. coli* were incubated aerobically with agitation at 200 rpm. Antibiotics were used at the following concentrations: ampicillin (Sigma), 100 μg/mL; carbenicillin (Sigma), 50 μg/mL; erythromycin (Gibco-BRL), 350 μg/mL for *E. coli* and 2 μg/mL for *S. dysgalactiae*; kanamycin (Sigma), 100 μg/mL for *E.coli* and 40 μg/mL for *S. dysgalactiae*. Plasmids used are shown in Table 4-1.

Minimal medium, used to culture sco recombinants, was a modified Tris minimal succinate (TMS) medium (Sebulsky et al., 2000) (100 mM NaCl, 50 mM KCl, 0.1 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2.6H_2O$, 20.5 mM NH_4Cl, 0.05 mM $FeSO_4.7H_2O$, 142 mM Na_2SO_4, 2 mM KH_2PO_4, 100 mM Tris, pH 7.4). The medium was autoclaved

Table 4-1 Plasmids used in this study

Plasmid	Genotype/Phenotype	Source or reference
pMF403ci	Ω(Km^r) cassette; Ap^r	Dr. M.C. Fontaine (VIDO, Canada)
pET-GapC	pET15b containing 1.01-kb *S. dysgalactiae gapC*; Ap^r	Dr. A. Bolton (VIDO, Canada)
pETgk	pET-GapC *gapC*::Ω(Km^r) cassette; Ω(Km^r) cassette from pMF403ci digested with *Bam*HI, blunted, and cloned into the *Nsi*I site of pET-GapC; Ap^r Km^r	This study
pG$^+$host9	pGK12 containing *repA*^{ts}; Erm^r	Maguin et al., 1996
pGhgk	pG$^+$host9 *gapC*::Ω(Km^r) cassette; *gapC*::Ω(Km^r) cassette from pETgk, digested with *Nde*I and *Eco*RI, blunted, and cloned into the *Sma*I site of pG$^+$host9; Erm^r Km^r	This study

and mixed with a 0.2 micron-filter-sterilized amino acid solution (1 g/100mL casamino acid, 0.05 mg/100mL pantothenic acid, 0.01 mg/100mL biotin, 1.68 mg/100 mL thiamine, 1.66 g/100 mL succinic acid).

4.2.2 General genetic manipulations

Plasmid DNA was prepared from *E.coli* by the alkaline lysis method (Birnboim and Doly, 1979). Chromosomal DNA was prepared from *S. dysgalactiae* using the CloneTech Nucleospin Tissue Kit (Clonetech) with the following modification. One hundred and fifty units of mutanolysin were added to the first buffer (20 mM Tris-Cl, pH8, 2 mM EDTA, 1% (v/v) Triton X-100, and 2 mg/mL lysozyme). Restriction endonucleases and DNA ligase were used according to the manufacturers' recommendations (Stratagene, New England Biolabs, Invitrogen, Amersham Pharmacia Biotech). When required, incompatible DNA restriction fragments were joined by ligation after treatment with the Klenow fragment of DNA polymerase I (Stratagene) to produce blunt fragments ends.

Electroporation of *E. coli* TG1(dev) was performed as follows: *Escherichia coli* were grown in 500 mL of LB-broth with vigorous shaking at 37°C to an A_{600} of 0.45-0.6 (taking cells still in mid-log growth phase). The cultures were chilled for 30-45 minutes on ice and cells were harvested by centrifuging at 4000xg for 15 minutes at 4°C. The ionic strength of the suspension was reduced by extensive washing with 500 mL of cold ddH_2O, 250 mL of cold 15%(v/v) glycerol, and 10 mL of cold 15% glycerol, respectively, and finally suspended in 1 mL of 15% glycerol. This concentrated suspension was aliquoted, frozen on dry ice/95% EtOH, and stored at –

70°C. For electroporation, 50 μL of cells were mixed with 1-5 μL of DNA solution to give a final concentration from 10 pg/mL to 7.5 μg/mL. The cell/DNA mixture was placed in a cold 0.1 cm-electroporation cuvette, and the pulse applied at 1.8 kV/0.1 cm, 200 ohms in a Gene Pulser apparatus (BIO-RAD). Following the pulse, the cells were immediately mixed into 1 mL of SOC medium (2 g% Bacto tryptone, 0.5 g% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). The sample was incubated, with shaking at 200 rpm for 1 hour at 37°C. The cells were collected by centrifugation, diluted and plated on LB-agar containing 350 μg/mL erythromycin to select for transformants (Dower et al., 1988). All the temperature sensitive plasmids were transformed into *E. coli* TG1(dev) which has the wild type *repA* gene to complement the temperature sensitive mutation. Therefore, the *E.coli* can be cultured at 37°C.

The preparation of competent *S. dysgalactiae* and electroporation of *S. dysgalactiae* was modified from that of Perez-Casal et al. (1993). *Streptococcus dysgalactiae* was grown in 100 mL of THYE supplemented with 60 mM glycine and 68 μg/mL hyaluronidase at 37°C with 5% CO_2 to an A_{600} of 0.2-0.3 (thus the cells were in mid-log growth phase). The cells were harvested by centrifuging at 2100 xg for 10 minutes at 4°C. The ionic strength of the suspension was reduced by extensive washing twice with 25 mL of cold 15% glycerol, and finally resuspended in 250 μL of 15% glycerol. Seventy microliters of competent cells were mixed with 5 μg of DNA. The cell/DNA mixture was placed in a cold 0.1 cm-electroporation cuvette, and the pulse applied at 2.5 kV/0.1 cm, 200 ohms in a Gene Pulser apparatus. Following the pulse, the cells were immediately mixed into 10 mL of THYE medium. Samples were incubated

for 1 hour at 37°C with 5% CO_2. The cells were centrifuged, diluted and plated on THYE-agar containing 2 μg/mL erythromycin to select for transformants.

Southern blot hybridizations were performed essentially as described by Sambrook et al. (1989). DNA samples digested with the appropriate restriction endonucleases were separated on 1% agarose gels, vacuum transferred to nitrocellulose paper, and hybridized with probes at 42°C. DIG-labeled Ω Km^r and *gapC* probes (Roche) were prepared according to the manufacturer's instructions. All other DNA manipulations were as described by Sambrook et al. (1989).

4.2.3 Construction of pGhgk plasmid

Plasmid pET-GapC was linearized with *Nsi*I at a single site in the *gap*C gene and ligated with a 2.17-kB *Bam*HI fragment of the Ω Km^r cassette that was blunted with the Klenow fragment of DNA polymerase I. The resulting plasmid pETgk was confirmed by digestion with *Ssp*I, *Sap*I and *Ava*I, *Xba*I and *Hpa*I. The plasmid was propagated in *E. coli* EC768. The plasmid pETgk was cut with *Nde*I and E*co*RI, and the *gapC*::ΩKm^r fragment was purified and ligated with pG^+host9 plasmid digested at the *Sma*I site. The resulting plasmid, pGhgk (Fig.4-1) was digested with *Bam*HI and *Hind*III, analyzed by agarose gel electrophoresis, and sequenced to verify the construct.

4.2.4 Allele exchange mutagenesis

The method for allele exchange was modified from Biswas et al. (1993). A mutant strain of *S. dysgalactiae* was constructed by chromosomal insertion of the thermosensitive vector containing the altered *gapC* gene (pGhgk). One microgram of

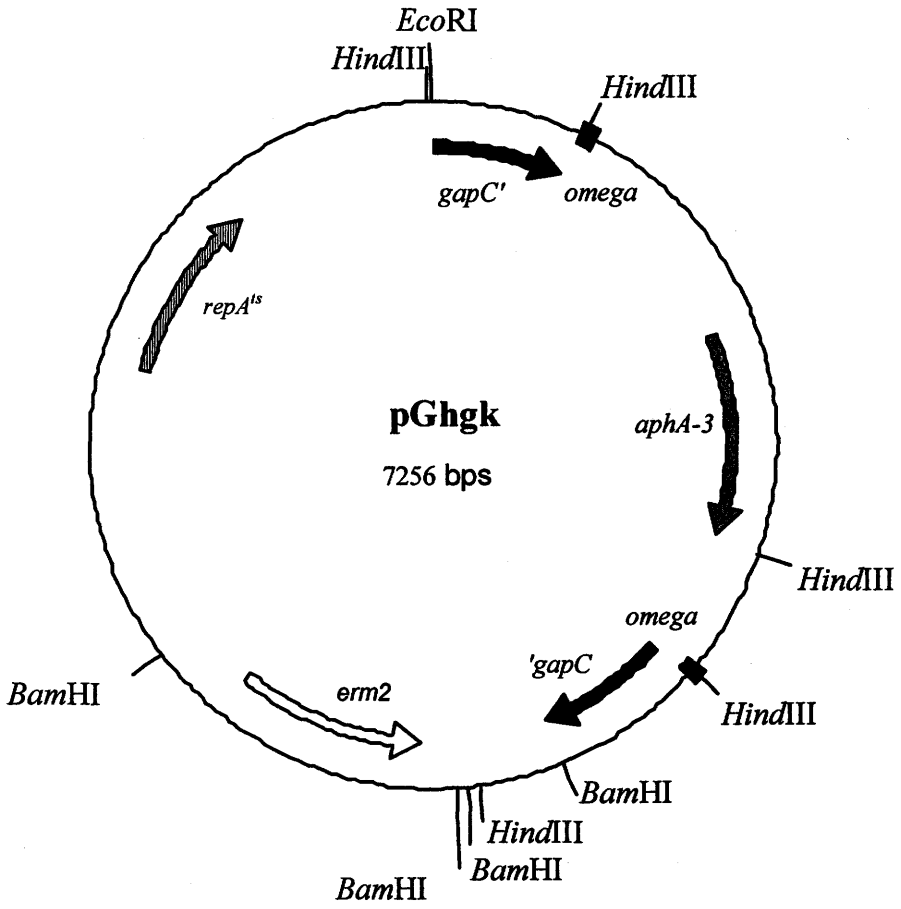


Fig. 4-1 Plasmid map of pGhgk. *Bam*HI and *Hind*III restriction sites are indicated. *erm2*: erythromycin resistance gene; *repA*^{ts}: gene encoding a temperature-sensitive RepA; *aphA3*: kanamycin resistance gene

purified plasmid DNA was used to transform *S. dysgalactiae*, followed by selection for colonies resistant to erythromycin at 30°C. Transformants were verified as *S. dysgalactiae* by the API 20 Strep diagnostic kit (BioMérieux, Quebec, Canada) and analyzed with the APILAB Plus software provided by the same supplier. To verify the integrity of pGhgk in *S. dysgalactiae*, plasmid DNA was isolated from *S. dysgalactiae* and transformed back into *E. coli* TG1(dev), followed by selection for colonies resistant to erythromycin. Plasmid DNA was then isolated and digested with *Bam*HI, and analyzed by agarose gel electrophoresis.

To isolate chromosomal integrants of the plasmid, one of the *S. dysgalactiae* isolates containing plasmid pGhgk was grown overnight in THYE medium at 30°C. Samples were diluted, plated on media containing kanamycin and incubated at 37°C. Kanamycin resistant colonies at 37°C were potential sco recombinants which had plasmids integrated into the chromosomes. In order to confirm that a sco event had occurred, colonies resistant to kanamycin at 37°C were screened for resistance to erythromycin at 37°C. The mutant *S. dysgalactiae* was verified by the API 20 Strep diagnostic kit and analyzed with the APILAB Plus software. The frequency of integrations per cell (ipc) was estimated as the ratio of the number of Km^r cells at 37°C to the number of viable cells at 30°C. The integrants isolated at 37°C were routinely maintained at 37°C in THYE medium containing erythromycin.

To excise the integrated plasmid from the *S. dysgalactiae* chromosome and obtain a dco recombinant (Fig. 4-2), two approaches were tried. Since GapC functions as GAPDH in the glycolysis pathway, we supplied a downstream product of the pathway, phosphoenolpyruvate (PEP), and used an alternate carbon source, succinate, to

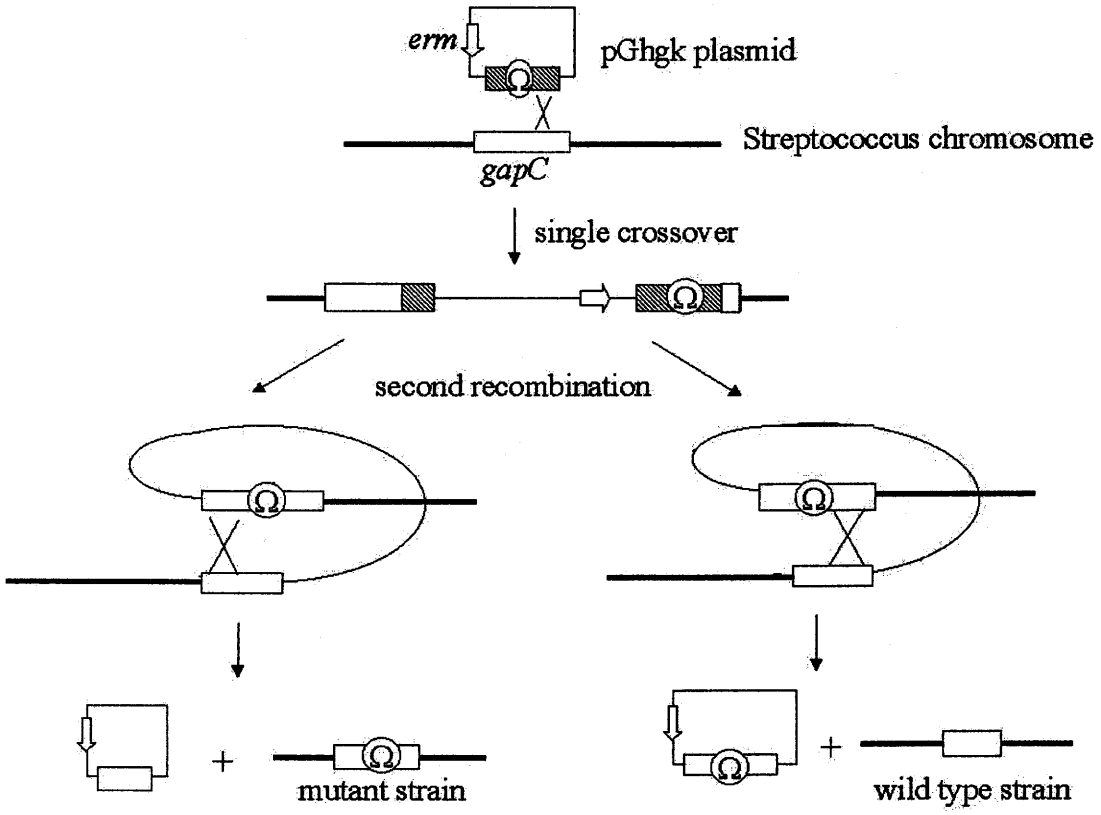


Fig. 4-2 Diagram of gene replacement method. The initial recombination event occurs between homologous sequences of the plasmid (*gapC* gene in pGhgk vector) and the chromosome of *S. dysgalactiae* and results in the formation of a cointegrate. Cointegrates are identified by plating transformed cells at 37°C (the nonpermissive temperature for plasmid DNA replication) onto medium that selects for the plasmid-encoded antibiotic resistance gene (*erm*). After growth at 30°C (the permissive temperature for plasmid DNA replication), the cointegrates undergo a second recombinantion event, regenerating plasmid in the cell. If the marker on the incoming plasmid (Ω Km^r; Ω) remains in the chromosome, the mutant strain will be obtained.

generate energy for bacteria. We added 10 mM PEP to media used in growing sco bacteria at 30°C, since the direct product of GAPDH in the glycolysis pathway, 1,3-bisphosphoglycerate, was not commercially available. PEP is a downstream intermediate in the glycolysis pathway and is also important in glucose transport into the cells via the phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) (Thompson, 1978). Bacteria were subcultured for 6 days at 30°C. Colonies were screened for dco recombinants on THYE agar plates containing PEP and kanamycin or erythromycin at 37°C. We also attempted to enrich for dco recombinants by using a modified Tris-minimal succinate medium. A Gap$^-$ mutant strain of *S. aureus* was successfully constructed by insertional inactivation using bacteria grown in modified Tris-minimal succinate medium (Sebulsky et al., 2000; Taylor and Heinrichs, 2002). The strategy was to use minimal media supplemented with succinate as a carbon source, an important intermediate in the Krebs cycle. Therefore, though bacteria did not have GAPDH, they were able to generate energy by succinate catabolism and thus were viable (Taylor and Heinrichs, 2002). The growth media-specific phenotype was also reported in other GAPDH mutants, specifically *E. coli* (Hillman and Fraenkel, 1975; Irani and Maitra, 1974) and *Salmonella typhimurium* (Fraenkel and Horecker, 1964).

4.2.5 Effect of the GAPDH inhibitor iodoacetate on bacterial GapC

To study the effect of iodoacetate (IAA) on bacterial GapC protein, stationary-phase *S. dysgalactiae* were diluted 1:50 and grown in THYE at 37°C with CO_2 until the A_{600} was about 0.45-0.55. Bacteria were harvested, washed once with 0.1 M PBS, pH 7.2 and resuspended in supplemented DMEM without antibiotic at a density of 10^7

cfu/mL. One milliliter of bacteria suspension was incubated with 0.25-2.5 mM IAA at 37°C with CO_2 for ½ hour. After incubation, cells were diluted and plated on THYE-agar and viable counts were determined. The experiment was performed once.

4.3 Results

4.3.1 Allele exchange mutagenesis

During the generation of sco recombinants, the number of Km^r colonies at 37°C (non-permissive temperature for the vector) was $1.3x10^4$ cfu/mL whereas the viable count at 30°C (permissive) was $1x10^9$ cfu/mL. Therefore, ipc was estimated as $1.3x10^{-5}$. The selected clones showed integration of plasmids downstream of the wild type *gapC* as verified by Southern blot analysis (Fig. 4-3, 4-4).

Southern hybridization analysis was also performed on sco chromosomal DNA to examine whether the genome contained a single or multiple copies of the *gapC* gene. DNA hybridization analysis of chromosomal DNA using a *gapC* probe showed (i) 5.2- and 6.4-kb of *Eco*RI chromosome fragments of sco and 4-kb fragment of wild type bacteria (Fig.4-3B), (ii) 5.4- and 9.0-kb of *Sap*I chromosome fragments of sco and 6.4-kb fragment of wild type bacteria (Fig.4-3B), (iii) 4.6 and 4.8-kb of *Eco*RI-*Sap*I chromosome fragments of sco and 2.5-kb fragment of wild type bacteria (Fig.4-3B), (iv) 5.0- and 13.0-kb of *Aat*II-*Kpn*I chromosome fragments of sco and 9.6-kb fragment of wild type bacteria (Fig.4-4B), (v) 20.0-kb of *Stu*I chromosome fragment of sco and 13.5-kb fragment of wild type bacteria (Fig.4-4B). DNA hybridization analysis of chromosomal DNA using the Ω Km^r cassette probe showed 5.2 - kb of *Eco*RI

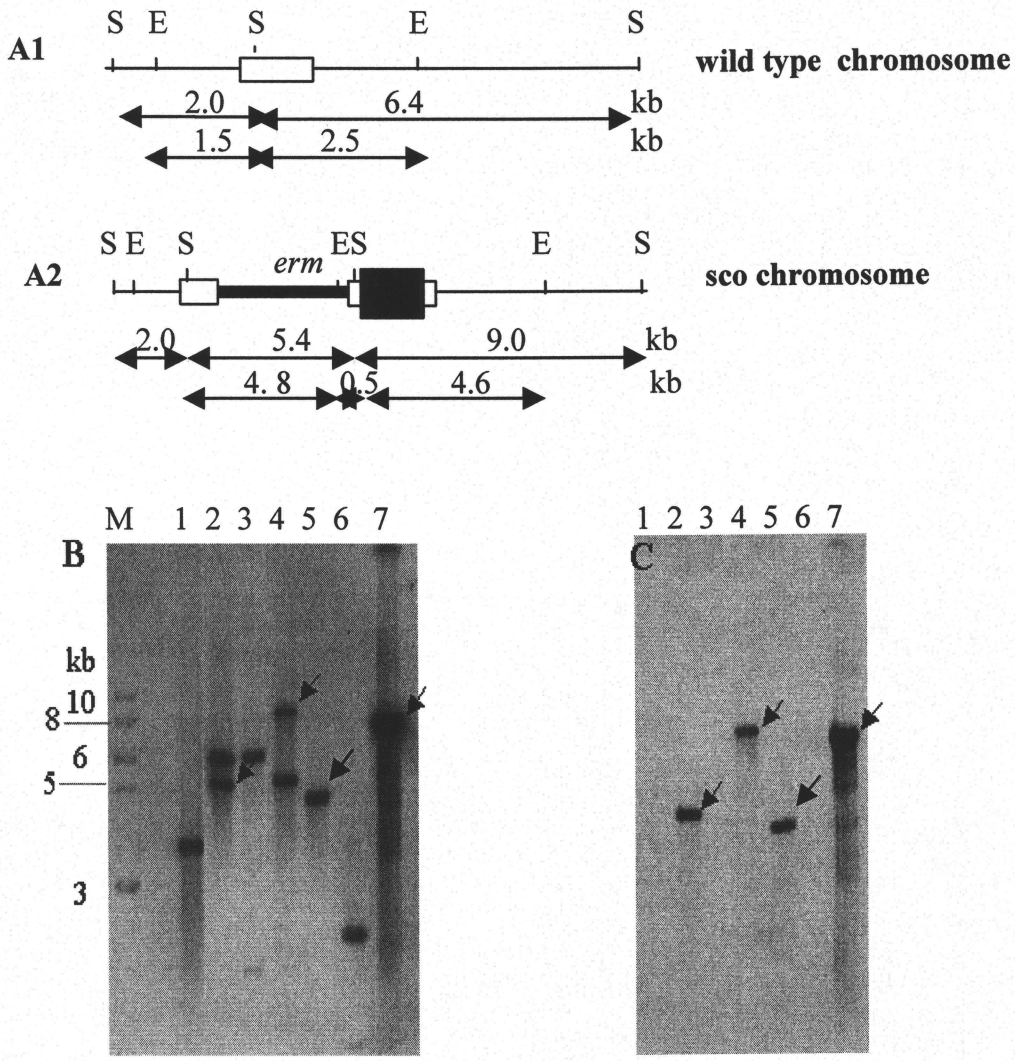


Fig. 4-3 Restriction maps and Southern blot analysis of sco and wild type *S. dysgalactiae* chromosomal DNA digested with *Eco*RI, *Sap*I, and *Eco*RI-*Sap*I. (A1,A2) Restriction maps of the *Eco*RI, *Sap*I, and *Eco*RI-*Sap*I chromosome fragments of sco and wild type *S. dysgalactiae*. Restriction enzymes: E, *Eco*RI; S, *Sap*I. The white box represents *gapC* gene. The black box represents Ω Km^r cassette. The thin line represents streptococcal chromosome and the heavy black line represents integrated plasmid. (B and C) Southern blot analysis of sco and wild type *S. dysgalactiae*. (B) DIG-labeled *gapC* fragment was used as the probe. (C) DIG-labeled Ω Km^r cassette was used as the probe. DNA from wild type *S. dysgalactiae* (lanes 1, 3, and 6), sco mutant (lanes 2, 4, and 5) and pGhgk plasmid (lane 7) was digested with *Eco*RI (lanes 1, 2, and 7), *Sap*I (lanes 3 and 4), and *Eco*RI-*Sap*I (lanes 5 and 6). Size standards are shown on the left margin (lane M). Arrows show fragments of *gap*C interrupted with Ω Km^r cassette.

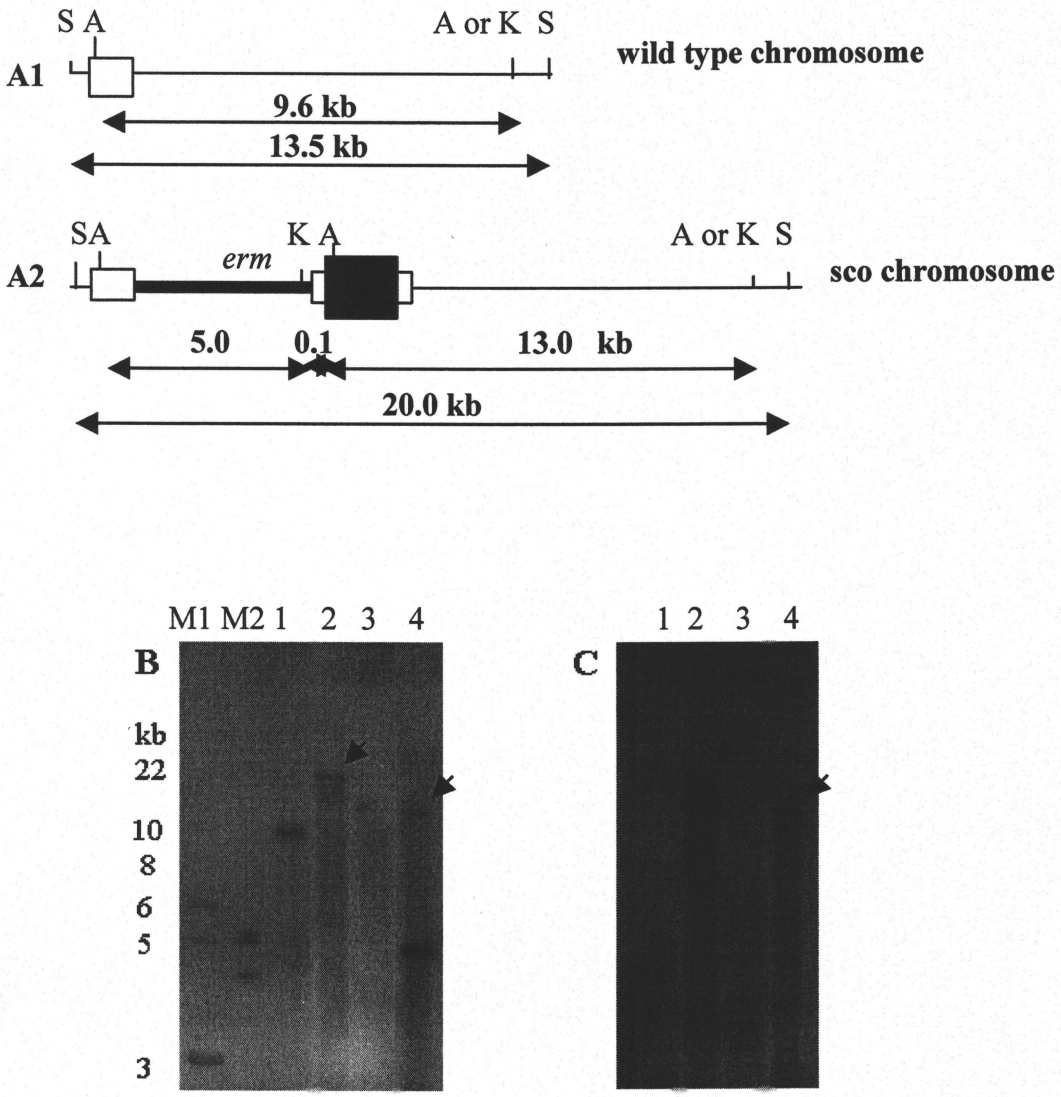


Fig. 4-4 Restriction maps and Southern blot analysis of sco and wild type *S. dysgalactiae* chromosomal DNAs digested with *Aat*II-*Kpn*I, and *Stu*I. (A1,A2) Restriction maps of the *Aat*II-*Kpn*I, and *Stu*I chromosome fragments of sco and wild type *S. dysgalactiae*. Restriction enzymes: A, *Aat*II; K, *Kpn*I; S, *Stu*I. The white box represents *gapC* gene. The black box represents Ω Km^r cassette. The thin line represents streptococcal chromosome and the heavy black line represents integrated plamid. (B and C) Southern blot analysis of sco and wild type *S. dysgalactiae*. (B) DIG-labeled *gapC* fragment was used as the probe. (C) DIG-labeled Ω Km^r cassette was used as the probe. DNA from wild type *S. dysgalactiae* (lanes 1 and 3) and sco mutant (lanes 2 and 4) was digested with *Aat*II-*Kpn*I (lanes 1 and 4) and *Stu*I (lanes 2 and 3). Size standards are shown on the left margin (lanes M1 and M2). Arrows show fragments of *gap*C interrupted with Ω Km^r cassette.

chromosome fragment of sco bacteria (Fig.4-3C), 9.0-kb of *Sap*I chromosome fragment of sco bacteria (Fig.4-3C), 4.6-kb of *Eco*RI-*Sap*I chromosome fragment of sco bacteria (Fig.4-3C), 13.0-kb of *Aat*II-*Kpn*I chromosome fragment of sco bacteria (Fig.4-4C), and 20-kb of *Stu*I chromosome fragment of sco bacteria (Fig.4-4C). The results verified that a single copy of *gapC* was present in *S. dysgalactiae* as in GAS (Lottenberg et al.,1992; Winram and Lottenberg, 1996) and that the plasmid had integrated directly downstream of the *gapC* gene.

In order to obtain a dco recombinant, we used PEP, a downstream intermediate in the glycolysis pathway, as a supplement. Bacteria were subcultured for 6 days and colonies were screened at 37°C for Erm^s and Km^r dco recombinants. One thousand colonies were screened for dco in each condition. However, no dco recombinants were isolated. The frequency of a dco event was estimated to be lower than 1/1000. The numbers of viable wild type cells and sco mutants were $3.3x10^8$ and $1.5x10^8$ cfu/mL, respectively, when PEP was added to the cultures. Therefore, there were no significant differences when PEP was added to the cultures. PEP, used at 10 mM, might not be enough to support the growth of bacteria. If *gapC* was an essential gene and present as a single copy on the chromosome, then it might not be possible to isolate a knockout mutant by allele replacement.

We also tried to use minimal medium which was successfully used to produce a Gap^- mutant strain of *S. aureus*. However, *S. aureus* has two copies of *gap* gene and only one was knocked out by allele replacement (Taylor and Heinrichs, 2002). We first determined if *Streptococcus dysgalactiae* could grow in minimal medium using

succinate as a carbon source for growth of cells. However, *Streptococcus dysgalactiae* could not grow in the modified minimal medium, using succinate as a carbon source.

4.3.2 Effect of GAPDH inhibitor iodoacetate on bacterial GapC

Since one of the GapC functions is as GAPDH in glycolysis pathway, IAA which is a specific GAPDH inhibitor, was chosen to study the role of GapC in bacterial viability. The number of bacteria treated with 0.25 mM IAA, which is a specific concentration to inhibit GAPDH activity (Campbell-Burk et al., 1987), at 37°C for 30 minutes was decreased compared to the control (Table 4-2), suggesting IAA killed bacteria and *gapC* probably played an essential role in bacterial viability. As the experiment was performed once, this is a preliminary data.

4.4 Discussion

A direct method for examining the role of protein as a virulence factor in bacteria would be to generate an isogenic mutant lacking an active copy of the gene. In our laboratory, a *mig* mutant strain was constructed by allele replacement mutagenesis of *S. dysgalactiae* and it was found that the mutant strain was significantly less resistant to phagocytosis and killing by bovine neutrophils than the wild type strain, suggesting that the Mig protein of *S. dysgalactiae* plays a role in virulence of the bacteria by preventing phagocytosis by bovine neutrophils (Song et al., 2001). In this study, I attempted to insertionally inactivate the *gapC* gene with a DNA cassette containing a Km^r marker and it was possible to isolate sco recombinants in which the plasmid had

Table 4-2 Number of viable bacteria after treatment with various concentrations of iodoacetate (IAA)

Sample	No. of viable bacteria ($x10^6$cfu/mL)*
Bacteria alone	113.33
Bacteria + 0.25 mM IAA	19.33
Bacteria + 2.5 mM IAA	2.78

* This experiment was performed once.

integrated directly downstream of the *gapC* gene. It was not possible to isolate either the recombinant of a dco or a sco mutants of which Ω Km cassette integrated upstream of *gapC* gene. Therefore, this suggested that the upstream integration might interrupt the normal expression of this gene and this gene may be essential for viability in strain ATCC 43078, as is the cases in *S. pyogenes* (Winram and Lottenberg et al., 1998) and *S. equisimilis* (Gase et al., 1996). The sco isolation indicated that *Streptococcus dysgalactiae* does contain a functional recombination system with the frequency of integration of $1.3x10^{-5}$. In order to obtain dco, the number of colonies to screen might have to be equal or higher than 10^5.

Therefore, alternative strategies to generate an conditional mutant might be tried such as mutating nucleotide bases encoding amino acid residues that mediate protein binding to host cells but are not essential for GAPDH activity. From the work of Winram and Lottenberg (1998), they could generate an altered *plr* gene encoding a plasmin-binding deficient, but glycolytically active, Plr protein by directed mutagenesis of the recombinant *plr* gene. They subsequently identified the unique amino acid residues that mediate plasmin binding but are not essential for GAPDH activity.

Another possible strategy to generate an isogenic *gapC* mutant would be to transform *S. dysgalactiae* with a plasmid containing *E. coli gapA*. The *gapA* gene is considered to be the only active GAPDH-encoding gene in *E. coli* (Branlant et al., 1983; Della Seta et al., 1997; Hillman and Fraenkel, 1975) and the gene product is not translocated to the cell surface. It is possible that *E. coli* gapA would function as GAPDH complementary to *S. dysgalactiae* GapC and make it possible to make a viable mutant of *S. dysgalactiae* that does not have GapC on the cell surface. Using the gene

replacement procedure, Brown et al. (1995) have shown that an essential gene, *mur*A, can be replaced on the *E. coli* chromosome with a deletion allele, as long as the deletion is complemented by another copy of the essential gene.

An alternative approach to demonstrate that *gapC* might be an essential gene was to use the GAPDH specific inhibitor, IAA. The inhibition mechanism of GAPDH from rabbit muscle by IAA was characterized by Amelunxen and Carr (1975) and more recently by Nagradova et al. (1996). It consists of a covalent binding of IAA to the active site cysteine, preventing the enzyme/NAD^+ complex formation and the charge transfer between cysteine and NAD^+ which occurs during the dehydrogenation of GAP. The concentration of IAA that produces a significant yet selective inhibition of GAPDH was determined by conducting ^{31}P nuclear magnetic resonance (NMR) and O_2 consumption measurements on yeast cell suspensions treated with IAA at concentrations ranging from 0.05 to 1 mM. At concentrations less than 0.5 mM, IAA did not significantly affect the activities of alcohol dehydrogenase (ADHE), acetate kinase (AK), lactate dehydrogenase (LDH), pyruvate formate lyase (PFL), and pyruvate kinase (PK). Therefore the effect of IAA can be considered to be specific for GAPDH at concentrations below 0.5 mM (Webb, 1966; Campbell-Burk et al., 1987). The number of viable bacteria remaining after treatment with IAA was decreased compared to the control (Table 4-2) and since the concentration of IAA used was reported to specifically inhibit GAPDH, this supports the hypothesis that GAPDH might be an essential gene for *S. dysgalactiae*.

Another strategy to study the effect of GapC on bacterial infection was adopted. Bacterial adherence/penetration on/into MAC-T cells were observed in the presence of GapC-specific polyclonal antibody, which is described in detail in chapter 5.

4.5 Conclusion

In this study, a single-crossover recombinant containing both wild type and altered *gapC* alleles, which occurred downstream of wild type *gapC*, was isolated while a double-crossover mutant could not be isolated at frequencies of less than 1 in 1000. Also iodoacetate had a significantly impact on viability of *S. dysgalactiae.* All these evidence suggest the *gapC* is an essential gene.

5.0 THE EFFECT OF GapC ON *S. dysgalactiae* ADHERENCE / PENETRATION *in vitro*

5.1 Introduction

Bacterial adherence to the surfaces of epithelial cells in the mammary gland and entry into mammary epithelial cells may be important virulence mechanisms of pathogens that cause mastitis. Invasion of bacteria into epithelial cells could result in protection from both host defense mechanisms and the action of antimicrobial agents and could also affect the secretory function of mammary cells (Calvinho and Oliver, 1998).

Streptococcus dysgalactiae frequently causes mastitis in dairy cows. However, little is known about both bacterial and host factors that contribute to the establishment and persistence of intramammary infection caused by this organism. The bacterium used in this study was *S. dysgalactiae* which adheres to epithelial cells from the bovine mammary gland and to extracellular matrix proteins *in vitro* (Calvinho et al., 1996; Filippsen et al., 1990; Frost et al., 1977; Mamo et al., 1987). The bacterium also invades MAC-T cells (Almeida and Oliver, 1995), a clonal cell line produced from primary bovine mammary alveolar cells by stable transfection with SV-40 large T-antigen. MAC-T cells show a population doubling time of approximately 17 hours and can be cultured for more than 350 passages without showing any sign of senescence (Huynh et

al., 1991). The clonal nature of the cells, their immortality, and their ability to uniformly differentiate and secrete casein proteins make this cell line unique (Huynh et al., 1991). Coculture of MAC-T cells with *S. dysgalactiae* did not appear to affect cell viability (Almeida and Oliver, 1995). However, lactate dehydrogenase increased in coculture supernatants, suggesting that some cellular damage was induced following bacterial invasion (Almeida and Oliver, 1995).

To investigate the effect of GapC on *S. dysgalactiae* adherence/penetration of bovine mammary epithelial cells, the GapC-specific polyclonal antibody was incubated with *S. dysgalactiae* on MAC-T cells. The viable counts of bacteria after adherence/penetration on/into MAC-T cell were measured.

5.2 Materials and Methods

5.2.1 MAC-T cell culture

MAC-T cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 25mM HEPES, 0.37% $NaHCO_3$, 10% heat-inactivated fetal bovine serum (Invitrogen), 5 μg/mL insulin (Sigma), 1 μg/mL hydrocortisone (Sigma), and 50 U/mL gentamicin (Invitrogen). Cells were subcultured twice per week at $1x10^5$ cells/mL in 75 cm^2- culture flasks. Cells were seeded to 24-well culture plates ($1x10^5$ cells/well) (Costar) and incubated overnight at 37°C under 5% CO_2 in DMEM plus supplements before adding bacteria.

The method used to assess the viability of MAC-T cells was a trypan blue exclusion method. Briefly, 100 μl of 0.1% trypan blue (Invitrogen) was added to 100 μl

of cell suspension, followed by examination with a hemocytometer under an inverted microscope. Cells which excluded the dye were considered viable.

5.2.2 Estimation of GapC number on the *S. dysgalactiae* cell surface

5.2.2.1 Purification of native GapC protein

GapC was purified from *S. dysgalactiae* using a sonication protocol modified from the QIAexpressionist (QIAGEN). Two hundred and fifty milliliters of *S. dysgalactiae* at 2.21×10^8 cells/mL were pelleted at 3500 rpm for 30 minutes, resuspended in 4 mL of 50 mM Tris-Cl pH 8, and sonicated on ice at 5-second pulses for 3 minutes, using a sonicator equipped with a microtip. Cell debris was pelleted at 12000 rpm for 30 minutes, and washed 3 times with 25 mL of 0.1 M phosphate buffer saline (PBS), pH 7.2 at 7000 rpm for 10 minutes each. The pellet was resuspended in 0.5 mL of PBS, and incubated with 170 units/g wet weight mutanolysin for 2 hours at 37°C and GapC was purified with a HiTrap Blue column (Amersham Pharmacia Biotech) according to the manufacturer's protocols.

5.2.2.2 Determination of specific GapC activity

The purified native GapC preparations and total membrane extracts were tested for GAPDH activity according to the method originally described by Winram and Lottenberg (1998) with some modifications. Diluted sample protein in 5 μL volumes were added to 95 μL of reaction buffer and substrates (40 mM triethanolamine, 50 mM Na_2HPO_4, 5mM EDTA, 1mM β-NAD, and 2 mM DL-glyceraldehyde 3-phosphate). Negative control assays were performed as above but without the addition of DL-glyceraldehyde 3-phosphate. The reduction of NAD^+ to NADH was monitored

spectrophotometrically at 37°C at 340 nm; absorbances were recorded at 20 s intervals for 15 minutes using an ELISA plate reader. NADH standards at known concentrations were included to set a standard curve. The absorbance values of samples were converted to μmol NADH by comparison with the standard curve. One unit activity in this study was defined as the ability of GAPDH to produce 1 μmol NADH per minute at 37°C under the conditions used. Protein concentrations were determined using a D_C protein assay (BIO-RAD) to calculate specific activities expressed as unit per mg protein.

5.2.3 Purification of the recombinant GapC protein and determination of specific GapC activity

Stationary-phase *E. coli* strain BL21(DE3) carrying the pETgk plasmid was diluted 50-fold and grown in LB broth containing 50 μg/mL carbenicillin until the A_{600} = 0.6. The expression of the recombinant *gapC* gene was induced by adding 1mM IPTG. After 4 hours, cells were pelleted, sonicated, and purified by nickel-chelate affinity chromatography under native conditions with a 50%Ni-NTA slurry (QIAGEN, Hilden, Germany) according to the manufacturer's protocols (QIAGEN). The purified GapC preparations were tested for GAPDH activity as described above in 5.2.2. Protein concentrations were determined using a D_C protein assay (BIO-RAD) to calculate specific activities expressed as unit per mg protein.

5.2.4 Preparation of the *S. dysgalactiae* GapC-specific polyclonal antibody

An NHS (N-hydroxysuccinimide)-activated Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) column was conjugated with recombinant GapC according to the

manufacturer's instructions (Amersham Pharmacia Biotech) and packed by gravity. The column volume was 4 mL. The column was equilibrated with 500 mL of wash/binding buffer (0.15 M PBS containing 5 mM EDTA, pH 7.2). Thirty seven point five milliliters of AS00-143 bovine serum from cattle immunized with recombinant His-GapC protein was circulated through column. The column was washed with 500 mL of wash/binding buffer to remove unbound protein. Finally, the GapC-specific polyclonal antibody was eluted with 50 mL of 0.2 M glycine pH 2.4-2.8 for 4-7 hours. The pH of eluent was immediately adjusted to be 7.5 with 1 M Tris-Cl pH 7.5 using a PD-10 desalting column (Amersham Pharmacia Biotech). The column was re-equilibrated with 500mL of wash/binding buffer and pH was monitored to a pH of 7.2. The flowthrough serum was recirculated through the column one more time and treated as previously described.

5.2.5 Effect of the GapC-specific polyclonal antibody on bacterial adherence/penetration on/into MAC-T cells.

Streptococcus dysgalactiae culture of mid-log phase, A_{600} of 0.45-0.55, was harvested, washed once with 0.1 M PBS , pH 7.2 and resuspended in supplemented DMEM without antibiotics at a density of 10^4 cfu/mL. The MAC-T cell monolayers were washed 3 times with supplemented DMEM without antibiotics. One milliliter of bacteria was incubated with 10 μg of the GapC-specific polyclonal antibody or 10 μg of bovine IgG antibody at 4°C for 1 hour before being added to prewashed MAC-T cells [multiplicity of infection (moi), 0.1]. After incubation at 37°C with 5%CO_2 for ½ hour, cells were washed and detached from the plates by treatment for 5 minutes at 37°C with 0.25% trypsin solution and then lysed by adding 0.5% saponin. Cell lysates were

serially diluted 10-fold and plated on THYE agar to quantify adhering/penetrating streptococcus. The percentage of bacterial adherence/penetration on/into MAC-T cells, compared to the total number of bacteria in the inoculum, was calculated. The experiment was performed once in triplicate.

5.3 Results

5.3.1 Estimation of GapC number on the *S. dysgalactiae* cell surface

The number of GapC molecules on bacterial cell surface could be estimated from the specific GAPDH activity of purified native GapC, compared to that of GapC in the total membrane extract. The purified native GapC, which appeared as a single band on SDS-PAGE (Fig. 5-1A) and was recognized by bovine anti-GapC antibody on Western blot analysis (Fig. 5-1B), was determined to be 10.53 units/mg of specific activity.

Due to the fact that 5.52×10^{10} cells of *S. dysgalactiae* contained a total GAPDH activity of 4202 units, as determined from the total membrane extract, each cell thus contained 7.61×10^{-8} units of GAPDH activity, which equals to 7.23×10^{-9} mg of purified GapC. Since the molecular weight of GapC is 35.73 kDa, considered as a monomer, it could be estimated that there are 2.02×10^{-16} mol or 1.22×10^{8} molecules of GapC monomer presented on the surface of each bacterium.

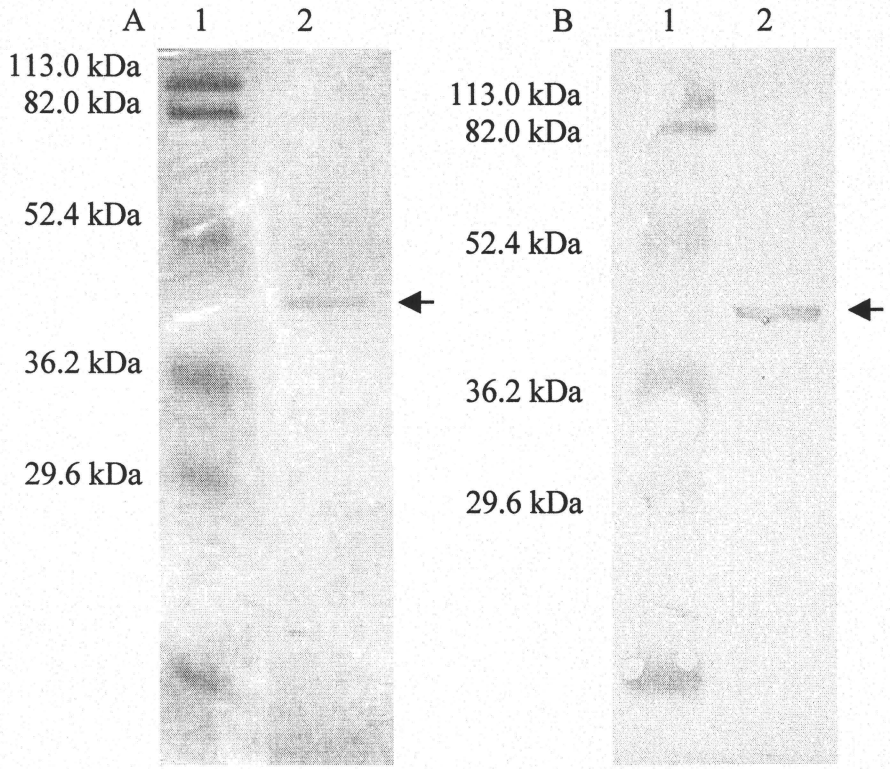


Fig. 5-1 Purification of *S. dysgalactiae* cell surface GapC. Purification was done as described, and was monitored by SDS-PAGE on a 12% gel (A) and Western blot analyzed with bovine anti-*Streptococcus dysgalactiae* GapC polyclonal antibody (B). Lane 1, a low range SDS-PAGE molecular weight marker; lane 2, native GapC protein

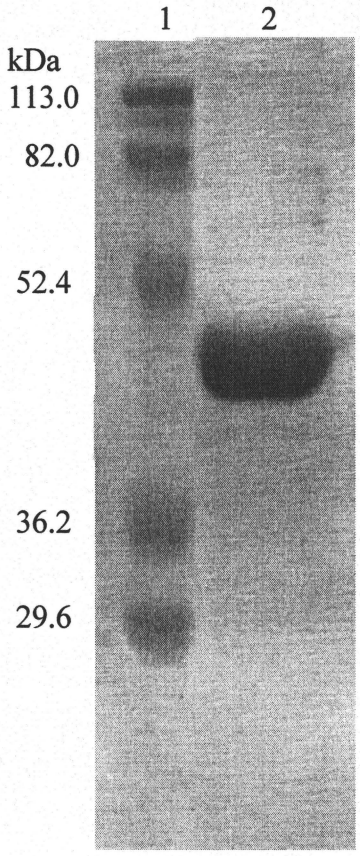
5.3.2 The effect of the GapC-specific polyclonal antibody on bacterial adherence/penetration on/into MAC-T cells.

The GapC-specific polyclonal antibody was an alternative method to demonstrate a role of GapC in bacterial infection into MAC-T cells. The antibody was purified by an affinity column against purified His-Gap protein. The purity of His-Gap protein was monitored by SDS-PAGE on a 12% gel (Fig. 5-2). The specific GAPDH activities of recombinant His-Gap obtained were 58.5 units/mg.

The GapC-specific polyclonal antibody at a concentration of 10 μg/mL did not directly affect bacterial adherence/penetration on/into MAC-T cells (Table 5-1). As the experiment was performed once in triplicate, this is a preliminary data. The concentration of purified antibody used, which was equal to 6.58×10^{-11} mol or 3.96×10^{13} molecules, were considered to be in excess of the number of bacterial cell surface GapC in the system, 2.02×10^{-12} mol.

5.4 Discussion

Since the GapC$^-$ mutant isolation was unsuccessful, antiserum which is specific to GapC was used to investigate the specific role of GapC in bacterial infection of epithelial cells. In this study, the number of GapC on the bacterial cell surface was estimated in order to verify that the antibody used was in excess of the number of cell surface GapC. Based on the assumption that all the cells were broken, the number of cell surface GapC was approximately 2.02×10^{-16} mol per bacterial cell which is comparable to the number of SDH, GapC related protein, observed for *S. pyogenes*



Fig, 5-2 Purification of recombinant His-GapC. Purification was done as described, and was monitored by SDS-PAGE on a 12% gel. Lane 1, a low range SDS-PAGE molecular weight marker; lane 2, purified recombinant His-GapC

Table 5-1 Percentage of bacterial adherence/penetration on/into MAC-T cells after treatment with the GapC-specific polyclonal antibody and bovine IgG at 37°C for 30 minutes

Sample	% of bacterial adherence/penetration (means +/- SD)
Bacteria + GapC-specific polyclonal antibody*	53.81+/-1.82
Bacteria + bovine IgG (Sigma)	51.07+/-4.19
Bacteria	51.24+/- 2.53

* Although I did not test the ability of the purified GapC-specific polyclonal antibody to bind to the bacterium, the antibody did react with GapC as determined by Western blotting (unpublished data).

($1.83x10^{-17}$ mol per bacterial cell; Pancholi and Fischetti, 1992 and Vijaykumar Pancholi, personal communication). As a viable count was not done before and after sonication, it is a possibility that not all cells were broken. Therefore, the reported value may even be an overestimate but none-the-less as excess of antibody was used.

An excess amount of the antibody was used in the experiment shown by the relative number of the antibody ($6.58x10^{-11}$ mol) to that of bacterial GapC ($2.02x10^{-12}$ mol). The native GapC of bacteria was extracted and its integrity was verified by SDS-PAGE (Fig.5-1A) and its ability to bind to the bovine anti-GapC antibody (Fig.5-1B). However, treatment of *S. dysgalactiae* with the GapC–specific polyclonal antibody or bovine immunoglobulin G did not affect uptake of this organism by MAC-T cells (Table 5-1). This suggested that (i) GapC might play an important role in adherence/penetration of bacteria into MAC-T cells and the antibody might be able to bind GapC but did not block these activities. The antibody used may not have recognized the region involved in adherence/penetration on/into *S. dysgalactiae*, or (ii) GapC might play a role in adherence/penetration, but the antibody might not be able to access GapC, or (iii) GapC did not directly play a role in adherence/penetration of *S. dysgalactiae* on/into MAC-T cells.

5.5 Conclusion

The GapC-specific polyclonal antibody, purified from cattle immunized with recombinant GapC, in excess quantity could not directly inhibit *S. dysgalactiae* adherence/penetration on/into MAC-T cells.

6.0 STUDY OF HOST RESPONSE TO *S. dysgalactiae* INFECTION BY BOVINE MICROARRAYS

6.1 Introduction

The response of host cells to infection can be monitored using two main groups of techniques. The first group targets known genes (e.g. quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (Ichikawa et al., 2000) or Northern blotting (Alwine et al., 1977)), which allows the study of one or a few genes at a time, and the other group targets genes with differential regulation that have unknown sequence and identity (e.g. differential display (Liang and Pardee, 1992), subtractive hybridization (Kuang et al., 1998) or representational difference analysis (Lisitsyn and Wigler, 1993)). However, these techniques are limited by the amount of data obtained from one experiment and are time consuming to perform. Recently, genomic tools to analyze gene expression on a global basis have been developed, including the use of macro- and microarrays (Cohen et al., 2000; Schena et al., 1995; Cummings and Relman, 2000; Staudt and Brown, 2000; Yowe et al., 2001).

DNA microarrays consist of the ordered placement of individual cDNA fragments or oligonucleotides onto a solid support. Labeled probe(s) (radioactive, luminescent, or fluorescent) are hybridized against the microarray and the number of spots on the array, usually consisting of cDNA, which generate a signal are quantified (Duggan et al., 1999).

The CattleArray3800 is the first commercial microarray for food animal species (Pyxis Genomics, Chicago, Illinois). The CattleArray3800 contains 3820 selected cDNA clones printed in duplicate on a single glass slide. The array elements were selected from over 7000 cDNA clones derived from expressed sequence tags (ESTs) of bovine spleen and normalized placenta libraries. Three thousand and seventy two placenta ESTs and 748 spleen ESTs representing distinct genes were chosen. The ESTs representing distinct genes were determined via sequence similarity searches against human UniGene sequences using an E value of $e10^{-5}$ as a threshold. The CattleArray3800 also contains spots for positive cattle controls: beta actin (ACTB); phosphoglycerate kinase 1; (PGK1); hypoxanthine phosphoribosyltransferase (HPRT) and beta-2-microglobulin (β2M). Two exogenous plant genes, soy rubisco small chain 1 (RBS1) and chlorophyll a/b binding protein (CAB) can be used as negative or spiking controls (Pyxis Genomics, Chicago, Illinois). Thus, this microarray offers an alternative tool to study bovine gene expression.

In this study, bovine microarrays were used to study the effects of *S. dysgalactiae* on MAC-T cells in order to investigate the mechanisms involved in early host-pathogen interactions.

6.2 Materials and Methods

6.2.1 MAC-T gene expression following exposure to *S. dysgalactiae*

The methods to infect MAC-T cells were similar to those described in chapter 5 with the following changes. Two hundreds microliters of approximately $1x10^7$ cfu *S.*

dysgalactiae were added to prewashed MAC-T cells (moi, 100). After incubation for 1 hour at 37°C with 5%CO_2, the monolayers were then washed 3 times with Hybri-Max® SFPF (serum-free and protein-free) hybridoma media (Sigma) to remove non-adhered bacteria. The cells were continuously incubated in 1 mL of HybriMax SFPF media containing 100 μg/mL of gentamicin for 4 hours at 37°C with 5%CO_2. SFPF media was chosen to reduce the chance that bacteria would leave cells and multiply in the media (supernatant) again. Furthermore, when using SFPF media, gentamicin was used at a concentration that would kill any remaining extracellular bacteria. The monolayers were trypsinized and 1 mL of cells was taken for viable counts and 100 μL of cells was taken to quantitate host cells by trypan blue exclusion. Total RNA was extracted from the remaining cells using an RNeasy Mini kit (QIAGEN). RNA samples were treated with the RNase-free DNase Set (QIAGEN) and cleaned up with the RNeasy Mini kit according to the manufacturer's instructions. The concentration of RNA was determined by absorbance measurement at 260 and 280 nm(A_{260} and A_{280}) (Sambrook et al., 1989). RNA samples were subjected to electrophoresis through 1% formaldehyde-agarose (FA) gel at 5-7 V/cm to show integrity of RNA.

Purified total RNA samples from MAC-T cells and MAC-T cells infected with *S. dysgalactiae* were aminoallyl labeled for microarray studies according to the methods from The Institute For Genomic Research (TIGR) and the Ontario Cancer Institute (OCI) with some modifications. Two micrograms of oligo (dT) (Life Technologies) was added to 15 μg of each purified total RNA and the final volume was brought up to 12.9 μL. The mixtures were incubated at 70°C for 10 minutes and chilled on ice for 1 minute. The RNA reaction mixtures contained 1x First Strand buffer, 0.01

M DTT, 0.5 mM of each dATP, dCTP, and dGTP, 0.3 mM of dTTP (Amersham Pharmacia Biotech), 0.2 mM aadUTP (Sigma), 20 U Superase In (Ambion). The mixtures were incubated at 42°C for 2 minutes and 400 U SuperScript II (Life Technologies) was added into each tube. The mixtures of thirty microliters were incubated at 42°C for 3 hours. RNA was hydrolysed with 10 μL of 0.5M EDTA and 10 μL of 1M NaOH and heated at 65°C for 15 minutes. Ten microliters of 1 M HCl was added to neutralize the pH. Unincorporated nucleotides were removed by the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions except that the wash step was done twice with phosphate wash buffer (5 mM KPO_4, pH 8.0 and 80% EtOH) and cDNA was eluted with 60 μL phosphate elution buffer (4mM KPO_4, pH 8.5). The concentration and purity of cDNAs were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer. The cDNAs were completely dried in a speed vac concentrator and resuspended in 4.5 μL freshly prepared 0.1M Na_2CO_3, pH 9.0.

cDNA samples from MAC-T cells and MAC-T cells infected with bacteria were labeled with Cy5-dUTP and Cy3-dUTP, respectively for 1 hour. Probes were purified with QIAquick PCR Purification Kit. The total volume of purified probes was 60 μL. The concentration, purity, and dye incorporation of probes were determined by measuring the absorbance at 260, 280, 320, and 550 nm for Cy3-labeled cDNA and at 260, 280, 320, and 650 nm for Cy5-labeled cDNA. Absorbance measurements could be used to analyze the labeling reaction. In general, the total picomoles of cDNA synthesized of each sample could be calculated using :

$$\text{pmol nucleotides} = \frac{[A_{260}\text{x vol } (\mu L) \text{ x } 37 \text{ ng}/\mu L \text{x } 1000 \text{ pg/ng}]}{324.5 \text{ pg/mol}}$$

* 1 A_{260} = 37 ng/μL for cDNA; 324.5 pg/pmol represents the average molecular weight of a dNTP

The total picomoles of dye incorporation (Cy3 and Cy5 accordingly) of each sample could be calculated using :

$$\text{pmol Cy3} = \frac{A_{550} \text{ x vol } (\mu L)}{0.15}$$

$$\text{pmol Cy5} = \frac{A_{650} \text{ x vol } (\mu L)}{0.25}$$

$$\text{nucleotides/dye ratio} = \frac{\text{pmol cDNA}}{\text{pmol Cy dye}}$$

The probes were mixed and 20 μg bovine Hybloc (Applied Genetics Laboratories) was added. Probes were completely dried in a speed vac concentrator in the dark and suspended in 10 μL ddH_2O. One μL of 20 μg/μL poly(A) DNA (Amersham Pharmacia Biotech) was added. Probes were heated at 95°C for 3 minutes and 90 μL DIG Easy Hyb solution (Roche Molecular Biochemicals, Germany) was added to each probe.

The CattleArray3800 (Pyxis Genomics), containing 3820 selected cDNA clones printed in duplicate on a single glass slide, was used in this study. The array label was removed and the array was placed in 95°C ddH_2O for 2 minutes, rinsed with 95% EtOH, spin dried at 500 rpm for 1 minute and incubated in pre-warmed prehybridization

buffer (DIG Easy Hyb solution) at 37°C for 45 minutes. The array was plunged 4-5 times in 5 separate beakers in ddH_2O, rinsed in isopropanol and spin dried at 500 rpm for 1 minute. The probe mixture was slowly loaded onto the array. A HyBriSlip (Schleicher&Schuell) was laid over the array, carefully avoiding air bubbles. Plain clean microscope slides were placed at every 2^{nd} or 3^{rd} position in the hybridization chamber to create a platform onto which the hybridization arrays were placed. The chamber was incubated at 37°C for 16-20 hours. The array was plunged 4-5 times in 1x SSC to allow the HyBriSlip to fall off and was then washed once in pre-warmed 1x SSC containing 0.1% SDS solution at 37°C for 10 minutes. The slide was plunged 4-5 times in 1x SSC and spin dried at 500 rpm for 10 minutes. The array was kept in the dark until scanning with an Affymetrix 428 Array Scanner.

The array is physically scanned to produce a digital record of the red and green fluorescence emissions at each point on the array. Each spot on the slide was located and identified automatically by the Jaguar 2.0 Software (Affymetrix). The Jaguar variable circle size algorithm was chosen to draw a circle to identify each spot in the array. The algorithm computes circle size (diameter) independently for each spot in the array which is useful when the spot sizes vary across the image. The foreground and background signal intensities were classified with the easy algorithm threshold in which signal threshold was the 75^{th} $percentile_{BG}$ + (1.5x IQR_{BG}) where the 75^{th} $percentile_{BG}$ was background pixel intensity at which 75% of the background pixels are dimmer and 25% of the background pixels are brighter, and IQR (interquartile range of the background) was 75^{th} $percentile_{BG}$ - 25^{th} $percentile_{BG}$. The easy algorithm threshold would ease the threshold used to detect the spots on the array.

After red and green foreground (*Rf* and *Gf*, respectively) and background intensities (*Rb* and *Gb*, respectively) for each spot were obtained, they were analyzed by B-statistic to select differentially expressed genes (Lonnstedt and Speed, 2002). The background-corrected intensities are *R* and *G* where usually $R = Rf - Rb$ and $G = Gf - Gb$. The log-differential expression ratio is $M = \log_2 R / G$ for each spot. The log-intensity of the spot is $A = \frac{1}{2}\log_2 RG$, a measure of the overall brightness of the spot. (The letter *M* is a mnemonic for *minus* as $M = \log R - \log G$ while *A* is amnemonic for *add* as $A = (\log R + \log G) / 2$. It is convenient to use base-2 logarithms for *M* and *A* so that *M* refers to units of 2-fold change and *A* is in units of 2-fold increase in brightness. On this scale, $M = 0$ represents equal expression, $M = 1$ represents a 2-fold change between the RNA samples, $M = 2$ represents a 4-fold change, and so on. Any negative values of R or G were excluded from any analysis on the logarithmic scale (Smyth et al., 2002).

The graphical display of data from a microarray slide was illustrated using an MA-plot of Dudoit et al. (2002) which has the M-values on the vertical axis and the intensity A-values on the horizontal axis. Subject to the parametric assumptions being valid for the data, values for the B-statistic greater than zero correspond to a greater than 50-50 chance that the gene in question was differentially expressed (DE). The B-statistic is equivalent for the purpose of ranking genes to the penalized t-statistic

$$t = \frac{\text{average M}}{((a + s^2)/n)^{1/2}}$$

where *t* is a B value, average *M* is the mean of the *M* values for any particular gene across replicate arrays, *n* is a series of replicate arrays on which samples A and B

have been hybridized and we wish to identify which genes are DE, s^2 is the sample variance of the M-values across the replicates for the gene, and the penalty a is estimated from the mean and standard deviation of the sample variance s^2 (Lonnstedt and Speed, 2002). The experiments were performed in triplicate.

6.2.2 Northern blots

cDNA was prepared from total RNA purified from MAC-T cells using the same conditions as for the microarray experiments (see above). The following primer pairs were designed to amplify portions of the indicated MAC-T cDNA samples to produce templates for probe synthesis: succinate dehydrogenase complex, subunit A, flavoprotein, 5'-GCTGCAGGAAGGCTGTGAGAA-3', 5'-GCTTCCTCCAGTGCTGC TCAA-3'; myosin, light polypeptide kinase, 5'-GAGGCTGTCGCTGAGGAGAA-3', 5'-TCATCCCCGCAGACATCACTAA-3'; ORFs, 5'-GACGGCTCTGGAGAGTCTC A-3', 5'-TGGCTAATGGCTCATCCACATC-3'; GAPDH, 5'-CGGCACAGTCAAGG CAGAGAA - 3', 5' - GGCGTGGACAGTGGTCATAA - 3'. PCR products were sequenced using the above primers and labeled with DIG with the PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals). Northern blots were performed with samples separated on 1% formaldehyde agarose (FA) gels essentially as described in the RNeasy Kit (QIAGEN). Fifteen micrograms of total RNAs from infected and non-infected MAC-T cells were separated by electrophoresis at 50 volts for 6-6.5 hours, capillary transferred to a positively charged nylon membrane (Invitrogen) and cross-linked with short-wave UV light. Hybridization with labeled probes was done in the DIG Easy Hyb Solution at 50°C according to the manufacturer's instructions. The

probe-target hybrids were detected by a chemiluminescent reaction followed by exposure to X-ray film. Total RNA samples were hybridized with a probe of *gapdh* fragment. The probe was stripped off and membranes were rehybridized with probes of 3 gene fragments and without probes to verify the stripping process. The membranes were stripped by rinsing thoroughly with double distilled water, washing twice at 80°C in Stripping buffer (50% deionized formamide, 5% SDS, and 50 mM Tris-HCl, pH 7.5) for 1 hour, and rinsing again with 2xSSC for 5 minutes. The stripped membranes were kept wet in 2xSSC until the next use. The experiments were performed in duplicate.

6.3 Results

6.3.1 MAC-T gene expression following exposure to *S. dysgalactiae*

Microarrays have been used to study the host's response to bacterial infection using several *in vitro* models for pathogenesis. The bovine microarray is an alternative method to study gene expression of MAC-T cells after infection with *S. dysgalactiae*. The number of viable infected and non-infected MAC-T cells were not significantly altered after infection. Total RNA samples were extracted from infected and non-infected MAC-T cells. The average RNA concentration from approximately 4.8 x 10^6 MAC-T cells was 1.38 μg/μL with A_{260}/A_{280} ratios of 1.85-1.9. RNA samples were reverse transcribed and experimental and control cDNA samples obtained were labeled with Cy5 and Cy3, respectively. The analysis of the labeling reaction from 3 cDNA samples used for microarrays is shown in Table 6-1. The graphical display of data from

Table 6-1 Analysis of labeling reaction of cDNA samples with Cy3 and Cy5 dyes

	Experiment 1		Experiment 2		Experiment 3	
	MAC-T	infected MAC-T	MAC-T	infected MAC-T	MAC-T	infected MAC-T
Nucleotides (pmol)	595.19	540.46	472.05	950.94	677.29	451.53
Cy3 (pmol)	-	41.2	-	29.2	-	23.2
Cy5 (pmol)	9.84	-	18.96	-	13.2	-
Nucleotides /dye ratio	60.49	13.11	24.90	32.57	51.31	19.46

a microarray slide is shown in an MA-plot (Fig. 6-1, 6-2, 6-3). The MA-plot serves to increase the room available to represent the range of differential expression and makes it easier to see non-linear relationships between the log intensities. It also displays the important relationship between differential expression and intensity which is used in later analysis steps. The B values of the first 25 genes with the highest B values are shown in Table 6-2. The names of these genes corresponding accession numbers are shown in Table 6-3.

6.3.2 Confirmation of array data using Northern blots

Three transcripts with the highest ranks in differential expression from B statistic, succinate dehydrogenase complex, subunit A, flavoprotein, ORF, and myosin, light polypeptide kinase, respectively (Table 6-2) , were relatively quantified by Northern blots. The result of duplicate experiments showed no significant differences in signal intensities of all three transcipts between RNA samples from infected and non-infected MAC-T cells (Fig. 6-4, 6-5).

6.4 Discussion

A CattleArray3800 microarray was used to study the effect on MAC-T cells after infection by *S. dysgalactiae*. Since MA plots showed variability among triplicate arrays, the data needed to be analyzed statistically to search for genes with the highest possibility of differential expression. The B-statistic is a suitable compromise between the average *M* and t-statistic as the absolute level of average *M* is known to be

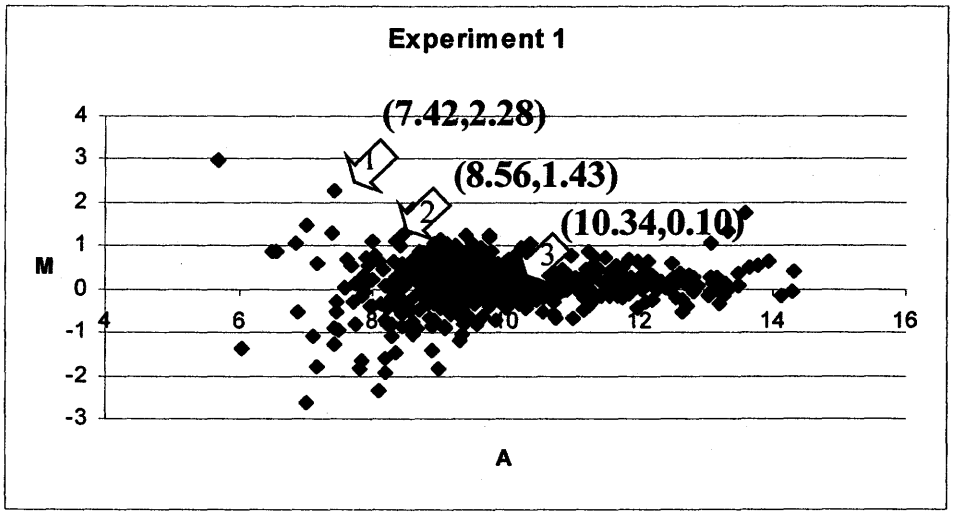


Fig. 6-1 MA plot of data from Experiment 1. M represents the log-differential expression ratio and A represents the log-intensity of the spot. Arrows represent the MA data of top three genes selected to be confirmed by Northern blots. Numbers in the arrows represent the rank of genes from the highest B value. Numbers in the parentheses represent A and M values, respectively.

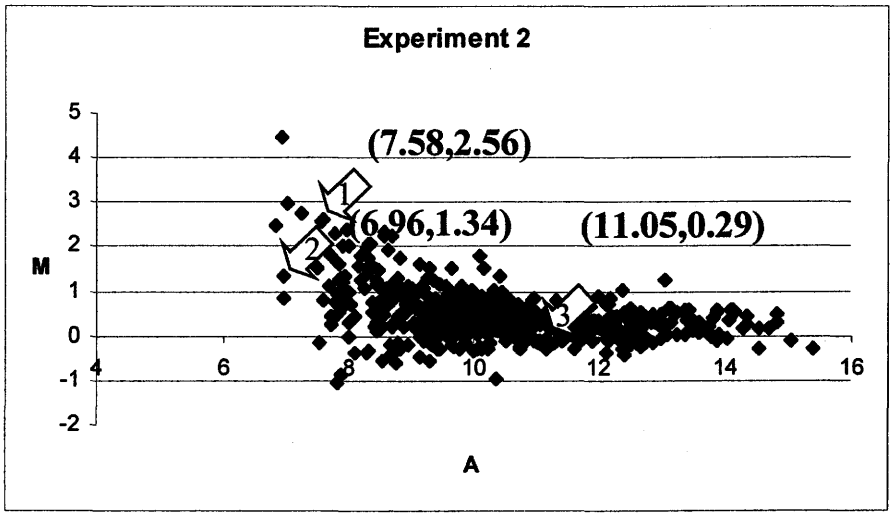


Fig. 6-2 MA plot of data from Experiment 2. M represents the log-differential expression ratio and A represents the log-intensity of the spot. Arrows represent the MA data of top three genes selected to be confirmed by Northern blots. Numbers in the arrows represent the rank of genes from the highest B value. Numbers in the parentheses represent A and M values, respectively.

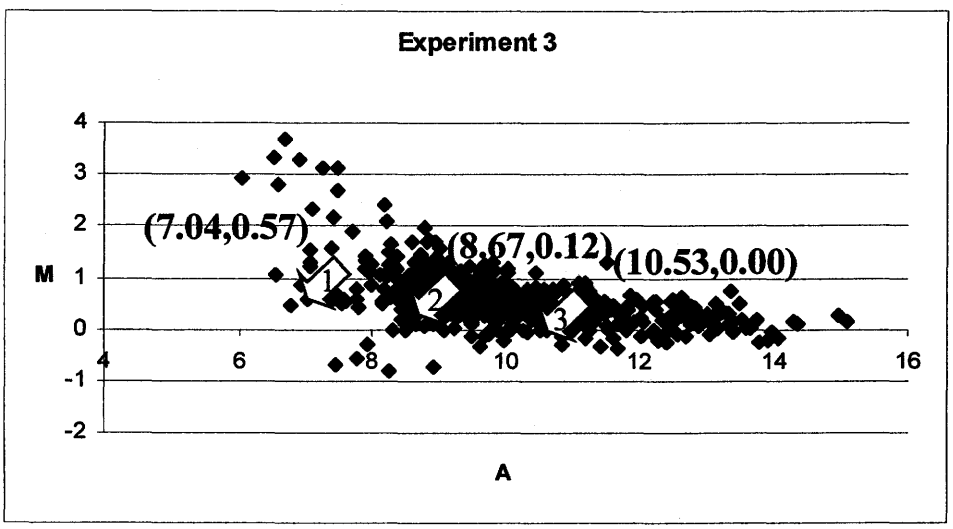


Fig. 6-3 MA plot of data from Experiment 3. M represents the log-differential expression ratio and A represents the log-intensity of the spot. Arrows represent the MA data of top three genes selected to be confirmed by Northern blots. Numbers in the arrows represent the rank of genes from the highest B value. Numbers in the parentheses represent A and M values, respectively.

Table 6-2 B , A, and M values of the top 25 genes with the highest possibility of differential expression

Accession Number	B value	Experiment 1		Experiment 2		Experiment 3	
		A	M	A	M	A	M
BF046015	1.14	7.42	2.28	7.58	2.56	7.04	0.57
AW465934	1.13	8.56	1.43	6.96	1.34	8.67	0.12
AW463818	1.12	10.34	0.10	11.05	0.29	10.53	0.00
AW461457	1.10	9.41	1.23	8.58	0.97	8.63	0.13
AW465061	1.10	8.21	-0.42	9.81	1.09	9.26	0.02
AW461632	1.09	5.67	2.99	8.20	1.26	6.95	0.86
AW465616	1.08	8.44	-0.33	6.94	4.47	8.59	0.29
AW461404	1.08	7.73	0.11	9.11	0.98	9.01	0.06
AW461536	1.07	9.26	0.25	10.11	0.42	10.07	0.02
AW464188	1.06	8.28	-0.18	8.59	2.34	8.97	0.15
AW462270	1.06	8.40	1.01	9.35	0.65	8.46	0.18
AW464606	1.05	9.77	0.60	9.73	0.72	8.63	0.15
AW463816	1.05	8.38	-0.40	8.96	1.14	8.79	0.07
AW465214	1.04	6.53	0.88	9.04	1.06	7.54	0.51
AW463776	1.04	9.10	0.84	7.91	2.00	8.15	0.52
AW466188	1.04	13.32	1.36	10.37	-0.95	10.62	0.01
AW463521	1.04	9.08	0.21	10.92	0.82	10.14	0.05
BF040189	1.04	8.59	1.85	8.57	1.04	7.07	1.23
AW462758	1.04	8.03	0.78	7.80	2.28	9.18	0.32
AW465524	1.03	8.94	0.31	7.78	0.42	8.92	0.11
AW461969	1.03	9.42	0.92	9.54	0.64	7.58	0.58
AW463057	1.03	8.71	0.50	8.20	1.81	8.60	0.42
BF044049	1.03	8.66	0.52	7.68	1.13	8.89	0.27
AW462297	1.03	9.11	-0.16	7.68	1.82	7.77	0.58
BF040873	1.03	7.39	1.29	8.52	0.37	7.18	0.89

B values greater than zero correspond to a greater than 50-50 chance that the gene in question was differentially expressed. *M* values refers to units of 2-fold change and *A* is in units of 2-fold increase in brightness.

Table 6-3 Names of genes with the highest B values

Accession Number	Description
BF046015	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
AW465934	myosin, light polypeptide kinase
AW463818	ORF
AW461457	hypothetical protein FLJ22965
AW465061	hypothetical protein MGC2560
AW461632	empty spiracles (Drosophila) homolog 2
AW465616	transcriptional intermediary factor 1
AW461404	ATPase, H+ transporting, lysosomal (vacuolar proton pump), beta polypeptide, 56/58kD, isoform 2
AW461536	hypothetical protein DKFZp761J139
AW464606	interleukin 10 receptor, beta
AW463776	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)
AW466188	Homo sapiens, Similar to deoxyguanosine kinase, clone MGC:2111 IMAGE:2989352, mRNA, complete cds
AW461969	diptheria toxin resistance protein required for diphthamide biosynthesis (Saccharomyces)-like 2
AW463057	diaphorase (NADH/NADPH) (cytochrome b-5 reductase)
BF044049	adenine phosphoribosyltransferase
AW462297	inhibitor of growth family, member 1

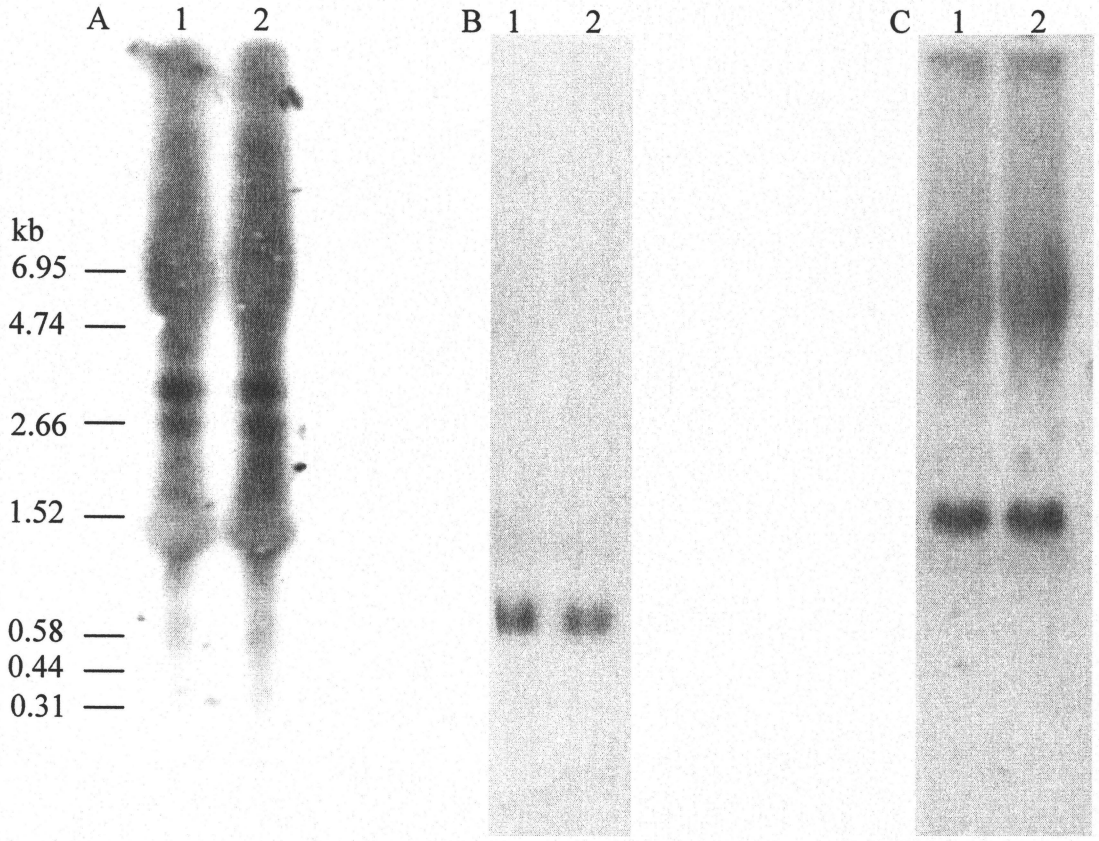


Fig. 6-4 Northern blot analysis of 3 selected mRNA expression in MAC-T cells after *S. dysgalactiae* infection. The genes selected were the ones that showed the highest trends to differentially express from B-statistic. Fifteen micrograms of total RNA from non-infected (Lane 1) or *S. dysgalactiae* infected (Lane 2) cells 5 hours postinfection was hybridized with probes from the DIG-labeled PCR products. Results from duplicate experiments are shown. A, mRNA hybridized with a probe derived from myosin, light polypeptide kinase gene (accession number AW465934); B, mRNA hybridized with a probe derived from an unknown ORF (accession number AW463818); C, mRNA hybridized with a probe derived from succinate dehydrogenase complex, subunit A, flavoprotien gene (accession number BF046015). Size standards are shown on the left margin.

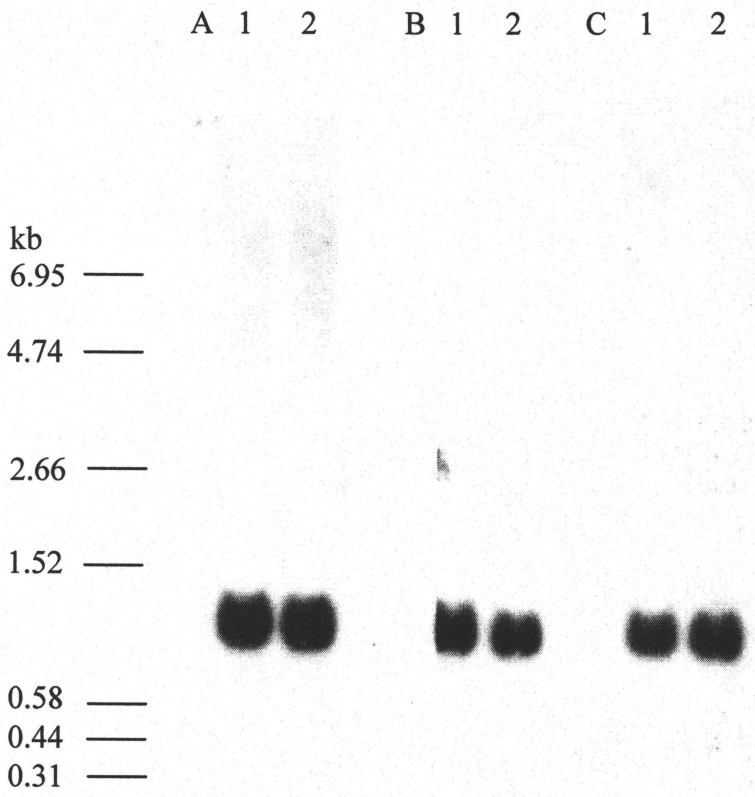


Fig. 6-5 Northern blot analysis of *gapdh* mRNA expression in MAC-T cells after *S. dysgalactiae* infection. Fifteen micrograms of total RNA from non-infected (Lane 1) or *S. dysgalactiae* infected (Lane 2) cells 5 hours postinfection was hybridized with a probe derived from *gapdh* PCR products. Results from duplicate experiments are shown. Size standards are shown on the left margin.

a poor choice as it does not take into account the variability of the expression levels for each gene. The shortcoming of the method is that the variability of the M-values over replicates is not constant across genes and genes with larger variances have a good chance of giving a large average M. A better choice is to rank genes according to the absolute value of the t-statistic. However, the ordinary t-statistic is still not ideal because a large t-statistic can be driven by an unrealistically small value for standard deviation. The shortcoming of the t-statistic is the opposite of that of average *M.* Genes with small sample variances have a good a chance of giving a large t-statistic even if they are not differentially expressed. The review of microarray analysis was described by Smyth et al. (2002) and Lonnstedt and Speed (2002).

The expression of 3 genes with the highest possibility of differential expression from B-statistic, coding for (i) succinate dehydrogenase complex, subunit A, flavoprotien (accession number BF046015), (ii) myosin, light polypeptide kinase (accession number AW465934), and (iii) ORF (accession number AW463818), were confirmed with Northern blot analysis. None of the selected genes showed differential expression between infected and non-infected MAC-T cells. Two bands could be observed using the myosin, light polypeptide kinase probe even though the probe was gel purified. This might be because the myosin kinase of MAC-T had 2 isoforms as reported in bovine endothelium (Garcia et al., 1997)

The B values of all three genes were higher than those of the remaining genes (Table 6-2). The higher the value of B, the higher the probability that the gene is differentially expressed. However, one of the possible reasons that data from cDNA array experiments could not be confirmed by other methods of mRNA assessment, was

error of B-statistic analysis. To achieve a reliable B-statistic analysis, the data should be obtained from dye-swap pair experiment in which two RNA samples (extracted from infected and non-infected MAC-T cells) are labeled with the opposite dye, and each set of data should be obtained from 3-5 independent experiments. In this study, due to the limit available of microarray slides, the dye-swap pair experiment was omitted, and the data obtained were from only triplicate experiments.

The use of cDNA arrays can yield a moderate degree of false positive results and underlined the need for confirmation of cDNA array results by other methods. False positive findings may result from the inability of hybrid selection methods to discriminate between genes that are closely related and show a high degree of sequence homology when uniform hybridization conditions are used for all genes (Eckman et al., 2000). In addition, a disadvantage of spotted microarrays is the variation in the size of the spot and the amount of DNA contained in every spot. Also, variations in length and composition of sequence and variations in post-processing and hybridization result in less consistency and reproducibility within and across experiments than high-density oligonucleotide arrays (Kato-Maeda et al., 2001). The choice of confirmation methods could also contribute to discrepancies between cDNA array analysis and other approaches. Analysis of mRNA expression using Northern blots or RNase protection assays can detect splice variants more reliably but is less sensitive than quantitative RT-PCR (Eckman et al., 2000).

To decrease the chance of false positive and false negative results from technical issues associated with the use of cDNA array, dye-swap pairs, as described above, is recommended to minimize the effects of any gene-specific dye-bias. Furthermore, self-

self hybridization experiments, in which two identical total RNA samples are labeled with different dyes and hybridized to the same slide provide useful controls for data analysis. Although there is no differential expression and one expects the red and green intensities to be equal, the red intensities often tend to be lower than the green intensities. Furthermore, the imbalance in the red and green intensities is usually not constant across the spots within and between arrays, and can vary according to overall spot intensity, location on the array, slide origin, and possibly other variables (Smyth et al., 2002).

Another technical issue is that of adequate controls for the reproducibility of reverse transcription and hybridization and detection of specific hybridization. To control for these variables, expression levels for house keeping genes are commonly used as standards relative to which the expression levels of all other genes are assessed (Eckman et al., 2000). In this experiment, GAPDH was used as a house keeping gene which showed similar signal intensities in Northern blots of both RNA samples, extracted from infected and non-infected cells.

Analyses of changes in gene expression in response to microbial infection or microbial products are only the beginning in our quest to fully understand complex issues in host-microbe pathogenesis. A more complete understanding will ultimately require not only quantitative and qualitative knowledge of gene expression, but also a complete analysis of changes in cellular functions, including protein production, post-trancriptional events, and post-translational protein modifications, and knowledge of protein-protein interactions that occur within cells after their interaction with infecting microbes and microbial products.

6.5 Conclusion

Bovine microarray analyses showed a number of genes with the possibility of differential expression following infection of MAC-T cells with *S. dysgalactiae*. The first three genes that showed the highest possibility of differential expression, ranked by B-statistic, were (i) succinate dehydrogenase complex, subunit A, flavoprotein (accession number BF046015), (ii) myosin, light polypeptide kinase (accession number AW465934) and (iii) ORF (accession number AW463818), respectively. Northern blot analysis of the RNA extracts showed no differential expression of the selected genes between infected and non-infected MAC-T cells under the conditions described.

7.0 GENERAL DISCUSSION AND CONCLUSION

The objectives of this thesis were to determine the role of GapC in the pathogenesis of *S. dysgalactiae* infection and to determine the host gene expression profile in response to *S. dysgalactiae* infection. The work accomplished during this study includes: construction of a sco recombinant of *S. dysgalactiae* by disrupting the *gapC* gene by insertional inactivation with a Ω Km^r cassette, characterization of GapC with GapC-specific polyclonal antibody and the GAPDH inhibitor iodoacetate, an investigation of gene expression by MAC-T cells following infection with *S. dysgalactiae* by microarray analysis, and finally confirmation of the microarray results by Northern blotting. This chapter will briefly interpret the results obtained and present a conclusion of the work.

From this study, I found that there was only one copy of *gapC* in *S. dysgalactiae* as occurs in the closely related species, *S. equisimilis* (Gase et al., 1996; Vandamme et al., 1996). The *plr* of *S. pyogenes*, which is highly similar to *gapC* of *S. dysgalactiae*, was also reported to have only one copy (Winram and Lottenberg, 1998). None of these genes could be inactivated by allele replacement (Gase et al., 1996; Winram and Lottenberg, 1998). The inability to isolate a dco mutant lacking *gapC* and the effect of IAA on *S. dysgalactiae* viability suggests that, as expected, the gene product is essential for cell viability. Thus, a specific inhibitor of the surface-localized molecule which does not affect the intracellular glycolytic activity represents one method of circumventing

this problem. However, the addition of affinity-purified anti-GapC antibody did not affect bacterial adherence/penetration on/into MAC-T cells. This suggests that GapC might not play a direct role in bacterial adherence/penetration on/into host cells. However, the role of GapC in other steps of pathogenesis, if any, remains unclear.

Plr, GapC related protein, in group A streptococci was supposed to play a role in bacterial infection of host cells as a plasmin binding protein. However, Plr did not account for all of the plasmin binding to group A streptococci as a mutation of the *plr* gene resulting in alteration of plasmin binding site did not reduce plasmin binding activity of intact group A streptococci (Winram and Lottenberg, 1998). In addition, an IgM monoclonal antibody which inhibited plasmin(ogen) binding to the recombinant Plr protein did not inhibit binding of plasmin(ogen) to intact group A streptococci (D'Costa et al., 1997).

Northern blot analysis did not show any differential gene expression of 3 selected genes of MAC-T cells after infection with *S. dysgalactiae* under the conditions used (ranked by B-statistic). Therefore, it might not be useful to investigate the differential gene expression of MAC-T cells after adding GapC under the same conditions. Interestingly, the genes related to necrosis and inflammation, which are the important characteristics of mastitis, together with a gene coding for lactoferrin which is the protein released from mammary epithelial cells after bacterial infection, and genes encoding for interleukin-1 (IL-1) and IL-8 which are proteins reported to be released from MAC-T cells after bacterial infection (Boudjellab et al., 2000), were not on the list of 25 genes with the highest possibility to be differentially expressed (Table 6-3). The ratios of Cy5/Cy3 signal intensities of those genes were shown in Table 9-1. The failure

to detect those genes which were related to pathogenesis may be due to an error of B-statistic since dye-swap pairs and self-self hybridization experiments were not performed as described in chapter 6. In addition, the conditions used did not mimic the pathogenesis of the disease *in vivo.* Although MAC-T cells are derived from mammary epithelial cells, they are immortal so they may have some different characteristics from natural mammary epithelial cells. Therefore, the response to bacterial infection might be different. It is suggested to use RNA extracted from infected udders to investigate differential gene expression by microarray. Furthermore, bacteria might not be virulent due to long-term laboratory passage. The virulence of bacteria should be characterized as to their ability to cause mastitis in cattle. However, this appeared to be unlikely because we have used this strain to challenge cattle a number of times (Andrew A. Potter, personal communication). The environment under which bacteria were cultured might not be suitable for bacteria to show their virulence. Several environmental cues signal the entry of the microbe into host tissue and thereby influence the expression of virulence factors such as low iron concentration and elevated temperature. In addition, physical-chemical parameters such as osmolarity, pH, oxygen, CO_2, or ions besides iron could play a similar role (Mekalanos, 1992). The types of DNA chips used might also affect the result. Cohen et al. (2000) compared the transcriptional response of THP1 cells to *Listeria monocytogenes* infection using commercially available oligonucleotide chip technology (Oligonucleotide HU6800 Set, Affymetrix) versus two macroarray systems (Atlas human cDNA array I, Clonetech; and Gene Discovery Array Human I, Genome Systems). Oligonucleotide chip technology was shown to detect 3-6 fold more

genes than either of the macroarray systems when genes common to each of these arrays were compared.

In conclusion, although GapC is essential for bacterial viability, the result of this study does not support the role of GapC in direct *S. dysgalactiae* adherence/penetration on/into host cells at least *in vitro*. However, the possibility that GapC plays a role in pathogenesis by other mechanisms cannot be ruled out.

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9.0 APPENDIX

Table 9-1 Intensity ratios of the top 25 genes with the highest B values

accession no.	Experiment 1	Experiment 2	Experiment 3
BF046015	-2.41	-1.46	1.48
AW465934	-3.33	1.61	-1.13
AW463818	1.01	1.12	1.06
AW461457	-2.02	1.2	-1.02
AW465061	1.12	-1.5	1.16
AW461632	-1.29	-1.19	-1.17
AW465616	1.15	-1.57	1.72
AW461404	-1.12	-2.43	1.08
AW461536	-1.32	-1.07	1.23
AW464188	1.2	-1.81	-1.01
AW462270	-1.14	1.17	-1.16
AW464606	-1.38	-1.29	1.25
AW463816	-1.19	1.04	-1.11
AW465214	-1.38	-1.21	-1.14
AW463776	-1.99	-1.6	1.41
AW466188	-2.2	1.9	-1.15
AW463521	1.17	-1.14	1.29
BF040189	-2.92	-1.04	-1.42
AW462758	-1.44	2.12	2.54
AW465524	-1.39	2.16	2.1
AW461969	-1.77	-1.41	1.43
AW463057	-1.79	-1.18	1.56
BF044049	-1.2	-1.08	1.01
AW462297	1.16	-1.23	1.58
BF040873	-1.89	1.15	1.08

After red and green foreground and background intensities for each spot was obtained, the signal intensities were used to calculate the intensity ratio by Jaguar Software 2.0 (Affymetrix) as :

Ratio = channel 2 intensity/channel 1intensity, if channel 2 intensity ≥ channel 1 intensity

or Ratio = -1/(channel 2 intensity/channel 1 intensity), if channel 2 intensity < channel 1 intensity

* Ratios were restricted to a minimum of –100 and a maximum of 100.
* Channel 1 and channel 2 intensities = Cy 5 and Cy3 signal intensities, respectively