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DISSERTATION

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Understanding the Pathogenicity of *Mycoplasma agalactiae* through *In vitro* and *In vivo* Infection Studies

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DEDICATION

In dedication to my mother for making me be who I am, and my wife for supporting me all the way!

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1 Abbreviations

A	adenin
A.	*Acholeplasma*
aa	amino acid
Abs	antibodies
Amp	ampicillin
AO	attachment organelle
ATCC	American Type Culture Collection
ATP	adenosin triphosphate
bp	base pairs
BEND	Bovine Endometrium cell line
BLAST	Basic local alignment search tool
BLF	Bovine Lung Fibroblast
BSA	bovine serum albumin
C	cytosin
CA	contagious agalactia
Cam	chloramphenicol
CCU	color changing unit
CDS	coding sequence
CFU	colony forming units
COG	conserved orthologue group
ddH2O	double-distilled H2O
DIF	double immunofluorescence
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco’s phosphate buffered saline
DNA	deoxyribonucleic acid
dNTP	deoxynucleotid triphosphate
dsDNA	double stranded DNA
DT	doubling time
E	endonuclease

E.	*Escherischia*
ECM	extra cellular matrix
EDTA	ethylene diamine tetraacetic acid
EtOH	ethanol
FBS	fetal bovine serum
FCS	fetal calf serum
Fig.	figure
FITC	fluoresceinisothiocyanate
G	guanine
GI	growth index
Gm	gentamicin
H_2O_2	hydrogen peroxide
HA	heamadsorption
HEPES	4-(2-hydroxyethyl)-piperazineethanesulfonic acid
HGT	horizontal gene transfer
IgG	immunoglobulin G
IHC	Immunohistochemistry
INV	invasive
IS	inverted sequences
IVET	In vivo expression technology
kb	kilobase pairs
kDa	kilodalton
lac	lactose
LB	Luria broth
LDH	lactate dehydrogenase
LN	lymph node
M.	*Mycoplasma*
MA	*Mycoplasma agalactiae*
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-of Flight
MAb	monoclonal antibody
MEM	minimum essential medium

MOI	multiplicity of infection
mRNA	messenger RNA
NA	not applicable
ND	not done
NEB	New England Biolabs
nt	nucleotide
OD	optical density
OIE	World Organization for animal health
oriC	origin of replication
ORF	open reading frame
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDHC	pyruvate dehydrogenase complex
PEG	polyethylene glycol
PLM	phase locked mutants
PSI-BLAST	Position assisted iterated BLAST
QTD	quantitative target display
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAP	RNA polymerase
rpm	rounds per minute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcription PCR
S.	*Spiroplasma*
SD	standard deviation
SDS	sodium dodecyl sulfate
SOC	super optimal broth with catabolite repression
SSM	signature sequence mutagenesis
ssDNA	single stranded DNA

STM	signature tagged mutagenesis
T	thymin
TBS	tris-buffered saline
Tet	tetracycline
tetR	casette consiting of the *tetM* under *tetPO*
Tn	transposon
Tn*4001*mod	Modified version of Tn*4001*
tRNA	transfer RNA
Trp	trpyptophan amino acid
U	unit
US	United States
vpma	variable surface proteins of *Mycoplasma agalactiae*
X-gal	5-bromo-4-chloro-3-indolyl b-D-galactopyranoside

2 Abstract

2.1 Abstract (English)

Mycoplasma agalactiae is an important pathogen of small ruminants, but the molecular basis of its pathogenesis is largely unknown. This lack of knowledge is mainly attributed to the previous lack of tools to genetically manipulate this agent. In the current study, pathogenicity determinants of *M. agalactiae* were identified by *in vitro* and *in vivo* screening of a Tn-mutant library of 200 sequenced mutants. Following extensive *in vitro* studies, pools of selected mutants were injected into lactating sheep via the intramammary route. The injected mutant population was compared with the recovered population to identify the attenuated mutants by using a negative selection method called Signature Sequence Mutagenesis. A total of 14 mutants were identified during initial screening in sheep and were then confirmed for 100% absence in a second round of sheep infection studies that were carried out in triplicate. The latter identified 7 mutants with insertions in MAG1050, MAG2540, MAG3390, *uhpT, eutD, adhT*, and MAG4460. Importantly, the attenuated mutants belong to different functional classes including many hypothetical genes implying their previously unknown role in *M. agalactiae* pathogenicity.

The *in vitro* analysis comprised growth profiles, cell invasion studies, co-cultivation and cytotoxicity studies in mammalian cell lines, revealing an important role of certain genes, as also confirmed for some mutants by complementation studies using a newly developed expression vector. These studies demonstrated for the first time *M. agalactiae*'s ability to invade

cultured mammalian cells. Furthermore, cell invasion was also demonstrated *in vivo* during experimental sheep infection. The detection of *M. agalactiae* in the cytoplasm of epithelial cells and macrophages clearly provided the first formal proof of *M. agalactiae*'s capability to translocate across the mammary epithelium and systemically disseminate to distant inner organs, a possible explanation for its persistence and the chronicity of disease caused by this agent.

Altogether, this study has identified many different factors involved in *M. agalactiae* pathogenesis and has provided a potential basis in developing successful intervention and prevention strategies

2.2 Abstract (Deutsch)

Mycoplasma agalactiae ist ein wichtiger bei kleinen Wiederkäuern vorkommender Krankheitserreger, dessen Pathogenesemechanismen auf dem molekularen Level jedoch weitgehed unverstanden sind, da bis vor kurzem ein Mangel an Möglichkeiten bestand, *M. agalactiae* genetisch zu manipulieren. In der vorliegenden Arbeit wurden Pathogenitätsdeterminanten von *M. agalactiae* durch *In vitro*- und *In vivo*- Screeningexperimente anhand von 200 sequenzierten Transposon-Mutanten identifiziert. Pools aus ausgewählten Mutanten wurden im Schaf-Infektionsmodell unter Anwendung der intramammären Infektionsroute auf Attenuierung einzelner Mutanten geprüft. Dazu wurden die für die Infektion verwendete Mutantenpopulation und die reisolierte Mutantenpopulation mittels Signatur-Sequenz-Mutagenese als Negativselektionsmethode verglichen.

Während eines initialen *In Vivo*-Screeningexperiments wurden insgesamt 14 Mutanten identifiziert und ihre Attenuierung in einem zweiten Infektionsexperiment aufgrund ihrer 100%igen Abwesenheit unter den Reisolaten bestätigt. Unter den attenuierten Mutanten befanden sich 7 Mutanten mit Insertionen in den Genen MAG1050, MAG2540, MAG3390, *uhpT, eutD, adhT* und MAG4460, die verschiedenen Funktionsklassen einschließlich hypothetischen Genen zugeordnet sind, deren pathogenetische Rolle bei *M. agalactiae* bisher unbekannt war.

Im Rahmen der *In vitro*-Untersuchungen wurden unter Einsatz verschiedener Zelllinien Wachstumsprofile erstellt sowie Zellinvasions-, Ko-Kultivierungs-

und Zytotoxizitätsstudien durchgeführt, anhand derer eine wichtige Rolle einzelner Gene aufgezeigt werden konnte. Diese wichtigen Funktionen konnten bei einigen Mutanten auch durch Komplementationsstudien unter Anwendung eines neu-entwickelten Expressionsvektors bestätigt werden. Darüber hinaus haben diese Untersuchungen erstmalig die Zellinvasionsfähigkeit von *M. agalactiae* nachgewiesen, und zwar sowohl *in vitro* im Zellkultursystem wie auch *in vivo* im Schaf-Infektionsmodell. Die Detektion von *M. agalactiae* im Zyotoplasma von Epithelzellen und in Makrophagen ist der erste formale Nchweis der Befähigung von *M. agalactiae* zur Translokation des mammären Epitheliums und nachfolgender systemischen Disseminierung unter Einbeziehung entfernt liegender innerer Organe, eine mögliche Erklärung für die Persistenz dieses Erregers und für die Chronizität der durch ihn hervorgerufenen Erkrankung.

Insgesamt konnten im Rahmen dieser Arbeit viele verschiedene für die Pathogenese relevante Faktoren von *M. agalactiae* identifiziert werden, wodurch eine mögliche Basis gelegt wurde, erfolgreiche Bekämpfungs- und Präventionsstrategien zu entwickeln.

3 Introduction

3.1 Features of Mycoplasmas

Mycoplasmas are the simplest and smallest (0.3-0.8 µm diameter) self-replicating bacteria with low GC content (~30 mol%) belonging to the class *Mollicutis* (*mollis*- soft, *cutis*- skin) and are taxonomically defined to have evolved from Gram-positive bacteria through reductive evolution (1, 2). The trivial name “mycoplasmas” is used for any species included in the class *Mollicutes* even though they are categorized under different genera, such as *Mycoplasma*, *Acholeplasma*, *Spiroplasma*, *Anaeroplasma* and *Ureaplasma* (3). The *M. genitalium* genome is one of the smallest prokaryotic genomes known and contains barely 500 protein-coding genes (4). Owing to their small genomes, mycoplasmas are fastidious and require complex media to grow under laboratory conditions. Mycoplasmas lack a rigid cell wall (Fig. 1A) and show a typical “fried egg” colony morphology (Fig. 1B) when grown on solid agar media. Due to the absence of a cell wall, they are resistant to beta lactam antibiotics that are otherwise used to inhibit bacterial growth. Another striking feature of mycoplasmas is their unusual codon usage. The UGA codon that functions as a stop codon in other bacteria is a tryptophan codon in mycoplasmas. This results in truncated gene products when mycoplasma genes are cloned in heterologous systems (5). Because of their smaller genome size and content, mycoplasmas provide an opportunity to study fundamental aspects of biology, such as the minimal set of essential genes, and also provide an

experimental platform to explore and create the first organism controlled by an artificially synthesized genome (6, 7).

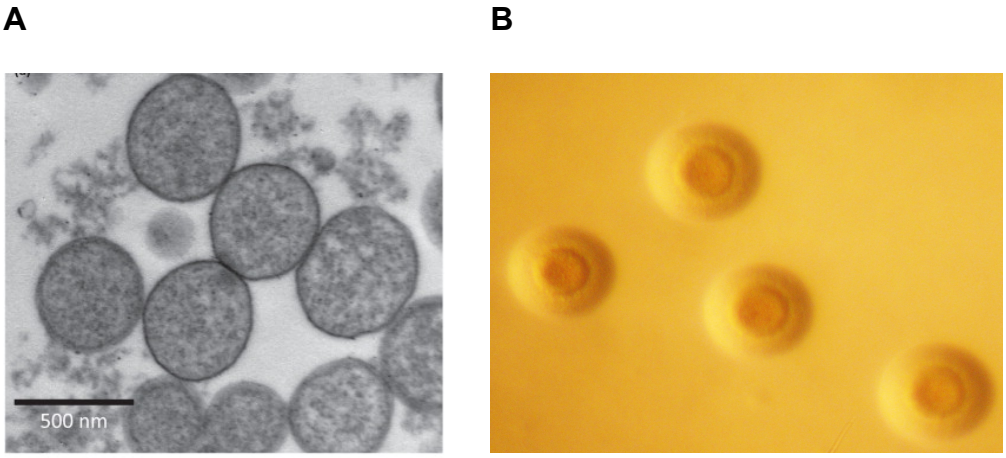

Fig.1. (A) Transmission electron microscopy of *M. agalactiae* illustrating cell morphology and size (8). (B) *M. agalactiae* colonies displaying typical “fried egg” morphology after 7 days of incubation on SP-4 agar.

The *Mycoplasma* genus includes more than 120 well-described species, many of which are established etiological agents of disease in man and animals (9-11). Due to their limited biosynthetic capabilities, these organisms are dependent on their infected hosts for nutrition and survival. They exhibit strict host and tissue specificity with a preference for the urogenital tract and the respiratory tract, as well as for the joints (10, 11). Mycoplasma infections are usually chronic and difficult to eradicate, but sometimes can also lead to acute disease (10). Mycoplasmas are regarded as ‘ideal parasites’ as they live in harmony with their host. Despite their medical and economical importance, the molecular basis for mycoplasma pathogenicity is largely unknown.

3.2 Mycoplasma Pathogenicity

Mycoplasmas are simple organisms, yet their pathogenicity is very complex. Due to their limited biosynthetic capabilities, mycoplasmas use host metabolic precursors such as lipids and nucleotides to grow in the in-host environment (10). Some mycoplasmas are capable of entering and surviving inside host cells by mechanisms that are up to now only poorly understood. As for most of the pathogenic bacteria, adhesion is a primary step for entering into the host cells. Loss of adhesion, either due to mutations or due to the blocking of adhesion proteins with specific antibodies, resulted in the loss of infectivity (12). Well-characterized human mycoplasmas, such as *M. pneumoniae* and *M. genitalium* use their surface bound adhesion proteins to gain access into the host cells (13). The P1 protein of *M. pneumoniae* was one of the first mycoplasma adhesins discovered and shown to play an important role in its infectivity (14) (Fig. 2). As the P1 adhesin of *M. pneumoniae*, the P140 protein of *M. genitalium* was found to be similarly important for host colonization (15). Mycoplasmas that share a common host, also share common properties between their virulence factors, such as the P1 adhesin of *M. pneumoniae* and the P140 adhesin of *M. genitalium,* which cross-react with antibodies raised against each other (13). Apart from the surface localized proteins involved in adhesion, some cytoplasmic proteins, such as the enolase, pyruvate dehydrogenase beta subunit (PDHB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have also been reported to be involved in cytadherence (16-19). Even though these proteins are involved in major metabolic activities inside the cell, their additional role in cytadhesion indicates the clever utilization of a minimal repertoire of proteins by mycoplasmas.

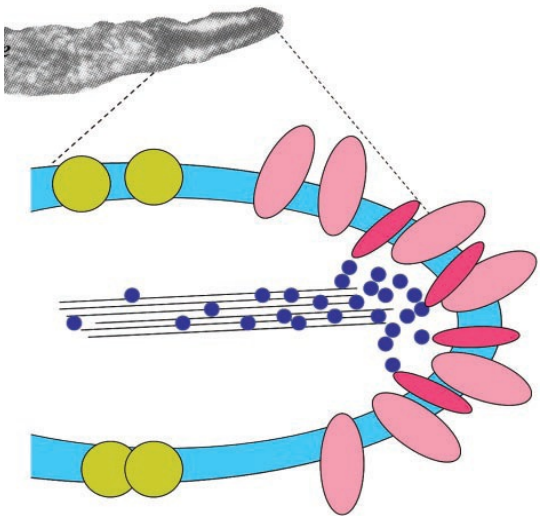

Fig. 2. Schematic depiction of the major cytadherence and accessory proteins in *M. pneumoniae* (12).

For many years, it was believed that mycoplasmas are obligate extracellular pathogens. After the first discovery of *M. fermentans* inside cells of the tissue samples from AIDS patients (12), several *Mycoplasma* species were found to be capable of invading non-phagocytic eukaryotic cells (20). The intracellular residence is a favored niche as it is protected from immune response and action of several antibiotics. Bacterial cell invasion is generally mediated by host surface receptors that facilitate the interaction between bacteria and host cells. Mycoplasmas have been shown to bind host cell proteins such as fibronectin and plasminogen that act as molecular bridges to facilitate invasion (20, 21). As found in other bacterial invasion studies, host cytoskeleton re-arrangements seems to play an important role in mycoplasma cell invasion (12).

Like many other successful pathogens, mycoplasmas possess sophisticated surface antigenic variation systems to evade the host immune response and to

adapt to the different host niches (2, 22). Surface variation of lipoproteins has been demonstrated to be advantageous to mycoplasmas for binding to a vast number of host surface proteins. Mechanisms such as frame shift mutation, site-specific recombination and unidirectional or reciprocal gene recombination drive the variation of surface lipoproteins in mycoplasmas (22, 23). Several mycoplasma species use these mechanisms to change their surface characteristics and persist within the host (Table.1).

Mycoplasmas cause host cell damage like other pathogenic bacteria. But unlike other bacterial pathogens, mycoplasmas do not release toxins. However, they can produce toxic metabolic products to affect host cells. Depending on the metabolic capabilities of specific mycoplasmas, the released products can differ in their mode of action and nature of damage to host cells. Some of the toxic metabolites like H_2O_2, urea, and hydroxyl-free radicals are capable of damaging host cell membranes (13). Biofilm formation, a common characteristic of many pathogenic bacteria, has also been implicated in mycoplasma persistence and protection against complement (24, 25). Mycoplasmas lack flagella but many species exhibit gliding motility supported by a tip structure or by cell surface proteins that act as tiny legs by connecting to the surface (26, 27). For motile mycoplasma pathogens, mutants with reduced motility were demonstrated to be less virulent (28). Gliding motility might facilitate access to host cell receptors as it was shown in one report that mycoplasma colonization of host epithelium requires this movement (29).

Based on the current knowledge it is clear that mycoplasma pathogenesis is very complex and diverse. To better understand the pathogenesis of mycoplasmas defined studies are needed to control the economic losses caused by mycoplasma infections in the dairy and meat industry and to understand and treat human mycoplasma.

Table 1. Mechanisms governing antigenic variation in mycoplasmas (22)

mechanism of variation	**genetic event**	**mycoplasma species**	**gene family**
ON/OFF switching	DNA slippage involving SSR in promoter regions	*M. hyorhinis*	*vlp*
		M. gallisepticum	*vlhA*
		M. capricolum subsp. capricolum	*vmc*
	site-specific recombination (gene rearrangement)	*M. pulmonis*	*vsa*
		M. bovis	*vsp*
		M. agalactiae	*vpma*
		U. parvum	*mba*
	site-specific recombination (promoter inversion)	*M. penetrans*	*mpl*
size variation	DNA slippage involving short direct repeats within CDSs	*M. agalactiae*	*vpma*
		M. hyorhinis	*vlp*
		M. pulmonis	*vsa*
		M. bovis	*vsp*
		U. urealyticum	*mba*
domain shuffling	gene conversion (unidirectional)	*M. synoviae*	*vlha*
	reciprocal recombination	*M. genitalium*	*mgp*
	DNA recombination	*M. agalactiae*	*vpma*
		M. bovis	*vsp*
other	gene or locus duplication	*M. agalactiae*	*vpma*
CDS: coding sequence; SSR: single sequence repeat.			

3.3 *Mycoplasma agalactiae*

3.3.1 *M. agalactiae* infection in small ruminants

Mycoplasma agalactiae is a causative agent of the CA syndrome in small ruminants. This organism belongs to the hominis phylogenetic group that also includes *M. synoviae*, *M. pulmonis*, *M. hyopneumoniae* and *M. mobile* (30). *M. agalactiae* is believed to have acquired 18% of its genome via horizontal gene transfer (HGT) from mycoplasmas belonging to the phylogenetically distant mycoides cluster that infect the same ruminant hosts (30). CA is mainly characterized by mastitis, conjunctivitis and arthritis (Fig. 3) as predominant symptoms of a localized infection (31-35). But, cases of septicemia, arthritis and sporadic genital infections are not uncommon (31). This is an indication that the pathogen is capable of crossing the epithelial barrier to reach distant host niches, as also evidenced in a recent report where naturally infected asymptomatic male goats were shown to harbour *M. agalactiae* in atypical inner organs like brain and heart (36). *M. agalactiae* is estimated to be responsible for annual losses in excess of US$ 30 million, especially in European countries around the Mediterranean, primarily due to losses in the dairy industry (35, 37). The CA syndrome is included in the list of transmissible diseases notifiable to the 'Office International des Epizooties' (OIE) that are considered to be of socio-economic and/or of public health importance (http://www.oie.int/en/animal-health-in-the-world/the-world-animal-health-information-system/oldclassification-of-diseases-notifiable-to-the-oie-list-b/). Although the disease is predominant in the Mediterranean region, it is also widespread in other countries (33, 35).

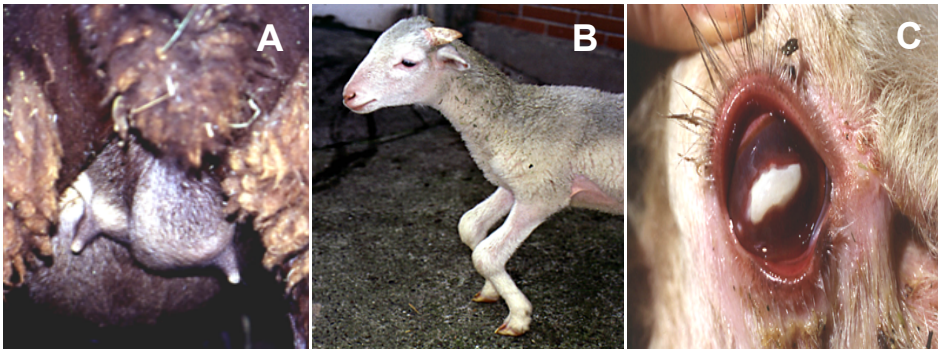

Fig. 3. Typical symptoms of (A) mastitis, (B) arthritis and (C) conjunctivitis, as observed in sheep showing the Contagious Agalactia syndrome (38)

Shedding of the pathogen by diseased or asymptomatic carriers continues for months or years after the initial infection with a potential risk of infecting susceptible animals (31, 33, 35). Antibiotic therapy tends to reduce clinical signs but promotes the carrier state (37). Attempts to minimize the clinical manifestation of CA by antibiotic therapy, culling and/or vaccination are not always successful and, in fact, no single vaccine against CA has been universally adopted (http://www.oie.int/doc/ged/D6448.PDF). Chronically infected but serologically negative herds with no signs of disease are a common clinical-epidemiological situation in endemic areas (39). Such animals easily escape disease control and eradication measures, and are capable of flaring up frequent CA outbreaks under stress conditions leading to huge economic losses (35, 39).

3.3.2 Current research on *M. agalactiae*

Despite its agronomical significance, *M. agalactiae's* pathogenic determinants and mechanisms of infection and persistence are largely unknown. The Vpmas (Variable Proteins of *M. agalactiae*) (Fig. 4) are the major abundantly expressed phase-variable lipoproteins of *M. agalactiae* that have been implicated in

pathogenesis (35, 40). Earlier studies done in our laboratory have reported about the mechanisms governing Vpma phase variation and the construction of phase-locked mutants (PLMs) (41, 42).

Like many other mycoplasmas, genetic manipulation of *M. agalactiae* was not possible for a long time until our laboratory achieved it through transposon mutagenesis (43). Later on, a random Tn mutant library was used by another research group to identify genes involved in *in vitro* host cell interaction through co-cultivation with mammalian cell lines (44, 45). The results obtained were then further evaluated *in vivo* to conclude that the NIF locus is important for its pathogenicity (46). Phenomenons like biofilm formation and H_2O_2 production, which are often implicated in pathogenesis, have also been shown in *M. agalctiae* (24, 47). Earlier efforts to identify factors involved in *M. agalactiae* pathogenesis were limited to *in vitro* studies, such as studies focusing on adhesion (48). However, later *in vivo* studies of naturally infected animals revealed several up-regulated genes in milk fat globules (49). In another natural infection study, *M. agalactiae* was isolated from several different locations of the host demonstrating its ability to spread systemically (36). Despite these efforts and the resulting progress of understanding the pathogenesis and involved factors, it is yet unclear how this pathogen successfully survives and persists inside the host for a prolonged time.

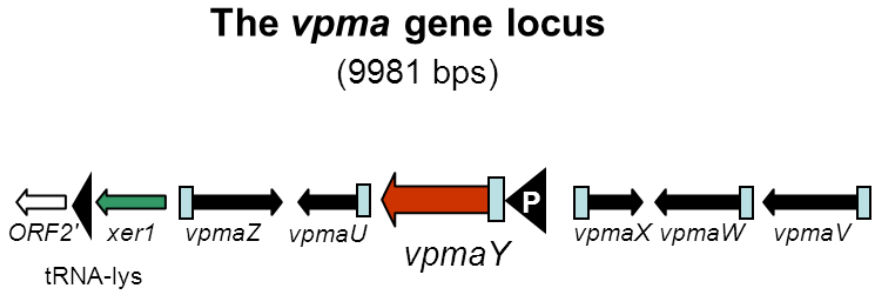

Fig. 4. Schematic representation of the *vpma* gene locus in the clonal variant 55-5 derived from *M. agalactiae* type strain PG2.

3.4 Genetic Manipulation of Mycoplasmas

3.4.1 Available genetic tools

Being important bacterial pathogens of humans and animals, thorough genetic analyses of pathogenic mycoplasmas are imperative in understanding their pathogenicity mechanisms and in identifying factors involved in disease progression. However, this has been hampered due to their long resistance to genetic manipulation and the lack of appropriate genetic tools for targeted gene disruptions and protein expression and analysis. In addition, the unusual property of mycoplasmas of using the general stop codon UGA as a tryptophan codon further poses problems in expressing mycoplasma proteins in heterogeneous systems like *E. coli* (50). In order to study the role of a mycoplasma gene or a set of genes in pathogenesis, it is important to mutate the same, either by homologous recombination or by random mutagenesis using transposons.

Targeted gene knockout. The targeted gene knock out through homologous recombination (HR) has been used in some mycoplasmas to disrupt specific

gene/s (41, 51, 52). The lack of HR in other mycoplasmas was assumed to be due to insufficient expression of genes involved in the initial recombination and resolution of Holiday junction (50). Proteomic results indeed showed that RuvAB is not expressed in *M. pneumonia*e, but other studies have shown that *M. pneumoniae* encodes proteins that bind ssDNA and promote homologous DNA recombination (53, 54). Another method to achieve targeted gene disruption is through the use of *ori*C plasmids and their integration into the chromosome (55). This method has been successfully used in *M. agalactiae* and other mycoplasmas to achieve targeted gene disruption (41, 52). Apart from this, transposition can also be used for the targeted disruption of specific genes (50).

Transposon mutagenesis. Random mutagenesis through transposition is widely used in mycoplasma pathogenesis studies. Transposon mutagenesis was initially applied to study the essential and minimal set of genes required in mycoplasmas as minimal bacteria (4, 7). Further studies using transposition have deciphered the role of different genetic factors involved in motility, cytadherence and also restriction modification systems (29, 56, 57). Large-scale transposon mutagenesis has been performed with a few species, and some of these studies additionally determined genes defective to mediate host colonization (58-60).

3.4.2 Genetic manipulation of *M. agalactiae*

Despite being a very important pathogen of the dairy industry, *M. agalactiae*'s pathogenic attributes are not yet understood. This is mainly due to the previous

lack of proper genetic tools for genetic manipulation. The first genetic modifications (43) and development of shuttle vectors for cloning and heterologous gene expression in *M. agalactiae* was reported by our laboratory (61). This *oriC*-based vector was then successfully used to disrupt *xer1* gene via homologous recombination to generate Vpma phase-locked mutants (PLMs) (41). As a first step towards manipulating the *M. agalactiae* genome, the wild type PG2 strain was transformed with the transposon Tn*4001*mod to randomly disrupt the genes (43). Later studies reported the development of a tetracycline inducible gene expression system for *M. agalactiae* indicating the further development of genetic tools to study pathogenesis of this ruminant pathogen.

3.5 Experimental Sheep Infection Models to study *M. agalactiae* Pathogenesis

In order to study pathogenesis, it is important to have a proper infection model that can simulate a natural infection. Previous *M. agalactiae* pathogenicity studies have been unsatisfactory due to the lack of appropriate animal models, especially small animal models for the convenient analysis of disease progression. The natural hosts, namely sheep and goats, have been used for experimental infections to study different aspects of *M. agalactiae* pathogenesis. Different infection models were used depending on the inoculation routes to study different aspects of disease, or to evaluate diagnostic tests, antibiotic therapies or vaccinations (62, 63). Lambs infected with *M. agalactiae* via the conjunctival route led to the constant colonization of the regional lymph nodes (LNs) (64). Later studies employing the intramammary route of infection showed

not only the lymphogenic but also the systemic spread of the pathogen in lactating ewes leading to the major symptoms of mastitis and agalactia (35, 65). Therefore experimental sheep intramammary infections were chosen for this dissertation project to assess the attenuation of *M. agalactiae* transposon mutants using negative selection methods described below.

3.6 *In vitro* and *In vivo* Genetic Analysis of Bacterial Virulence

The virulence of bacterial pathogens is very complex and involves several different genetic factors interacting during different stages of infection. Usually the sequence of events leading to persistent infection begins with adhesion leading to cell invasion and intracellular survival. In order to survive and persist inside the host, the pathogen should find a way to circumvent host immune responses. These processes involve several factors that are either secreted into the host cells or undergo direct interaction with them. Identifying these factors is a challenging task and needs sophisticated techniques to deal with sophisticated pathogens (66).

Several *in vitro* phenotypic assays have been developed to mimic the bacterial pathogenesis process that could facilitate identification of virulence factors. Assays focusing on adhesion, cell invasion, and cytotoxicity involving tissue culture have been used to simulate the in-host environment. Apart from these, the effect of different physiological factors namely pH, acid stress, salt stress and temperature have been used as a basis for the *in vitro* identification of different factors involved in pathogenesis.

Even though *in vitro* assays provide very useful information about mechanisms involved in bacterial pathogenesis, they cannot really imitate the *in vivo* host environment. Once inside the host, pathogens encounter very different environments that are very difficult to construct in the laboratory. In order to address this issue, several *in vivo* experimental models and methods have been developed (67, 68). Methods, such as the In Vivo Expression Technology (IVET) and the Signature Tagged Mutagenesis (STM) allow *in vivo* analysis of virulence factors (68, 69). IVET was designed to identify activated promoters *in vivo,* whereas STM involves the identification of specific attenuated mutants that are unable to survive inside the host amongst a total pool of infecting mutants (67). Out of these two methods, STM has been widely used to identify virulence factors for several important bacterial pathogens (70, 71).

3.6.1 Signature Tagged Mutagenesis (STM)

STM was developed as a method to simultaneously screen a large number of mutants *in vivo* using negative selection (69). This method allows rapid and unbiased identification of mutated virulence genes among a pool of mutants that are unable to survive in the infected host. Each mutated gene is marked with a 'Signature' tag to allow its identification among a pool of mutants in the animal host. Signature tags were originally designed as short DNA fragments containing variable 40 bp central regions flanked by invariable 'arms' that allow a transposon vector to further introduce mutations in the pathogen of interest. Each mutant is stored separately in microtitre plates and colony hybridization or dot blot studies are carried out for analysis. The mutants are pooled together to infect the host animal and those recovered from the animal tissue samples are

then compared to identify 'missing' ones. Mutants found absent in the output pool but present in the input pool are considered important for *in vivo* colonization. This method has been used in several pathogenic bacteria to identify important virulence genes (70). Over the years several technical modifications in the mutagenesis and detection methods led to the adaptation of this strategy to different hosts and pathogens as needed (Table. 2).

Table 2. Technical modification of STM (70)

Procedure modified	**Examples**	**Organisms**
For efficient mutagenesis		
Transposition	*In vivo* transposition	*Salmonella typhimurium, Mycbacterium tuberculosis, Vibrio cholerae, Yersnia enterocolitica, Legionella pneumophilia, Brucella suis, Escherichia coli*
	In vitro transposition	*Streptococcus pneumoniae, Neisseria meningitidis, Helicobactor pylori*
Mutagenesis without transposons	Shuttle mutagenesis	*N. meningitidis*
	Insertion-duplication mutagenesis	*S. pneumoniae*
	Gene-replacement by homologous recombination	*Saccharomyces cerevisiae*
	Non-homologous (illegitimate) recombination	*Candida globrata, Cryptococcus neoformans*
For efficient detection of tags		
Tag design	Longer tags	*Yersinia pestis, S. typhimurium.*
	Multiple tags	*Yersinia pseudotuberculosis, S. cerevisiae*
	Pre-selected pools of tags	*Staphylococcus aureus, Pseudomonas aeruginosa*
Detection methods	Hybridization to purified plasmids carrying tags	*S. aureus*
	Hybridization to PCR amplified tags	*S. pneumoniae*
	Non-radioactive probes	*S. aureus, Y. pseudotuberculosis*
	Direct detection of PCR product	*P. aeruginosa, group A Streptococcus, N. meningitidis*
	Microarray assembly of tags	*S. cerevisiae, Desulfovibrio desulfuricans, Shewanella oneidensis*
*The choice of a specific method of mutagenesis is depends on its applicability to organism of interest		

3.6.2 Signature Sequence Mutagenesis (SSM)

The Signature Sequence Mutagenesis (SSM), a variation of STM, is a transposon-based strategy that was employed to identify *M. gallisepticum* genes involved in the colonization of the chicken respiratory tract (59). Unlike STM, sequencing of transposon mutants prior to screening is necessary in SSM. Detection of attenuated mutants is through PCR instead of hybridization. Each mutant is identified by a 'signature sequence' generated by PCR reactions using a common Tn-specific primer and separate gene-specific primers, which are designed either upstream or downstream of the Tn-insertion site (59). Input pool PCR generated from the input pool of mutants injected into the chickens was compared with the output PCR corresponding to the mutants recovered from the animals at necropsy. Mutants absent in the output PCR but present in the input PCR represent attenuation *in vivo*. *M. gallisepticum* is the only mycoplasma where negative selection-based strategies have been used *in vivo* in the natural host to identify virulence genes using a pool of mutants (59, 60). In this project, the SSM PCR technique has been employed to identify *M. agalactiae* mutant/s that are unable to survive in experimentally infected sheep via the intramammary route.

3.7 Project Background

Genetic manipulation of *M. agalactiae* through transposon mutagenesis had been achieved and was employed to generate a transposon mutant library in the wild type strain PG2. Transposition was performed using the vector pISM2062 carrying the Tn*4001*mod transposon (72). Several different genomic

loci were found disrupted in the resulting mutants. More than 150 mutants were sequenced using a novel Quantitative Target Display (QTD) PCR-based method (73). The disrupted genes belonged to different functional categories, such as lipoproteins, transporters, metabolic enzymes and other important categories (Table 3). Genes with no known function were also disrupted and they constituted a significantly high number in the library. This transposon mutant library was used to study the role of the disrupted genes in *M. agalactiae* pathogenesis.

Several *in vitro* studies were performed to assess the role of each disrupted gene. Invasion into non-phagocytic cells has been shown for many mycoplasmas (12, 20) but not for *M. agalactiae*. As described above, cell invasion is an important strategy employed by pathogenic bacteria to persist inside the host cells and to avoid host immune response (2). Since *M. agalactiae* is known to cause chronic and persistent infections, its cell invasion capability was evaluated *in vitro* using different mammalian cell lines and *in vivo* during experimental sheep infection.

Table 3. Selected transposon mutants of *M. agalactiae* belonging to different functional categories that were used in experimental sheep infection studies

Locus tag	Gene description	Locus tag	Gene description
Lipoprotein/predicted lipoproteins		**Hypothetical Proteins**	
MAG2540	Vpma like lipoprotein	MAG1890	Hypothetical Protein
MAG7070	VpmaX	MAG4010	Hypothetical Protein
MAG7080	VpmaY	MAG2810	Hypothetical Protein
MAG6200	Predicted lipoprotein	MAG0110	Hypothetical Protein
MAG1450	Predicted lipoprotein	MAG0390	Hypothetical protein
MAG4460	Predicted lipoprotein	MAG3390	Hypothetical Protein
MAG6410	Lipoprotein	MAG3120	Hypothetical Protein
MAG1050	Lipoprotein	MAG0250	Hypothetical Protein
Transporters		MAG1570	Hypothetical Protein
MAG0370	Oligopeptide ABC transporter permease protein (OppB)	MAG3650	Hypothetical Protein
MAG0360	Oligopeptide ABC transporter permease protein (OppC)	**Metabolism**	
MAG0340	Oligopeptide ABC transporter permease protein (OppF)	MAG6330	L-ribulose-5-phosphate 4-epimerase
MAG4970	Hexose phosphate transport protein (UhpT)	MAG6300	Phosphoenolpyruvate phosphotransferase (PtsL)
MAG2320	Glycerol ABC transporter permease component (GtsB)	MAG0040	Esterase/Lipase (lip)
MAG5960	ABC transporter ATP-binding protein	MAG0940	Pyruvate dehydrogenase beta subunit (PdhB)
MAG6380	PTS system ascorbate-specific transporter subunit IIC (UlaA)	MAG1390	Phosphate acetyltransferase (EutD)
Other important genes		MAG4340	Alcohol dehydrogenase (AdhT)
MAG5920	Elongation factor G (FusA)	MAG0070	Lipoate protein ligase A (LplA)
MAG6730	Robonuclease HII (RnhII)	MAG0960	Dihydrolipoamide dehydrogenase (PdhD)
MAG3820	GTP-dependent nucleic acid-binding protein (EngD)	MAG2630	NADH oxidase (Nox)
MAG1790	DNA methylase	MAG0180	Deoxyguaosine kinase
MAG2930	F0F1 ATP synthase subunit alpha (AtpA)	MAG6360	Pentitol phosphotransferase enzyme II
MAG5520	Aminopeptidase		
MAG6310	Transcriptional regulator		
MAG3740	Cell division protein (MraZ)		
MAG1530	Type III restriction-modification system: methylase		

3.8 Project Description

The main goal of this dissertation project was to identify and study the factors involved in *M. agalactiae* pathogenesis. This project design was sub-divided into three parts.

1. Identification of genetic factors involved in host cell interaction

A previously prepared *M. agalactiae* transposon mutant library was used to screen the genes involved in colonization of the sheep udder. Pools of mutants were screened in an experimental sheep intramammary infection study using SSM PCR as a negative selection method. Pools of 15-19 mutants were screened in two successive cycles of sheep infection and resulted in the identification of a total of 14 different mutants that were unable to colonize the udder and other different host niches upon systemic spread. Among these 14 mutants, 7 were attenuated at local sites of infection, i.e. in udder, lymph nodes and milk samples, whereas the other 7 mutants were additionally found to be missing in distant body sites. Some of the attenuated mutants demonstrated growth deficiency during co-cultivation assays with HeLa cells and during cytotoxicity assays with murine macrophages as compared to the wild type strain. Taken together, these results imply a role of different set of genes in the pathogenesis of *M. agalactiae*.

2. *In vitro* and *in vivo* cell invasion and systemic spreading of *M. agalactiae* in the sheep infection model

The aim was to check the ability of *M. agalactiae* to invade non-phagocytic cells. This was assessed using HeLa cells as standard epithelial cell line and also using bovine endometrium (BEND) and bovine lung fibroblast (BLF) cell lines. The ability of *M. agalactiae* to invade these cell lines was determined using the gentamicin invasion assay and the double immunofluorescence technique (DIF) assay. With both methods the ability of *M. agalactiae* to invade non-phagocytic cells was demonstrated. When tested *in vivo* in the sheep intramammary

infection model using the wild type PG2 strain, the results showed spreading of *M. agalactiae* to distant body sites, such as brain, lungs, spleen and uterus. Dissemination to distant host niches and intracellular presence of *M. agalactiae* in epithelial and macrophage cells of the udder and other organs was clearly demonstrated by immunohistochemistry (IHC), culture and *M. agalactiae*-specific PCR. The results provided the first proof of *M. agalactiae*'s ability to invade host cells, both *in vitro* and *in vivo.*

3. Role of PDHB in *in vitro* growth and host cell invasion

Several *in vitro* assays were performed in order to assess the role of mutated genes in different cellular functions. Among the analyzed mutants, *pdhB* disrupted mutants demonstrated a growth deficiency in axenic medium. This mutant also showed deficiency in HeLa cell invasion as analyzed by DIF and exhibited different and smaller colony morphology as compared to the wild type strain. In order to confirm the role of the *pdhB* gene in growth, colony morphology and cell invasion phenotypes of *M. agalactiae*, the *pdhB* mutant was complemented with a copy of the intact *pdhB* gene. The complementation was performed by first constructing a new complementation vector carrying the *lacZ* reporter gene and a strong *vpma* promoter. The wild type copy of the *pdhB* gene was then cloned in this vector and transformed into the mutant to assess the functional complementation in terms of *in vitro* growth, colony morphology and cell invasion. The results demonstrated reversion to the wild type phenotypes indicating the role of the *pdhB* gene in cell invasion, growth and colony morphology.

3.9 References

1. **Sirand-Pugnet P, Citti C, Barre A, Blanchard A.** 2007. Evolution of *Mollicutes*: down a bumpy road with twists and turns. Res Microbiol **158:**754-766.
2. **Rosengarten R, Citti C, Glew M, Lischewski A, Droesse M, Much P, Winner F, Brank M, Spergser J.** 2000. Host-pathogen interactions in mycoplasma pathogenesis: virulence and survival strategies of minimalist prokaryotes. Int J Med Microbiol **290:**15-25.
3. **Rottem S, Barile MF.** 1993. Beware of mycoplasmas. Trends Biotechnol **11:**143-151.
4. **Hutchison CA, Peterson SN, Gill SR, Cline RT, White O, Fraser CM, Smith HO, Venter JC.** 1999. Global transposon mutagenesis and a minimal Mycoplasma genome. Science **286:**2165-2169.
5. **Dybvig K.** 1990. Mycoplasmal genetics. Annu Rev Microbiol **44:**81-104.
6. **Gibson DG, Glass JI, Lartigue C, Noskov VN, Chuang RY, Algire MA, Benders GA, Montague MG, Ma L, Moodie MM, Merryman C, Vashee S, Krishnakumar R, Assad-Garcia N, Andrews-Pfannkoch C, Denisova EA, Young L, Qi ZQ, Segall-Shapiro TH, Calvey CH, Parmar PP, Hutchison CA, 3rd, Smith HO, Venter JC.** 2010. Creation of a bacterial cell controlled by a chemically synthesized genome. Science **329:**52-56.
7. **Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, Hutchison CA, 3rd, Smith HO, Venter JC.** 2006. Essential genes of a minimal bacterium. Proc Natl Acad Sci U S A **103:**425-430.
8. **Citti C, Blanchard A.** 2013. Mycoplasmas and their host: emerging and re-emerging minimal pathogens. Trends Microbiol **21:**196-203.
9. **Frey J.** 2002. Mycoplasmas of animals, p. 73-90. *In* Razin S, Herrmann, R. (ed.), Molecular Biology and Pathogenicity of Mycoplasmas. Kluwer Academic/Plenum, Publishers, NewYork.
10. **Razin S, Yogev D, Naot Y.** 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiol Mol Biol Rev **62:**1094-1156.
11. **Rosengarten R, Citti C, Much P, Spergser J, Droesse M, Hewicker-Trautwein M.** 2001. The changing image of mycoplasmas: from innocent bystanders to emerging and reemerging pathogens in human and animal diseases. Contrib Microbiol **8:**166-185.
12. **Rottem S.** 2003. Interaction of mycoplasmas with host cells. Physiol Rev **83:**417-432.
13. **Tryon V, Baseman J.** 1992. Pathogenic Determinants and Mechanisms, p. 457-471. *In* Maniloff J (ed.), Mycoplasmas: Molecular Biology and Pathogenesis. American Society for Microbiology, Washington, DC.
14. **Razin S, Jacobs E.** 1992. Mycoplasma adhesion. J Gen Microbiol **138:**407-422.
15. **Razin S, Kahane I, Banai M, Bredt W.** 1981. Adhesion of mycoplasmas to eukaryotic cells. Ciba Found Symp **80:**98-118.

16. **Schreiner SA, Sokoli A, Felder KM, Wittenbrink MM, Schwarzenbach S, Guhl B, Hoelzle K, Hoelzle LE.** 2012. The surface-localised α-enolase of *Mycoplasma suis* is an adhesion protein. Vet Microbiol **156:**88-95.
17. **Song Z, Li Y, Liu Y, Xin J, Zou X, Sun W.** 2012. alpha-Enolase, an adhesion-related factor of *Mycoplasma bovis*. PLoS One **7:**e38836.
18. **Thomas C, Jacobs E, Dumke R.** 2013. Characterization of pyruvate dehydrogenase subunit B and enolase as plasminogen-binding proteins in *Mycoplasma pneumoniae*. Microbiology **159:**352-365.
19. **Dumke R, Hausner M, Jacobs E.** 2011. Role of *Mycoplasma pneumoniae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mediating interactions with the human extracellular matrix. Microbiology **157:**2328-2338.
20. **Fürnkranz U, Siebert-Gulle K, Rosengarten R, Szostak MP.** 2013. Factors influencing the cell adhesion and invasion capacity of *Mycoplasma gallisepticum*. Acta Vet Scand **55:**63.
21. **Yavlovich A, Katzenell A, Tarshis M, Higazi AA, Rottem S.** 2004. *Mycoplasma fermentans* binds to and invades HeLa cells: involvement of plasminogen and urokinase. Infect Immun **72:**5004-5011.
22. **Citti C, Nouvel LX, Baranowski E.** 2010. Phase and antigenic variation in mycoplasmas. Future Microbiol **5:**1073-1085.
23. **Browning GF, Marenda MS, Noormohammadi AH, Markham PF.** 2011. The central role of lipoproteins in the pathogenesis of mycoplasmoses. Vet Microbiol **153:**44-50.
24. **McAuliffe L, Ellis RJ, Miles K, Ayling RD, Nicholas RA.** 2006. Biofilm formation by *Mycoplasma* species and its role in environmental persistence and survival. Microbiology **152:**913-922.
25. **Simmons WL, Dybvig K.** 2007. Biofilms protect *Mycoplasma pulmonis* cells from lytic effects of complement and gramicidin. Infect Immun **75:**3696-3699.
26. **Jarrell KF, McBride MJ.** 2008. The surprisingly diverse ways that prokaryotes move. Nat Rev Microbiol **6:**466-476.
27. **Miyata M, Nakane D.** 2014. Gliding Mechanism of the *Mycoplasma pneumoniae* Subgroup, p. 237-254. *In* Browning GF, Citti C (ed.), Mollicutes: Molecular Biology and Pathogenesis. Caister Academic Press, Norfolk, UK.
28. **Szczepanek SM, Majumder S, Sheppard ES, Liao X, Rood D, Tulman ER, Wyand S, Krause DC, Silbart LK, Geary SJ.** 2012. Vaccination of BALB/c mice with an avirulent *Mycoplasma pneumoniae* P30 mutant results in disease exacerbation upon challenge with a virulent strain. Infect Immun **80:**1007-1014.
29. **Hasselbring BM, Page CA, Sheppard ES, Krause DC.** 2006. Transposon mutagenesis identifies genes associated with *Mycoplasma pneumoniae* gliding motility. J Bacteriol **188:**6335-6345.
30. **Sirand-Pugnet P, Lartigue C, Marenda M, Jacob D, Barre A, Barbe V, Schenowitz C, Mangenot S, Couloux A, Segurens B, de Daruvar A, Blanchard A, Citti C.** 2007. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. PLoS Genet **3:**e75.

31. **Bergonier D, Berthelot X, Poumarat F.** 1997. Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control. Rev Sci Tech **16:**848-873.
32. **Bergonier D, Poumarat F.** 1996. [Contagious agalactia of small ruminants: epidemiology, diagnosis and control]. Rev Sci Tech **15:**1431-1475.
33. **Corrales JC, Esnal A, De la Fe C, Sánchez A, Assunçao P, Poveda JB, Contreras A.** 2007. Contagious agalactia in small ruminants. Small Rumin Res **68:**154-166.
34. **DaMassa AJ, Wakenell PS, Brooks DL.** 1992. Mycoplasmas of goats and sheep. J Vet Diagn Invest **4:**101-113.
35. **Chopra-Dewasthaly R, Baumgartner M, Gamper E, Innerebner C, Zimmermann M, Schilcher F, Tichy A, Winter P, Jechlinger W, Rosengarten R, Spergser J.** 2012. Role of Vpma phase variation in *Mycoplasma agalactiae* pathogenesis. FEMS Immunol Med Microbiol **66:**307-322.
36. **Gomez-Martin A, De la Fe C, Amores J, Sanchez A, Contreras A, Paterna A, Buendia AJ, Corrales JC.** 2012. Anatomic location of *Mycoplasma mycoides subsp. capri* and *Mycoplasma agalactiae* in naturally infected goat male auricular carriers. Vet Microbiol **157:**355-362.
37. **Nicholas RA.** 2002. Improvements in the diagnosis and control of diseases of small ruminants caused by mycoplasmas. Small Ruminant Research **45:**145-149

.
38. **Bergonier D, Berthelot X, Rupp R, De Cremoux R, Lagriffoul G.** 2002. Le point Veterinaire **33:**43-45.
39. **Gomez-Martin A, Amores J, Paterna A, De la Fe C.** 2013. Contagious agalactia due to *Mycoplasma* spp. in small dairy ruminants: Epidemiology and prospects for diagnosis and control. Vet J **198:**48-56.
40. **Glew MD, Papazisi L, Poumarat F, Bergonier D, Rosengarten R, Citti C.** 2000. Characterization of a multigene family undergoing high-frequency DNA rearrangements and coding for abundant variable surface proteins in *Mycoplasma agalactiae*. Infect Immun **68:**4539-4548.
41. **Chopra-Dewasthaly R, Citti C, Glew MD, Zimmermann M, Rosengarten R, Jechlinger W.** 2008. Phase-locked mutants of *Mycoplasma agalactiae*: defining the molecular switch of high-frequency Vpma antigenic variation. Mol Microbiol **67:**1196-1210.
42. **Czurda S, Jechlinger W, Rosengarten R, Chopra-Dewasthaly R.** 2010. Xer1-mediated site-specific DNA inversions and excisions in *Mycoplasma agalactiae*. J Bacteriol **192:**4462-4473.
43. **Chopra-Dewasthaly R, Zimmermann M, Rosengarten R, Citti C.** 2005. First steps towards the genetic manipulation of *Mycoplasma agalactiae* and *Mycoplasma bovis* using the transposon Tn*4001*mod. Int J Med Microbiol **294:**447-453.

44. **Baranowski E, Guiral S, Sagne E, Skapski A, Citti C.** 2010. Critical role of dispensable genes in *Mycoplasma agalactiae* interaction with mammalian cells. Infect Immun **78:**1542-1551.
45. **Skapski A, Hygonenq MC, Sagne E, Guiral S, Citti C, Baranowski E.** 2011. Genome-scale analysis of M*ycoplasma agalactiae* loci involved in interaction with host cells. PLoS One **6:**e25291.
46. **Baranowski E, Bergonier D, Sagne E, Hygonenq MC, Ronsin P, Berthelot X, Citti C.** 2014. Experimental infections with *Mycoplasma agalactiae* identify key factors involved in host-colonization. PLoS One **9:**e93970.
47. **Khan LA, Miles RJ, Nicholas RA.** 2005. Hydrogen peroxide production by *Mycoplasma bovis* and *Mycoplasma agalactiae* and effect of in vitro passage on a *Mycoplasma bovis* strain producing high levels of H_2O_2. Vet Res Commun **29:**181-188.
48. **Fleury B, Bergonier D, Berthelot X, Peterhans E, Frey J, Vilei EM.** 2002. Characterization of P40, a cytadhesin of *Mycoplasma agalactiae*. Infect Immun **70:**5612-5621.
49. **Addis MF, Pisanu S, Ghisaura S, Pagnozzi D, Marogna G, Tanca A, Biosa G, Cacciotto C, Alberti A, Pittau M, Roggio T, Uzzau S.** 2011. Proteomics and pathway analyses of the milk fat globule in sheep naturally infected by *Mycoplasma agalactiae* provide indications of the in vivo response of the mammary epithelium to bacterial infection. Infect Immun **79:**3833-3845.
50. **Halbedel S, Stülke J.** 2007. Tools for the genetic analysis of Mycoplasma. Int J Med Microbiol **297:**37-44.
51. **Burgos R, Pich OQ, Querol E, Pinol J.** 2007. Functional analysis of the *Mycoplasma genitalium* MG312 protein reveals a specific requirement of the MG312 N-terminal domain for gliding motility. J Bacteriol **189:**7014-7023.
52. **Lee SW, Browning GF, Markham PF.** 2008. Development of a replicable *ori*C plasmid for *Mycoplasma gallisepticum* and *Mycoplasma imitans*, and gene disruption through homologous recombination in *M. gallisepticum*. Microbiology **154:**2571-2580.
53. **Sluijter M, Hoogenboezem T, Hartwig NG, Vink C.** 2008. The *Mycoplasma pneumoniae* MPN229 gene encodes a protein that selectively binds single-stranded DNA and stimulates recombinase A-mediated DNA strand exchange. BMC Microbiol **8:**167.
54. **Sluijter M, Spuesens EB, Hartwig NG, van Rossum AM, Vink C.** 2009. The *Mycoplasma pneumoniae* MPN490 and *Mycoplasma genitalium* MG339 genes encode reca homologs that promote homologous DNA strand exchange. Infect Immun **77:**4905-4911.
55. **Janis C, Lartigue C, Frey J, Wroblewski H, Thiaucourt F, Blanchard A, Sirand-Pugnet P.** 2005. Versatile use of *ori*C plasmids for functional genomics of *Mycoplasma capricolum* subsp. *capricolum*. Appl Environ Microbiol **71:**2888-2893.
56. **Dybvig K, Cao Z, French CT, Yu H.** 2007. Evidence for type III restriction and modification systems in *Mycoplasma pulmonis*. J Bacteriol **189:**2197-2202.

57. **Mudahi-Orenstein S, Levisohn S, Geary SJ, Yogev D.** 2003. Cytadherence-deficient mutants of *Mycoplasma gallisepticum* generated by transposon mutagenesis. Infect Immun **71:**3812-3820.
58. **French CT, Lao P, Loraine AE, Matthews BT, Yu H, Dybvig K.** 2008. Large-scale transposon mutagenesis of *Mycoplasma pulmonis*. Mol Microbiol **69:**67-76.
59. **Hudson P, Gorton TS, Papazisi L, Cecchini K, Frasca S, Jr., Geary SJ.** 2006. Identification of a virulence-associated determinant, dihydrolipoamide dehydrogenase (*lpd*), in *Mycoplasma gallisepticum* through in vivo screening of transposon mutants. Infect Immun **74:**931-939.
60. **Tseng CW, Kanci A, Citti C, Rosengarten R, Chiu CJ, Chen ZH, Geary SJ, Browning GF, Markham PF.** 2013. MalF is essential for persistence of *Mycoplasma gallisepticum* in vivo. Microbiology **159:**1459-1470.
61. **Chopra-Dewasthaly R, Marenda M, Rosengarten R, Jechlinger W, Citti C.** 2005. Construction of the first shuttle vectors for gene cloning and homologous recombination in *Mycoplasma agalactiae*. FEMS Microbiol Lett **253:**89-94.
62. **Kittelberger R, O'Keefe JS, Meynell R, Sewell M, Rosati S, Lambert M, Dufour P, Pepin M.** 2006. Comparison of four diagnostic tests for the identification of serum antibodies in small ruminants infected with *Mycoplasma agalactiae*. N Z Vet J **54:**10-15.
63. **Tola S, Manunta D, Rocca S, Rocchigiani AM, Idini G, Angioi PP, Leori G.** 1999. Experimental vaccination against *Mycoplasma agalactiae* using different inactivated vaccines. Vaccine **17:**2764-2768.
64. **Sanchis R, Abadie G, Lambert M, Cabasse E, Guibert JM, Calamel M, Dufour P, Vitu C, Vignoni M, Pépin M.** 1998. Experimental conjunctival-route infection with *Mycoplasma agalactiae* in lambs. Small Rumin Res **27:**31-39.
65. **de la Fe C, Castro-Alonso A, Herraez P, Poveda JB.** 2011. Recovery of *Mycoplasma agalactiae* from the ears of goats experimentally infected by the intramammary route. Vet J **190:**94-97.
66. **Lipsitch M, Moxon ER.** 1997. Virulence and transmissibility of pathogens: what is the relationship? Trends Microbiol **5:**31-37.
67. **Chiang SL, Mekalanos JJ, Holden DW.** 1999. In vivo genetic analysis of bacterial virulence. Annu Rev Microbiol **53:**129-154.
68. **Mahan MJ, Slauch JM, Mekalanos JJ.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686-688.
69. **Hensel M, Shea JE, Gleeson C, Jones MD, Dalton E, Holden DW.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400-403.
70. **Mazurkiewicz P, Tang CM, Boone C, Holden DW.** 2006. Signature-tagged mutagenesis: barcoding mutants for genome-wide screens. Nat Rev Genet **7:**929-939.
71. **Saenz HL, Dehio C.** 2005. Signature-tagged mutagenesis: technical advances in a negative selection method for virulence gene identification. Curr Opin Microbiol **8:**612-619.

72. **Knudtson KL, Minion FC.** 1993. Construction of Tn*4001* lac derivatives to be used as promoter probe vectors in mycoplasmas. Gene **137:**217-222.
73. **Sharma VM, Chopra R, Ghosh I, Ganesan K.** 2001. Quantitative target display: a method to screen yeast mutants conferring quantitative phenotypes by 'mutant DNA fingerprints'. Nucleic Acids Res **29:**E86-86.

4 Results and Publications

4.1 Publication: In vitro and in vivo cell invasion and systemic spreading of *Mycoplasma agalactiae* in the sheep infection model.

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In vitro and in vivo cell invasion and systemic spreading of *Mycoplasma agalactiae* in the sheep infection model

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ABSTRACT

Generally regarded as extracellular pathogens, molecular mechanisms of mycoplasma persistence, chronicity and disease spread are largely unknown. *Mycoplasma agalactiae*, an economically important pathogen of small ruminants, causes chronic infections that are difficult to eradicate. Animals continue to shed the agent for several months and even years after the initial infection, in spite of long antibiotic treatment. However, little is known about the strategies that *M. agalactiae* employs to survive and spread within an immunocompetent host to cause chronic disease. Here, we demonstrate for the first time its ability to invade cultured human (HeLa) and ruminant (BEND and BLF) host cells. Presence of intracellular mycoplasmas is clearly substantiated using differential immunofluorescence technique and quantitative gentamicin invasion assays. Internalized *M. agalactiae* could survive and exit the cells in a viable state to repopulate the extracellular environment after complete removal of extracellular bacteria with gentamicin. Furthermore, an experimental sheep intramammary infection was carried out to evaluate its systemic spread to organs and host niches distant from the site of initial infection. Positive results obtained via PCR, culture and immunohistochemistry, especially the latter depicting the presence of *M. agalactiae* in the cytoplasm of mammary duct epithelium and macrophages, clearly provide the first formal proof of *M. agalactiae's* capability to translocate across the mammary epithelium and systemically disseminate to distant inner organs. Altogether, the findings of these in vitro and in vivo studies indicate that *M. agalactiae* is capable of entering host cells and this might be the strategy that it employs at a population level to ward off the host immune response and antibiotic action, and to disseminate to new and safer niches to later egress and once again proliferate upon the return of favorable conditions to cause persistent chronic infections.

Introduction

Commonly described as the smallest and simplest bacteria, mycoplasmas are important pathogens of humans and animals with rather complex and sophisticated pathogenic attributes (Rosengarten et al., 2000). Having lost many of their metabolic pathways during a so-called degenerative evolution from a Gram-positive ancestor, these wall-less prokaryotes readily obtain their nutrition from host cells by mostly colonizing epithelial surfaces and thereby cause slow-progressing chronic diseases that are difficult to cure (Razin et al., 1998). Well-known for their antigenic variation systems, they have adapted sophisticated mechanisms to evade immune clearance, survive in the host and have evolved to infect new host niches (Rottem and Barile, 1993; Chopra-Dewasthaly et al., 2012; Citti and Blanchard, 2013). Mycoplasmas lack typical bacterial virulence factors like toxins, and the molecular determinants of their pathogenicity are largely unknown. This can be partially attributed to their fastidious and slow growth, relative recalcitrance to genetic manipulations, and also to their strict host-specificity that hinders the development of convenient small animal models (Razin et al., 1998; Citti and Blanchard, 2013).

Mycoplasma agalactiae is an economically important pathogen and the main etiological agent of contagious agalactia (CA) syndrome in sheep and goats, mainly characterized by mastitis,

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conjunctivitis and arthritis as predominant symptoms of a localized infection. Sporadically septicaemia, arthritis, pneumonia and reproductive disorders have also been reported (Bergonier et al., 1997; Gomez-Martin et al., 2013), indicating that the pathogen is capable of crossing the epithelial barrier to reach distant host niches, as also evidenced in a recent report where naturally infected asymptomatic male goats were shown to harbour *M. agalactiae* in atypical inner organs like brain and heart (Gomez-Martin et al., 2012). Nevertheless, *M. agalactiae* has so far been regarded as an extracellular parasite and it is unknown how it transforms local infections into systemic ones.

Persistence and chronicity are other hallmarks of *M. agalactiae* infections. Both diseased and asymptomatic animals continue to shed the pathogen for long periods of time, sometimes lasting up to several years (Bergonier et al., 1997). Antibiotic treatments are often unsuccessful as they can only reduce clinical symptoms but tend to promote the carriers that stay unaffected (Nicholas, 2002). Chronically infected and serologically negative herds with no signs of disease are a common clinical-epidemiological situation in endemic areas. Such animals easily escape disease control and eradication measures, and are capable of flaring up frequent CA outbreaks under stress conditions (Gomez-Martin et al., 2013) leading to huge economic losses. Despite such agronomical significance, *M. agalactiae's* pathogenic determinants and mechanisms of infection and persistence are largely unknown, a fact that can be attributed to its long resistance to genetic manipulation until 2005 (Chopra-Dewasthaly et al., 2005a, b), and also because it does not exhibit the more practical phenotypes associated with mycoplasma pathogenicity, such as hemadsorption and the presence of terminal tip structure as attachment organelle, and lacks convenient small animal models or cell lines for appropriate studies.

M. agalactiae demonstrates surface antigenic diversity via high-frequency switching of six immunodominant surface lipoproteins (Vpmas) caused by Xer1 recombinase encoded on the same pathogenicity island-like locus (Glew et al., 2002; Chopra-Dewasthaly et al., 2008; Czurda et al., 2010). Though lacking in concrete proof, such variable systems are often believed to play important roles in pathogenicity via host immune evasion and adaptation. Our data from a recent experimental infection study with Xer1-disrupted Vpma 'phase-locked' mutants (Chopra-Dewasthaly et al., 2008) clearly demonstrated that Xer1 is not a virulence factor of *M. agalactiae* and Vpma phase variation is not necessary for establishing infection though it might critically influence the survival and persistence of the pathogen under natural field conditions (Chopra-Dewasthaly et al., 2012). P40, a cytadhesin, and P48 with macrophage stimulatory activity, are two other lipoproteins, which seem to have important pathogenicity related attributes (Rosati et al., 1999; Fleury et al., 2002). Besides, production of hydrogen peroxide (Khan et al., 2005), biofilm formation (McAuliffe et al., 2006) and identification of genes involved in indirect host cell interactions (Baranowski et al., 2010) are also implicated in *M. agalactiae's* pathogenicity.

In view of the prevailing scenario, we tried to investigate whether *M. agalactiae* has the capacity to enter, survive and exit the eukaryotic host cells in a viable state, as this could explain the chronic, persistent and difficult-to-eradicate nature of its infections in spite of long antibiotic therapies. This phenomenon may also allow it to reach more favorable host niches by crossing the epithelial barrier as cell invasion is often considered a major factor for systemic spread (Cieri et al., 2002; Much et al., 2002). Here, we provide evidence for the first time that *M. agalactiae* is able to invade eukaryotic host cells whereby quantitative results are supported by the qualitative double immunofluorescence assay. Intracellular mycoplasmas were detected not only after in vitro infection but also in vivo in various tissue samples from experimentally infected sheep using immunohistochemistry. Also, by the isolation of mycoplasmas from various internal organs of sheep experimentally infected via the intramammary route we formally demonstrate that *M. agalactiae* has the capability to cross local epithelial barriers and to disseminate to distant body sites. The findings of this study, together with the sophisticated antigenic variation system, could explain the persistence and chronicity of *M. agalactiae* infections.

Materials and methods

Mycoplasma growth

M. agalactiae pathogenic type strain PG2 (Sirand-Pugnet et al., 2007) was used in this study and was previously isolated from an infected goat in Spain (Fleury et al., 2002). It was grown in Aluotto or SP4 medium supplemented with penicillin, pyruvate, and phenol red as indicator as described before (Chopra-Dewasthaly et al., 2005b). Mycoplasma cultures were grown for 48 h and diluted serially in minimal essential medium (MEM) supplemented with non-essential amino acids and 10% heat inactivated fetal bovine serum (FBS) (Gibco BRL, Life Technologies) prior to infection of cultured mammalian cells. Number of viable mycoplasmas at the time of infection was determined by plating serial dilutions on SP4 plates containing 1% (wt/vol) Difco Noble agar and counting colonies under BMS 74955 stereomicroscope after 4–5 days of incubation at 37 °C.

Cell culture

HeLa-229 (ATCC CCL-2.1), Bovine endometrium cell line BEND (ATCC CRL-2398) and Buffalo lung fibroblasts (BLF; ATCC IMR-31) were the cell lines used in this study and were purchased from the American Type Culture Collection (ATCC; Manassas, USA) and certified to be free of mycoplasmas. HeLa-229 was maintained in MEM, BLF in McCoy's 5a medium (Sigma) with 10% heat inactivated FBS, and BEND cells in 1:1 mixture of Hams F12 and Eagle's MEM with Earle's BSS (Sigma-Aldrich) as per the instructions of ATCC. Trypsin and PBS were purchased from PAA Laboratories GmbH, Pasching, Austria or Sigma-Aldrich. 1×10^4 cells/well were seeded into Lab-Tek II Chamber Slides (Nunc International, Naperville, IL) for immunofluorescence staining and 5×10^4 cells/well were seeded into 24-well plates (CELLSTAR® Greiner Bio-One GmbH, Germany) for the gentamicin invasion assay 48 h prior to infection to attain confluence. Cell cultures were regularly checked for mycoplasma contamination by culture and PCR.

Mycoplasma infection and gentamicin invasion assay

Gentamicin invasion assay was carried out as described before with some modifications (Elsinghorst, 1994; Winner et al., 2000). *M. agalactiae* was grown for about 48 h indicated by metabolic color change before pelleting at 10,000 × *g* at 4 °C for 10 min and resuspending in MEM. The cells were passed through 27-gauge needle for three to four times to disrupt any cell aggregates. Eukaryotic cells were infected with diluted cultures of mycoplasmas at a multiplicity of infection (MOI) of about 10–30 and incubated at 37 °C with 5% CO_2 for 4, 8, 16 and 24 h. Thereafter, extracellular bacteria were killed by incubation in MEM containing 400 μg/ml of gentamicin for an additional 3 h period. Although a concentration of 50 μg/ml gentamicin is known to be completely inhibitory for *M. agalactiae* growth (Chopra-Dewasthaly et al., 2005b), a higher concentration of 400 μg/ml was used to ensure the reliability of the assay and was experimentally determined to be sufficient to kill 100% of *M. agalactiae* in 3 h duration. After gentamicin treatment, supernatants were checked for the presence of any viable mycoplasmas by plating on SP4 agar. Subsequently, the cells were washed two to three times

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with PBS and trypsinized before making serial dilutions for plating on SP4 agar to quantify invaded mycoplasmas. Trypsinization did not cause any adverse effects on the mammalian cells as confirmed by the viable cell counts using trypan blue staining. Invasion frequency was calculated as percentage ratio of cfu of intracellular mycoplasmas to cfu of mycoplasmas added initially. Survival and exit of mycoplasmas from the eukaryotic cells was assessed by the same procedure as was described for invasion, except that after 24 h of infection, eukaryotic cells were further incubated in fresh MEM containing 50 μg/ml gentamicin for 8, 16 and 24 h. This lower concentration of gentamicin was checked to be sufficient to prevent the multiplication of mycoplasmas (Chopra-Dewasthaly et al., 2005b). Escape of invaded intracellular mycoplasmas from eukaryotic cells and their reinfection was monitored by incubating the cells in parallel wells under the same conditions in absence of gentamicin. Penicillin (50 μg/ml) was added here as a control antibiotic to prevent general contamination. Cells collected at 8, 16 and 24 h post gentamicin treatment were then serially diluted and plated on SP4 agar. The cfu obtained in presence and in absence of gentamicin were compared with the cfu of invaded mycoplasmas at 24 h pi. Viability of eukaryotic cells was checked regularly by trypan blue staining. All the above experiments were done in duplicates and performed at least thrice under the same conditions.

Raising M. agalactiae antiserum

M. agalactiae specific antiserum was generated in rabbits as described earlier (Ruhnke and Rosendal, 1994) by subcutaneous inoculation of 10^{10} cfu of *M. agalactiae* type strain PG2 at the Institute of Bacteriology, Mycology and Hygiene, Veterinary Medicine University of Vienna, Austria.

Double immunofluorescence assay

Double immunofluorescence assay was performed to visually detect *M. agalactiae* inside the eukaryotic cells. The assay was performed as originally described (Heesemann and Laufs, 1985) and previously applied on mycoplasmas (Winner et al., 2000) with minor modifications. Eukaryotic cells were seeded on eight-well Lab-Tek II Chamber Slides (Nalge Nunc International, Naperville, IL) in their respective media and confluent monolayers were infected with mycoplasmas at a MOI of approximately 20 as described above and incubated at 37 °C with 5% CO_2 for 24 h for optimal invasion. Unbound bacteria were washed away with PBS containing 2% BSA and chamber slides overlaid with 300 μl of 1:200 diluted rabbit anti-*M. agalactiae* serum and gently shaken for 30 min at room temperature. After washing away the excessive antiserum, the cells were covered with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G (IgG) (Invitrogen) as a secondary antibody for 20 min at room temperature with shaking to stain extracellular mycoplasmas. After air-drying, cells were permeabilized for antibody diffusion by successively treating with 50%, 70%, and 96% (vol/vol) ethanol and twice with 100% methanol for 1 min each. The cells were again air-dried prior to treatment with 300 μl of rabbit anti-*M. agalactiae* serum for 30 min before labeling with Texas Red-labeled goat antirabbit IgG (Invitrogen) as secondary antibody for 20 min to stain extracellular and intracellular bacteria. After washing, the nuclei were stained with 300 μl of 1:2000 diluted DAPI (Invitrogen). After final washing, the chambers were removed and cells mounted under a glass coverslip in 1:2 (vol/vol) glycerol-PBS containing 13% (wt/vol) Mowiol (Clariant, Muttenez, Switzerland) and 0.5% (wt/vol) *n*-propyl gallate (Sigma). The cells were examined under OLYMPUS AX 70 epifluorescence microscope with an oil immersion lens (magnification, ×100). Extracellular mycoplasmas were observed as green spots using FITC filter set, whereas the Texas Red filtering revealed both the extracellular and intracellular mycoplasmas as red spots. The two images were superimposed using the Soft Imaging System Cell* from Olympus, Muenster, Germany, whereby intracellular mycoplasmas appeared red and extracellular mycoplasmas appeared yellow due to the overlap of red and green color.

Systemic spread and reisolation of M. agalactiae from different inner organs of experimentally infected sheep

Clinically healthy lactating ewes of the local mountain breed, negative for major sheep pathogens (attested by routine bacteriological and PCR diagnostics) and also confirmed to be seronegative for *M. agalactiae* by two different commercial ELISA kits (Cypress Diagnostics, Langdorp, Belgium and IDEXX Montpellier SAS, Montpellier, France) were infected via the right teat canal as described earlier (Chopra-Dewasthaly et al., 2012). The inoculum, which consisted of 10^9 bacteria/sheep in 5 ml volume, was prepared as described before (Chopra-Dewasthaly et al., 2012) except that the pellet was resuspended in PBS (Gibco BRL, Life Technologies). The sheep were housed at the Veterinary Medicine University of Vienna, Austria, and infections carried out in accordance with the guidelines of the Austrian law for animal protection with the requisite official approval. Sheep were subjected to regular clinical and serological examination 1 week prior to the intramammary infection and during the entire experimental period of 2 weeks. Presence of bacteria in milk and in mucosa of eye, ear, nose, and genitals was checked regularly by culturing in Aluotto medium as described earlier (Chopra-Dewasthaly et al., 2012). The animals were humanely killed after a fortnight and various organs, such as spleen, lungs, kidneys, udders, heart, brain, uterus, liver and joint tissue, and also various lymph nodes including mandibular, parotid, medial and lateral retropharyngeal, superficial cervical, mediastinal, jejunal, mesenterial, medial iliac, popliteal and supramammary were obtained. A portion of each of these was immediately cultured and the rest of the samples stored at −80 °C in individual sterile vials for subsequent examination. Bacteria from these necropsied specimens were isolated by growing undiluted and diluted tissue samples in Aluotto medium at 37 °C for 7 days. Confirmation of *M. agalactiae* was made by standard biochemical and serological methods as described earlier (Chopra-Dewasthaly et al., 2012) and by performing *M. agalactaie* specific PCR based on 16S rRNA gene (Chavez Gonzalez et al., 1995). Host tissues found positive for *M. agalactiae* were cultured again in SP4 medium (Chopra-Dewasthaly et al., 2005b) for calculating quantitative mycoplasma loads per gram of tissue by excising, weighing and inoculating an estimated small part (about 1 cm^3) from the frozen −80 °C sample (Chopra-Dewasthaly et al., 2012).

In vivo detection of M. agalactiae by immunohistochemical analysis

Tissue samples collected from necropsied animals were fixed in 10% buffered formalin, alcohol dehydrated, embedded in paraffin wax and stained with haematoxylin-eosin and examined by light microscopy. Additional sections were stained for demonstration of mycoplasmas using a rabbit polyclonal antiserum raised against whole cell antigens of *M. agalactiae* (as described above in Section 2.4). Immunohistochemistry was performed using the HRP polymer method on a Lab Vision-Autostainer (Thermo Fisher Scientific, Fremont, CA). Briefly, the paraffin wax sections (2 μm) were mounted on positively charged glass slides (Superfrost plus; Menzel Glaeser, Braunschweig, Germany) and deparaffinized with Neo Clear® solution (Merck, Darmstadt, Germany) and rehydrated twice in 100% alcohol and once in 96% and 70% alcohol and finally in distilled water. Antigen retrieval was performed by heating the slides in citrate buffer (pH 6.0) in the Lab Vision PT Module (Thermo

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Fisher Scientific). To reduce nonspecific background staining due to endogenous peroxidase, slides were incubated in Hydrogen Peroxidase Block (Thermo Fisher Scientific) for 5 min, followed by a 10-min incubation in Ultravision Protein Block (Thermo Fisher Scientific). Subsequently, the sections were incubated with the primary antibody (rabbit anti – *M. agalactiae* serum 1:1500) for 30 min at room temperature, followed by the Primary Antibody Enhancer (Thermo Fisher Scientific) for 15 min, and finally with Large Volume HRP Polymer (Thermo Fisher Scientific) for 20 min. Large Volume DAB Plus Substrate System (Thermo Fisher Scientific) was used as chromogen for 5 min. After counterstaining with 1:8 diluted Mayer's Hematoxylin (Thermo Fisher Scientific) for 1 min, the sections were dehydrated in alcohols (70, 96, and 100%) and treated with Neo Clear® (Merck, Darmstadt, Germany) before mounting them in Neomount (Merck, Darmstadt, Germany) for microscopic examination.

Statistical analysis

Invasion rates are expressed as mean ± standard deviation (SD) of n independent values. The significance of differences between means of experiments was calculated by Student's t test using GraphPad Prism 5 (Graphpad Software Inc, CA, USA). Differences with $P < 0.05$ were considered significant.

Results

Entry of M. agalactiae into mammalian cells

The ability of *M. agalactiae* to invade mammalian cells was investigated by infecting three different mammalian cell lines with the pathogenic type strain PG2. Apart from HeLa, which is a standard epithelial cell line used in many mycoplasma invasion studies (Andreev et al., 1995; Winner et al., 2000; Yavlovich et al., 2004; Marques et al., 2010; Fürnkranz et al., 2013; Hopfe et al., 2013), gentamicin invasion assays were also performed on cultured ruminant cells, namely BEND and BLF. PG2 cells were incubated with these mammalian cells at an MOI of 10–30 for 4, 8, 16 and 24 h before subjecting them to gentamicin treatment to kill extracellular bacteria and then directly plated onto SP4 agar to enumerate viable intracellular bacteria by cfu counts. Each cfu represents an infected eukaryotic cell and might be actually corresponding to more than one mycoplasma cell residing in the same eukaryotic host cell. This implies that the actual invasion frequency might be higher than the calculated value as most infected cells were observed to harbor multiple mycoplasma cells in them (Fig. 1). The invasion frequency was expressed as percentage ratio of number of recovered intracellular bacteria to the number of bacteria used for the initial infection. For HeLa cells an increase in invasion frequencies was observed with increasing infection times (Fig. 2).

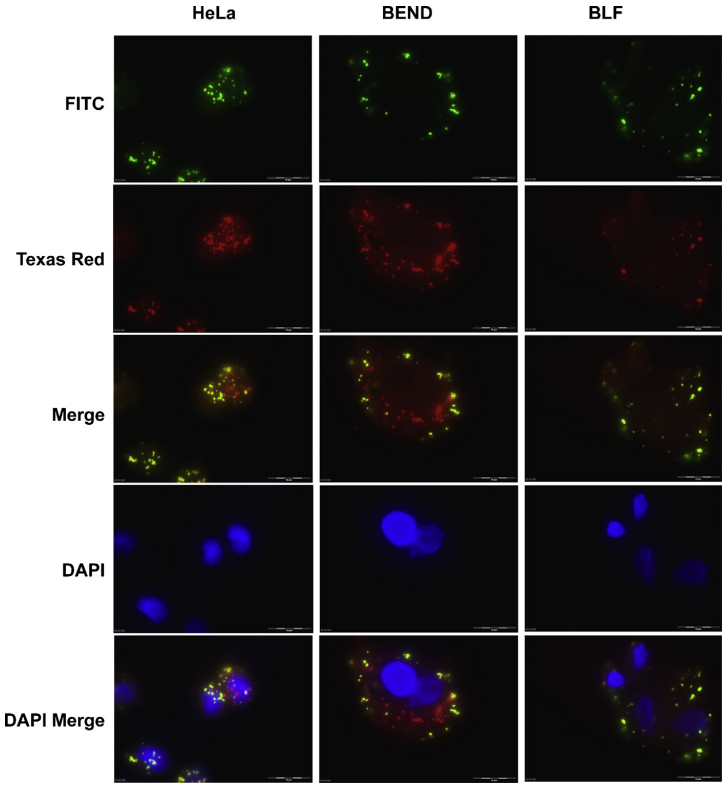

Fig. 1. Double immunofluorescence staining showing the invasion of *M. agalactiae* into HeLa-229 and BEND and BLF cells. The five panels correspond to the same area of the infected monolayer. FITC fluorescence showing extracellular mycoplasmas stained green, Texas Red fluorescence showing extracellular and intracellular mycoplasmas stained red, and DAPI fluorescence showing cell nuclei stained blue. Merged images indicating the localization of extracellular (yellow) and intracellular (red) mycoplasmas. Bars, 10 μm.

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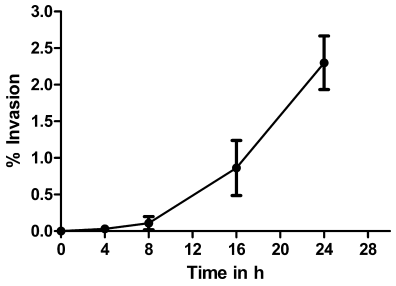

Fig. 2. Invasion of *M. agalactiae* type strain PG2 into cultured HeLa-229 cells at different times post infection. The percentage invasion was calculated by dividing the cfu value obtained after gentamicin treatment with the cfu value of total mycoplasmas added for infection and multiplied by 100. The data represent mean (±SD) of four independent experiments performed in duplicate.

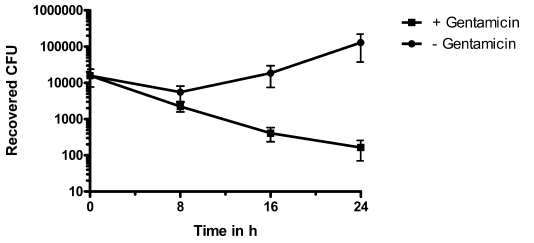

Fig. 3. Fate of *M. agalactiae* after entry into the eukaryotic cells. HeLa-229 cells were incubated with *M. agalactiae* type strain PG2 for 24 h followed by gentamicin treatment for 3 h at 37 °C. Cells were washed and incubated with fresh MEM with (black squares) or without (black circles) gentamicin for additional 8, 16 and 24 h, and then trypsinized and plated to enumerate the viable mycoplasmas. Mean values ± SD from three independent experiments performed in duplicate are indicated.

The invasion at 4 h post infection was quite low (0.032% ± 0.01) and thereafter increased exponentially with highest rates witnessed at 24 h post infection (2.28% ± 0.19). Such long infection periods and/or comparable invasion frequencies have earlier been reported for many bacterial pathogens, including mycoplasmas, which were predominantly known to be extracellular but were subsequently shown to have alternative intracellular lifestyle (Martin and Mohr, 2000; Dusanic et al., 2009; Marques et al., 2010; Buim et al., 2011; Hopfe et al., 2013; Lamberti et al., 2013). The percentage invasion frequency differed slightly for the three cell types. At 24 h post infection, the invasion frequency for BEND cells (2.14% ± 0.8) was comparable to HeLa but BLF cells demonstrated a relatively lower frequency of 1.14 ± 0.5. It was checked that incubation of mycoplasmas in MEM medium alone for this time period of 24 h yielded negligible or no increase in their numbers.

Invasion of *M. agalactiae* into non-phagocytic cells was further confirmed by double immunofluorescence staining as used for other mycoplasmas (Winner et al., 2000) with some modifications as mentioned above under Section 2.6. Since the gentamicin invasion assay had indicated optimal invasion rates at 24 h post infection, this time point was selected for fluorescence staining to enable proper readouts. The results clearly demonstrate the intracellular status of *M. agalactiae* as seen in Fig. 1, which shows five micrographs, each corresponding to the same area of infected HeLa, BEND and BLF cells. Extracellular mycoplasmas were observed as green spots using FITC filter set, whereas the Texas Red filtering revealed both the extracellular and intracellular mycoplasmas as red spots. The two images were superimposed and intracellular mycoplasmas appeared red. As controls, uninfected eukaryotic monolayers were also stained to rule out the possibility of any previous contamination with mycoplasmas and/or any cross-reactivity of antibodies. In agreement with the gentamicin invasion assay results, BLF cells showed comparatively fewer intracellular mycoplasmas via double immunofluorescence as compared to BEND and HeLa cells (Fig. 1).

Fate of M. agalactiae after entry into the eukaryotic cells

Cell invasion and subsequent intracellular survival, intermittent or prolonged, is an important strategy of many successful pathogens to evade the host immune response. Not just the entry but also the exit from the host cell is a critical step for an intracellular pathogen (Hybiske and Stephens, 2007, 2008; Friedrich et al., 2012; Lamberti et al., 2013). Therefore, fate of *M. agalactiae* was evaluated after its entry into HeLa cells. This was done by infecting HeLa cells with *M. agalactiae* for 24 h and further treating them with gentamicin to kill extracellular bacteria. This was followed by additional incubations for 8, 16, or 24 h in absence, as well as in presence of gentamicin at a concentration of 50 μg/ml. Viable intracellular bacteria were enumerated at these different times by cfu counts as described in Section 2.3. The results, as shown in Fig. 3, are very different for the cells incubated in presence of gentamicin as compared to those that were incubated in absence of the same. In presence of gentamicin, a continuous decrease in the cfu counts is observed until the last tested time point of 24 h of incubation. Compared to this, in absence of gentamicin the cfu count is not going down and rather shows an increase by 24 h of incubation. Two possibilities could explain the latter results whereby the bacteria are either replicating extracellularly or intracellularly. But intracellular multiplication is ruled out as parallel incubations in gentamicin show a continuous decrease in the cfu counts. Taken together, these results imply that intracellular *M. agalactiae* is released in a viable state into the surrounding medium and possibly multiplies extracellularly to reinfect new host cells, as observed in case of incubation in the absence of gentamicin. Since we did not see any cell death in parallel uninfected wells during 24 h infection, the exit of *M. agalactiae* is likely not due to cell lysis caused by culture overgrowth. As a control, presence of gentamicin was checked to have no adverse effects on the viability of eukaryotic cells.

In vivo systemic dissemination of M. agalactiae to distant body sites during experimental intramammary sheep infection

M. agalactiae causes chronic and persistent infections in small ruminants and the infected animals continue to shed the bacteria for several months, sometimes for several years (Bergonier et al., 1997; Gomez-Martin et al., 2013). Cell invasion is believed to play a major role in the systemic spreading of many pathogens, including mycoplasmas (Cieri et al., 2002; Much et al., 2002). Apart from a report describing the isolation of *M. agalactiae* from naturally infected goats (Gomez-Martin et al., 2012) there are hardly any such reports based on experimental infections of lactating sheep. However, sporadic isolation of *M. agalactiae* (P89) was observed in spleen and lungs of very few lambs experimentally infected via the conjunctival route (Sanchis et al., 1998). We wanted to formally address the ability of *M. agalactiae* to cross the epithelial barrier of the udder and disseminate throughout the body by demonstrating its isolation from various internal organs during an experimental intramammary sheep infection. Routine milk and mucosal swab samples, as well as the necropsied tissue and lymph node samples were checked for the reisolation of *M. agalactiae* via culture and PCR methods as described earlier (Chavez Gonzalez et al., 1995; Chopra-Dewasthaly et al., 2012). Data demonstrated the presence of

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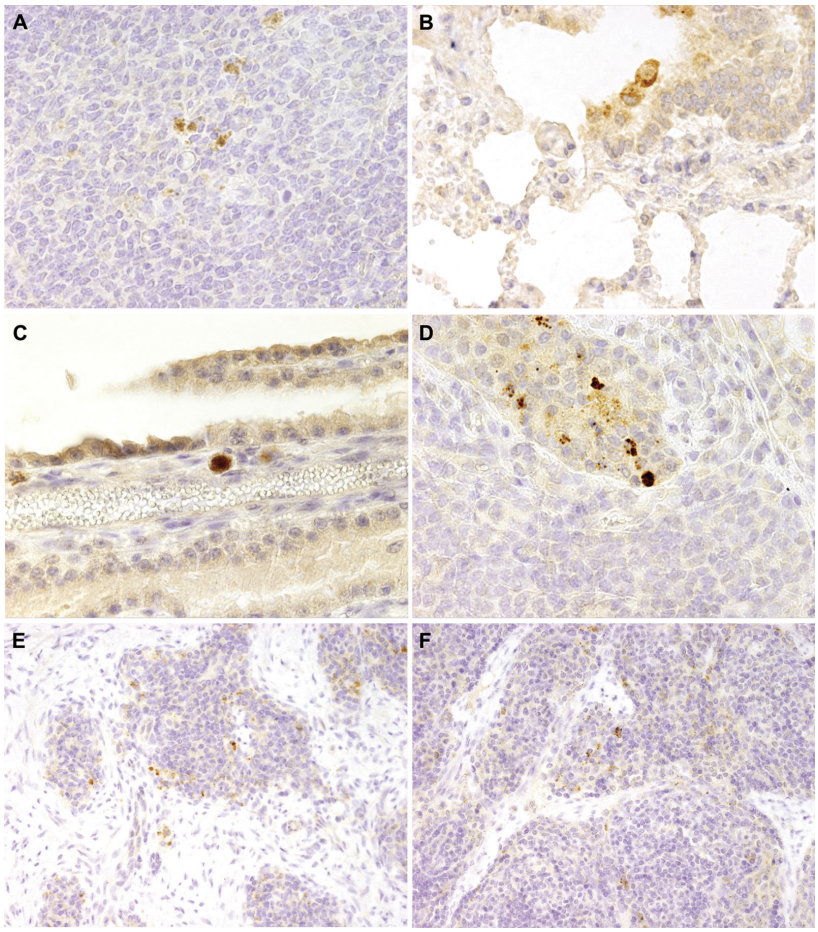

Fig. 4. Immunohistochemical detection of *M. agalactiae* in samples obtained from sheep experimentally infected via the intramammary route and necropsied at Day 16 p.i. *M. agalactiae* specific signals are observed in spleen (A), lung (B), choroid plexus of brain (C), udder (D) and, left (E) and right (F) popliteal lymph nodes respectively, using anti-*M. agalactiae* specific rabbit polyclonal antibodies. (A)–(D), ×40; (E) and (F), ×20.

M. agalactaie in various internal organs collected from infected animals (Table 1). Apart from the expected organs such as udders and lymph nodes, *M. agalactiae* was also detected in liver, lungs, uterus, kidneys, heart, brain, and carpal and knee joint tissues (Fig. 4). The results clearly illustrate the successful dissemination and bilateral spreading of the pathogen from the single site of infection, that is, the right teat canal, to several distant body sites, including heart and brain (see Table 1). Quantitative mycoplasma loads in organs, such as uterus, udder, popliteal lymph nodes and lungs varied between 10^3 and 10^5 cfu/g of tissue (Table 2). All samples found positive in *M. agalactiae* specific PCR did not show mycoplasma loads during quantitative analysis. This might be due to the already low mycoplasma count in these samples that die further during the sensitive freeze/thaw cycles needed for cfu enumeration. Overall, the results show for the first time the systemic spread of *M. agalactiae* to new anatomic sites during experimental intramammary infection of sheep.

Immunohistochemical demonstration of M. agalactiae's cell invasiveness during an in vivo infection

Detection of *M. agalactiae* in ovine mastitis by immunohistochemistry has not been reported so far. Various tissue samples obtained at necropsy were examined by immunohistochemistry using PG2 specific polyclonal antiserum to determine the presence of *M. agalactiae* in tissue/organs of infected animals. As illustrated in Fig. 4D, *M. agalactiae* antigens were clearly visible in the cytoplasm of mammary duct epithelium. Not only in the expected udder tissue, but also for the first time, *M. agalactae* was detected in the distant internal organs, such as the lungs (Fig. 4B), brain (Fig. 4C) and spleen (Fig. 4A) of experimentally infected sheep using immunohistochemistry technique. Interestingly, immunohistochemical staining of the lung (Fig. 4B) and brain tissue (Fig. 4C) demonstrated the presence of *M. agalactiae* in the cytoplasm of macrophages. Except for spleen, these results are in agreement

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Table 1
Qualitative bacteriological examination of lymph nodes and organs from sheep inoculated by the intramammary route with 10^9 viable cfu of *M. agalactiae* type strain (PG2) and necropsied at Day 16 p.i.

Organs/Tissue samples	PCR[a]	Culture[b]	Immunohistochemistry[c]
Supramammary LN[d] right	+	+	+
Parotid LN right	+	+	ND[e]
Parotid LN left	+	+	ND
Popliteal LN right	+	+	+
Popliteal LN left	+	+	+
Liver	+	+	–
Udder right	+	+	+
Udder left	+	+	–
Lung left	+	+	+
Lung right	+	+	+
Kidney right	+	+	ND
Spleen	–	–	+
Heart	+	+	–
Uterus (fallopian tube) right	+	+	–
Uterus left	+	+	–
Capsule (carpal joint) left	+	+	ND
Capsule right	+	+	ND
Brain	+	+	+
Synovial fluid (knee joint) right	+	+	ND

[a] *M. agalactiae* 16S rRNA specific PCR results.
[b] *M. agalactiae* colonies obtained on Aluotto agar by plating the broth subcultures of various tissue and lymph node (LN) samples.
[c] IHC: immunohistochemical analysis of selected *M. agalactiae* cultures.
[d] LN: lymph node.
[e] ND: not done.

Table 2
Quantitative bacterial examination of lymph nodes and organs from sheep inoculated by the intramammary route with 10^9 viable cfu of *M. agalactiae* type strain PG2 and necropsied at Day 16 p.i.

Organ/lymph node	Mycoplasma load[a]
Supramammary LN[b] Right	1.8×10^4
Parotid LN left	5×10^3
Parotid LN right	5×10^3
Uterus (fallopian tube) right	2.46×10^6
Popliteal LN left	5×10^3
Popliteal LN right	5×10^3
Udder right	4.79×10^6

[a] Mycoplasma load is represented as cfu/g of tissue/organ/lymph node.
[b] LN (lymph node).

with bacterial culture and PCR results, reconfirming *M. agalactiae's* capability to spread into anatomically distant body parts during experimental infections.

Discussion

Mycoplasmas are generally considered extracellular pathogens although in the last decades there have been quite a few reports providing sufficient evidence for some of these species to be capable of invading eukaryotic host cells (Fürnkranz et al., 2013). However, for *M. agalactiae*, which is well known for its chronic and persistent infections, cell invasion, and for that matter even a precise or direct account of its cytadherence capability, considering adherence to be a prerequisite for invasion, is yet to come. This is in accordance with the fact that it fails to show the more convenient phenotypes of hemadsorption and terminal tip structure unlike some other invasive *Mycoplasma* spp. (Rosengarten et al., 2000; Winner et al., 2000; Rottem, 2003). The data presented in this study provides the first evidence about the cell invasion capability of *M. agalactiae* pathogenic type strain PG2. Presence of *M. agalactiae* was demonstrated in the standard HeLa cells, as well as in two different ruminant cell lines, namely BEND and BLF, using the quantitative gentamicin invasion assay and the qualitative visual method of double immunofluorescence staining. Though the highest invasion frequency of *M. agalactiae* is calculated to be around 2.3% at 24 h post infection, it is comparable and even better than the reported invasion frequencies of some other pathogens, especially mycoplasmas (Martin and Mohr, 2000; Cieri et al., 2002; Dusanic et al., 2009; Marques et al., 2010; Buzinhani et al., 2011)

Furthermore, apart from the in vitro proof of *M. agalactiae* cell invasion, an in vivo experimental infection study was also performed, whereby the pathogen was demonstrated to be capable of crossing the epithelial barrier of the infected right udder and disseminating throughout the body as confirmed by its reisolation from various internal organs. Cell invasion capacity of pathogens is often believed to play a significant role in their systemic spread (Winner et al., 2000; Cieri et al., 2002; Much et al., 2002). Whether *M. agalactiae's* modest cell invasiveness actually correlates with the observed systemic infection needs to be formally demonstrated. But the fact that *M. agalactiae* is capable of entering and escaping cultured eukaryotic cells and its bilateral presence in many different host internal organs away from the site of initial in vivo infection can be well explained, and perhaps indirectly correlated, with its ability to invade local epithelial cells and/or to spread to draining lymph nodes before becoming systemic. This is supported by the immunohistochemical detection of *M. agalacatiae* in the cytoplasm of mammary duct epithelium (Fig. 4D) and macrophages present in different organs (Fig. 4B and C). To our knowledge this is the first study which has formally proved, not only the entry of *M. agalactiae* into host cells, but has also shown using immunohistochemistry staining, the inter- and intra-cellular residence of this pathogen in distant internal organs, such as lungs (Fig. 4B), spleen (Fig. 4A), brain (Fig. 4C), and in the knee and carpal joints (Fig. 4E and F) of sheep experimentally infected via the right teat canal. Especially interesting is the immunohistochemistry result where *M. agalactiae* is seen in the cytoplasm of macrophages in the lungs (Fig. 4B) and choroid plexus tissue of the brain (Fig. 4C). This implies that either the mycoplasma is capable of actively invading these phagocytic cells, or it has partially or fully survived the macrophage phagocytosis and subsequent immune clearance at the time of necropsy. Both possibilities are equally intriguing and would provide a fresh insight into the pathogenicity mechanisms of *M. agalactiae*.

An important finding of the present study is that the internalized *M. agalactiae* is released into the extracellular media in a perfectly viable state, though it is difficult to conclude anything about its intracellular replication under the given experimental conditions. After exiting the host cells, mycoplasmas tend to multiply and reinfect new host cells. This in vitro scenario could very well reflect the sequence of events occurring in vivo during the experimental intramammary infection, where the bacteria is speculated to have translocated through various host cell layers by cell invasion, exit and reinfection, thereby leading to the systemic spread of infection.

As for most other invasive mycoplasmas, the precise mechanism employed by *M. agalactiae* to enter non-phagocytic host cells is unknown. It is likely that surface proteins facilitating adhesion will have an effect on invasion, though adherence is not sufficient to trigger invasion events. *M. agalactiae* lacks a dedicated terminal tip structure where adhesion related molecules are localized for efficient cytadherence of some important mycoplasma pathogens (Rottem, 2003). Except for P40 (Fleury et al., 2002), no other cytadhesin has been identified in *M. agalactiae*, although the Vpma family of variable surface lipoproteins were shown to contain adhesion epitopes found in the homologous Vsp proteins of *M. bovis* (Glew et al., 2002), a close phylogenetic relative of *M. agalactiae* (Askaa and Ernø, 1976). Further investigations into the detailed invasion mechanisms of *M. agalactiae*, including the identification of mycoplasma adhesins and invasins and also eukaryotic host

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receptors will add to our understanding of this important ruminant pathogen.

It is thus clear that like many other bacterial pathogens, the division between intracellular and extracellular mycoplasmas is becoming increasingly blurred as more and more pathogens originally believed to be extracellular are now shown to have alternative intracellular lifestyles (Bower et al., 2005; Fürnkranz et al., 2013; Lamberti et al., 2013). This alternative capacity of *M. agalactiae*, even if it is exhibited by a very small subpopulation of total infected cells, might provide the pathogen with a gross advantage at the population level to hide from antibiotics and host immune responses and to navigate through the host body to reestablish infection in new host niches, thus causing persistent infections that are difficult to eradicate.

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References

Andreev, J., Borovsky, Z., Rosenshine, I., Rottem, S., 1995. Invasion of HeLa cells by Mycoplasma penetrans and the induction of tyrosine phosphorylation of a 145-kDa host cell protein. FEMS Microbiol. Lett. 132, 189–194.
Askaa, G., Ernø, H., 1976. Elevation of *Mycoplasma agalactiae* subsp. *bovis* to species rank: *Mycoplasma bovis* (Hale et al.) comb. nov. Int. J. Syst. Bacteriol. 26, 323–325.
Baranowski, E., Guiral, S., Sagne, E., Skapski, A., Citti, C., 2010. Critical role of dispensable genes in *Mycoplasma agalactiae* interaction with mammalian cells. Infect. Immun. 78, 1542–1551.
Bergonier, D., Berthelot, X., Poumarat, F., 1997. Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control. Rev. Sci. Tech. 16, 848–873.
Bower, J.M., Eto, D.S., Mulvey, M.A., 2005. Covert operations of uropathogenic *Escherichia coli* within the urinary tract. Traffic 6, 18–31.
Buim, M.R., Buzinhani, M., Yamaguti, M., Oliveira, R.C., Mettifogo, E., Ueno, P.M., Timenetsky, J., Santelli, G.M., Ferreira, A.J., 2011. *Mycoplasma synoviae* cell invasion: elucidation of the *Mycoplasma pathogenesis* in chicken. Comp. Immunol. Microbiol. Infect. Dis. 34, 41–47.
Buzinhani, M., Yamaguti, M., Oliveira, R.C., Cortez, B.A., Marques, L.M., Machado-Santelli, G.M., Assumpcao, M.E., Timenetsky, J., 2011. Invasion of *Ureaplasma diversum* in bovine spermatozoids. BMC Res. Notes 4, 455.
Chavez Gonzalez, Y.R., Ros Bascunana, C., Bolske, G., Mattsson, J.G., Fernandez Molina, C., Johansson, K.E., 1995. In vitro amplification of the 16S rRNA genes from *Mycoplasma bovis* and *Mycoplasma agalactiae* by PCR. Vet. Microbiol. 47, 183–190.
Chopra-Dewasthaly, R., Marenda, M., Rosengarten, R., Jechlinger, W., Citti, C., 2005a. Construction of the first shuttle vectors for gene cloning and homologous recombination in *Mycoplasma agalactiae*. FEMS Microbiol. Lett. 253, 89–94.
Chopra-Dewasthaly, R., Zimmermann, M., Rosengarten, R., Citti, C., 2005b. First steps towards the genetic manipulation of *Mycoplasma agalactiae* and *Mycoplasma bovis* using the transposon Tn4001mod. Int. J. Med. Microbiol. 294, 447–453.
Chopra-Dewasthaly, R., Citti, C., Glew, M.D., Zimmermann, M., Rosengarten, R., Jechlinger, W., 2008. Phase-locked mutants of *Mycoplasma agalactiae*: defining the molecular switch of high-frequency Vpma antigenic variation. Mol. Microbiol. 67, 1196–1210.
Chopra-Dewasthaly, R., Baumgartner, M., Gamper, E., Innerebner, C., Zimmermann, M., Schilcher, F., Tichy, A., Winter, P., Jechlinger, W., Rosengarten, R., Spergser, J., 2012. Role of Vpma phase variation in *Mycoplasma agalactiae* pathogenesis. FEMS Immunol. Med. Microbiol. 66, 307–322.
Cieri, M.V., Mayer-Hamblett, N., Griffith, A., Burns, J.L., 2002. Correlation between an in vitro invasion assay and a murine model of *Burkholderia cepacia* lung infection. Infect. Immun. 70, 1081–1086.
Citti, C., Blanchard, A., 2013. Mycoplasmas and their host: emerging and re-emerging minimal pathogens. Trends Microbiol. 21, 196–203.
Czurda, S., Jechlinger, W., Rosengarten, R., Chopra-Dewasthaly, R., 2010. Xer1-mediated site-specific DNA inversions and excisions in *Mycoplasma agalactiae*. J. Bacteriol. 192, 4462–4473.
Dusanic, D., Bercic, R.L., Cizelj, I., Salmic, S., Narat, M., Bencina, D., 2009. *Mycoplasma synoviae* invades non-phagocytic chicken cells in vitro. Vet. Microbiol. 138, 114–119.
Elsinghorst, E.A., 1994. Measurement of invasion by gentamicin resistance. Methods Enzymol. 236, 405–420.
Fleury, B., Bergonier, D., Berthelot, X., Peterhans, E., Frey, J., Vilei, E.M., 2002. Characterization of P40, a cytadhesin of *Mycoplasma agalactiae*. Infect. Immun. 70, 5612–5621.
Friedrich, N., Hagedorn, M., Soldati-Favre, D., Soldati, T., 2012. Prison break: pathogens' strategies to egress from host cells. Microbiol. Mol. Biol. Rev. 76, 707–720.
Fürnkranz, U., Siebert-Gulle, K., Rosengarten, R., Szostak, M.P., 2013. Factors influencing the cell adhesion and invasion capacity of *Mycoplasma gallisepticum*. Acta Vet. Scand. 55, 63.
Glew, M.D., Marenda, M., Rosengarten, R., Citti, C., 2002. Surface diversity in *Mycoplasma agalactiae* is driven by site-specific DNA inversions within the vpma multigene locus. J. Bacteriol. 184, 5987–5998.
Gomez-Martin, A., De la Fe, C., Amores, J., Sanchez, A., Contreras, A., Paterna, A., Buendia, A.J., Corrales, J.C., 2012. Anatomic location of *Mycoplasma mycoides* subsp. *capri* and *Mycoplasma agalactiae* in naturally infected goat male auricular carriers. Vet. Microbiol. 157, 355–362.
Gomez-Martin, A., Amores, J., Paterna, A., De la Fe, C., 2013. Contagious agalactia due to *Mycoplasma* spp. in small dairy ruminants: epidemiology and prospects for diagnosis and control. Vet. J. 198, 48–56.
Heesemann, J., Laufs, R., 1985. Double immunofluorescence microscopic technique for accurate differentiation of extracellularly and intracellularly located bacteria in cell culture. J. Clin. Microbiol. 22, 168–175.
Hopfe, M., Deenen, R., Degrandi, D., Kohrer, K., Henrich, B., 2013. Host cell responses to persistent mycoplasmas different stages in infection of HeLa cells with *Mycoplasma hominis*. PLoS One 8, e54219.
Hybiske, K., Stephens, R.S., 2007. Mechanisms of host cell exit by the intracellular bacterium Chlamydia. Proc. Nat. Acad. Sci. U.S.A. 104, 11430–11435.
Hybiske, K., Stephens, R.S., 2008. Exit strategies of intracellular pathogens. Nat. Rev. Microbiol. 6, 99–110.
Khan, L.A., Miles, R.J., Nicholas, R.A., 2005. Hydrogen peroxide production by *Mycoplasma bovis* and *Mycoplasma agalactiae* and effect of in vitro passage on a *Mycoplasma bovis* strain producing high levels of H_2O_2. Vet. Res. Commun. 29, 181–188.
Lamberti, Y., Gorgojo, J., Massillo, C., Rodriguez, M.E., 2013. *Bordetella pertussis* entry into respiratory epithelial cells and intracellular survival. Pathog. Dis. 69, 194–204.
Marques, L.M., Ueno, P.M., Buzinhani, M., Cortez, B.A., Neto, R.L., Yamaguti, M., Oliveira, R.C., Guimaraes, A.M., Monezi, T.A., Braga Jr., A.C., Machado-Santelli, G.M., Timenetsky, J., 2010. Invasion of Ureaplasma diversum in Hep-2 cells. BMC Microbiol. 10, 83.
Martin, D.W., Mohr, C.D., 2000. Invasion and intracellular survival of *Burkholderia cepacia*. Infect. Immun. 68, 24–29.
McAuliffe, L., Ellis, R.J., Miles, K., Ayling, R.D., Nicholas, R.A., 2006. Biofilm formation by mycoplasma species and its role in environmental persistence and survival. Microbiology 152, 913–922.
Much, P., Winner, F., Stipkovits, L., Rosengarten, R., Citti, C., 2002. *Mycoplasma gallisepticum*: influence of cell invasiveness on the outcome of experimental infection in chickens. FEMS Immunol. Med. Microbiol. 34, 181–186.
Nicholas, R.A., 2002. Improvements in the diagnosis and control of diseases of small ruminants caused by mycoplasmas. Small Ruminant Res. 45, 145–149.
Razin, S., Yogev, D., Naot, Y., 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiol. Mol. Biol. Rev. 62, 1094–1156.
Rosati, S., Pozzi, S., Robino, P., Montinaro, B., Conti, A., Fadda, M., Pittau, M., 1999. P48 major surface antigen of *Mycoplasma agalactiae* is homologous to a *malp* product of *Mycoplasma fermentans* and belongs to a selected family of bacterial lipoproteins. Infect. Immun. 67, 6213–6216.
Rosengarten, R., Citti, C., Glew, M., Lischewski, A., Droesse, M., Much, P., Winner, F., Brank, M., Spergser, J., 2000. Host–pathogen interactions in *Mycoplasma pathogenesis*: virulence and survival strategies of minimalist prokaryotes. Int. J. Med. Microbiol. 290, 15–25.
Rottem, S., Barile, M.F., 1993. Beware of mycoplasmas. Trends Biotechnol. 11, 143–151.
Rottem, S., 2003. Interaction of mycoplasmas with host cells. Physiol. Rev. 83, 417–432.
Ruhnke, H.L., Rosendal, S., 1994. Useful protocols for diagnosis of animal mycoplasmas. In: Whitford, H.W., Rosenbusch, R.F., Lauerman, L.H. (Eds.), Mycoplasmosis in Animals: Laboratory Diagnosis. Iowa State University Press, Ames, pp. 141–166.
Sanchis, R., Abadie, G., Lambert, M., Cabasse, E., Guibert, J.M., Calamel, M., Dufour, P., Vitu, C., Vignoni, M., Pépin, M., 1998. Experimental conjunctival-route infection with *Mycoplasma agalactiae* in lambs. Small Ruminant Res. 27, 31–39.
Sirand-Pugnet, P., Lartigue, C., Marenda, M., Jacob, D., Barre, A., Barbe, V., Schenowitz, C., Mangenot, S., Couloux, A., Segurens, B., de Daruvar, A., Blanchard, A., Citti, C., 2007. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. PLoS Genet. 3, e75.
Winner, F., Rosengarten, R., Citti, C., 2000. In vitro cell invasion of *Mycoplasma gallisepticum*. Infect. Immun. 68, 4238–4244.
Yavlovich, A., Katzenell, A., Tarshis, M., Higazi, A.A., Rottem, S., 2004. *Mycoplasma fermentans* binds to and invades HeLa cells: involvement of plasminogen and urokinase. Infect. Immun. 72, 5004–5011.

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4.2 Manuscript I (under review PLoS One)

Disruption of the *pdhB* pyruvate dehyrogenase gene affects colony morphology, *in vitro* growth and cell invasiveness of *Mycoplasma agalactiae*

Running title: *pdhB* disruption affects growth and invasiveness

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Keywords: *Mycoplasma agalactiae*; pyruvate dehydrogenase, colony morphology, cell invasion, transposon mutant, growth, intracellular

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Abstract

The utilization of available substrates, the metabolic potential and the growth rates of bacterial microbes can play significant roles in their pathogenicity. This study concentrates on *Mycoplasma agalactiae* that leads to significant economic losses by causing the contagious agalactia syndrome in small ruminants by as yet unknown mechanisms. This lack of knowledge is primarily due to its fastidious growth requirements and the scarcity of genetic tools available for its manipulation and analysis. Transposon mutagenesis of *M. agalactiae* type strain PG2 resulted in several disruptions throughout the genome. Among the sequenced genomic loci, a mutant defective in *in vitro* growth was found to have a transposon insertion in the *pdhB* gene component of pyruvate dehydrogenase complex. This growth difference was quite significant during the actively dividing logarithmic phase but a gradual recovery was observed as the cells approached stationary phase. The mutant also exhibited a different and smaller colony morphology compared to the wild type strain PG2. For complementation, *pdhAB* was cloned downstream of a strong *vpma* promoter and upstream of a *lacZ* reporter gene in a newly constructed complementation vector. The latter when transformed into the *pdhB* mutant recovered its normal growth and colony morphology. Interestingly, the *pdhB* mutant also demonstrated a significantly reduced invasiveness for HeLa cells as revealed by double immunofluorescence staining. This deficiency was recovered in the complemented strain whose invasion was found comparable to PG2. Taken together, the data indicate that pyruvate dehydrogenase might be an important player in the infection and colonization processes of *M. agalactiae.*

1 Introduction

Mycoplasmas are the simplest self-replicating organisms that have evolved from Gram-positive bacteria through reductive evolution (74, 75). As a result of their limited biosynthetic and metabolic capabilities, mycoplasmas rely on infected host cells for their nutrition and thus lead a parasitic life (10, 76). As successful pathogens they have developed mechanisms to invade and survive within the host cells. Surface lipoproteins play an important role in the adhesion and invasion of mycoplasmas and also in evading host immune response because of their variable nature (2, 77). Apart from surface lipoproteins some metabolic enzymes are also known to play a role in host-pathogen interactions (18, 19, 78, 79). Although there have been suggestions by some that metabolic enzymes should not be considered as virulence factors (80), several studies have identified and described them as important pathogenicity determinants besides their role in basic metabolism (81, 82). Genomes of mycoplasmas were among the first ones to be sequenced yet there is a lack of knowledge about their pathogenicity. This dearth of information is mainly attributed to insufficient availability of genetic tools and also because mycoplasmas are not so amenable to genetic manipulations. Additionally, only few mycoplasmas have self-replicating plasmids that otherwise could play an important role in their genetic studies. Nevertheless, several efforts have been made in the past in establishing genetic tools through the development of *oriC*-based shuttle vectors and studies have also been undertaken to genetically manipulate mycoplasmas using conventional methods, such as transposition and homologous recombination (50).

M. agalactiae is a pathogen of small ruminants and the main etiological agent of contagious agalactia syndrome that causes major economic losses every year, especially because it is hard to eradicate and carriers continue to shed and infect new herds for many years after the initial infection (33, 34). Although the disease characteristics and pathology are well documented, little is known about its pathogenicity determinants and mechanisms of infection and persistence. As a first step towards understanding the pathogenicity of *M. agalactiae*, *ori*C containing shuttle vectors were developed and genetic manipulation was achieved using Tn*4001*mod (43, 61). To understand the role of Vpmas (Variable surface proteins of *M. agalactiae*) in pathogenicity, phase locked mutants were generated through the targeted disruption of site-specific recombinase *xer1* (41) and it was shown that indeed Vpmas and Vpma phase variation play a role in *M. agalactiae's* pathogenesis (35). Other studies have elaborated on *M. agalactiae's* in vitro (45) and in vivo (83) interactions with host cells, biofilm formation (24) and host cell adhesion (48). Except for a handful of such reports, and despite the availability of its genome sequence since 2007 (30), functional analysis of *M. agalactiae* genes is largely unaccomplished.

Transposon mutagenesis of *M. agalactiae* PG2 strain resulted in a clone carrying an insertion in the *pdhB* gene (MAG_0940), which encodes the E1 beta subunit of the pyruvate dehydrogenase (PDH) complex involved in the conversion of pyruvate to acetyl-CoA (Fig. 1). The *M. agalactiae* PDH complex consists of 4 genes arranged as 2 operons, designated *pdhAB* and *pdhCD,* encoding the E1 and E2 subunits respectively. The current study reports the role of the E1 beta subunit (PDHB) in *in vitro* growth, colony

morphology and HeLa cell invasion of *M. agalactiae.* Recovery of these phenotypes was observed for the mutant when a wild type copy of *pdhAB* was introduced into it using a newly developed complementation vector. The data indicates that the PDH complex not only plays a conventional role in *M. agalactiae's* energy metabolism but also decreases its invasion capacity, which in turn might have an effect on its pathogenesis.

2 Material and Methods

2.1 Bacterial cultures and growth conditions *M. agalactiae* pathogenic type strain PG2 (30, 84) and *pdhB* transposon mutant were grown at 37° C in SP4 medium supplemented with penicillin, pyruvate, and phenol red as indicator as described before (43). Gentamicin sulphate (50µg/ml) was additionally added both in broth and agar plates for the propagation of *pdhB* transposon mutant. *E. coli* DH10B (Invitrogen GmbH, Lofer, Austria) transformants containing transposon vector pISM2062 or *M. agalactiae oriC* vector pMM21-7 were grown in LB broth (10 g tryptone, 5 g yeast extract, and 5 g of NaCl per liter) supplemented with ampicillin (100 µg/ml), gentamicin sulphate (7µg/ml) or tetracycline (100 µg/ml). *M. agalactiae pdhB* mutant containing complementation plasmid pPlacZ was grown in SP4 broth containing both tetracycline (2 µg/ml) and gentamicin (50µg/ml). To monitor *lacZ* expression on the basis of blue-white colonies, *M. agalactiae* clones were grown on SP4 agar supplemented with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) at a concentration of 160 µg/ml (42).

2.2 Transposon mutant library Transposon mutants were generated in *M. agalactiae* type strain PG2 using suicidal plasmid pISM2062 containing transposon Tn*4001*mod (72) as described previously (43). Transposon insertion sites were sequenced using a novel method based on ligation mediated QTD PCR (73). This was further modified and standardized for *M. agalactiae* genome to sequence the Tn mutant library (85).

2.3 DNA manipulations Preparation of plasmids and genomic DNA and the isolation of DNA fragments from agarose gels was carried out using suitable kits from Promega (Wizard SV gel and PCR clean-up system, Promega, Mannheim, Germany) and Qiagen (QIAamp DNA Mini kit; Qiagen GmbH, Hilden, Germany). Restriction and modifying enzymes were purchased from Promega and Fermentas Life Science and Antarctic phosphatase was purchased from New England Biolabs (Frankfurt am Main, Germany). Transformation of *E. coli* cells was performed by electroporation with a Bio-Rad Gene-Pulser II instrument (Bio-Rad Laboratories GmbH, Vienna, Austria) using 1.25 V voltage, 25 F capacitance, and 200 Ω resistance. Transformation of *M. agalactiae* cells was also carried out by electroporation as described previously (43). Oligonucleotide synthesis and sequencing was carried out respectively at Microsynth AG (Balgach, Switzerland) and Agowa Sequencing Service (LGC's AGOWA Genomics, Berlin, Germany). Standard molecular procedures were performed as described earlier (86).

2.4 In silico analysis Promoters in mycoplasmas are not well defined. Usually promoter regions lie upstream of transcription start site although the

positions of conventional -10 and -35 elements may vary slightly. In order to find the putative *vpma* promoter region, approximately 400 bp fragment upstream of *vpmaY* was retrieved from NCBI and analysed by Neural Network Promoter Prediction (NNPP) (http://www.fruitfly.org/seq_tools/promoter.html) and BPROM softwares (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). The highest score fragments predicted commonly by both these softwares in the same region of the genome were selected for cloning.

2.5 PCR amplification PCR amplification of *pdhAB* and *vpma* promoter region was carried out in a total volume of 50 µl using 200 ng of PG2 genomic DNA as template. The reaction mixture constituted 1 µM of the respective primers (Table 1), 25 mM $MgCl_2$, 0.2 µM of each deoxynucleoside triphosphate (dNTP), and 5 U of *Taq* DNA polymerase (Promega) in 1× PCR buffer supplied by the manufacturer. Cycling parameters consisted of initial denaturation of 3 min at 94°C, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 54°C and either 1 min (*vpma* promoter) or 2 min (*pdhAB*) at 72°C for extension, with a final extension step of 5 min at 72°C. The PCR products were either gel extracted or purified with the appropriate kit before proceeding to restriction digestion.

2.6 Plasmid construction

2.6.1 Construction of placZ and pPlacZ The plasmid placZ was generated by cloning *lacZ* reporter gene in *BamH*I and *Sma*I sites in pMM21-7 shuttle

vector containing *M. agalactiae oriC* (61). The pIL plasmid (42) containing *lacZ* was digested with *BamH*I and *Sma*I and ligated into the vector pMM21-7 digested with the same enzymes. The resulting placZ construct was transformed into *M. agalactiae* PG2 to check *lacZ* expression in absence of promoter and found that there were no blue colonies expressing *lacZ*. The placZ plasmid was further developed into a complementation vector by cloning a strong *M. agalactiae* promoter in it. The putative *vpma* promoter region, which is described in section 2.1, was amplified from *M. agalactiae* PG2 strain using primers PromfpB and PromrpN (Table 1) containing *Bam*HI and *Nco*I sites respectively and this PCR fragment was introduced into corresponding sites in placZ. The resulting 12.6 kb plasmid was designated as pPlacZ and transformed into *M. agalactiae* PG2 to check *lacZ* expression in presence of the cloned putative promoter.

2.6.2 Construction of pPpdhABlacZ The complementation vector pPlacZ containing the *vpma* promoter was used to clone *pdhB* gene. However, our repeated attempts to clone *pdhB* gene alone in pPlacZ were unsuccessful. Hence, it was decided to clone *pdhAB* as both *pdhA* and *pdhB* are found in tandem in the same operon. The 2.1 Kb region corresponding to *pdhAB* was amplified with primers pdhABfpN and pdhABrfN containing *Nco*I restriction sites (see Table 1) and the PCR fragment was ligated into pPlacZ vector after digestion with *Nco*I to generate pP*pdhABlacZ* plasmid. The resulting construct was analyzed for orientation of the *pdhAB* gene that was also verified by sequencing before transforming into *pdhB* mutant. *M. agalactiae* clones containing this plasmid were further confirmed for the episomal presence of

pdhAB via Southern blot analysis using 2.1 Kb DIG-labeled *pdhAB* PCR fragment (Roche) as described previously (61).

2.7. Growth curve analysis Growth of *pdhB* mutant in SP4 medium was compared with the wild type strain PG2 at 37 °C. Both the cultures were grown for 48 h with appropriate antibiotic in SP4 medium and growth curve was initiated by sub culturing 1:100 diluted cultures in SP4 medium in duplicates. Starting cfu of all the cultures were determined by plating suitable dilutions on SP4 agar plates. At 6, 12, 24 and 48 h post inoculation, small aliquots of each culture were diluted in SP4 medium and plated on SP4 agar plates. After 5-6 days of incubation at 37 °C, mycoplasma colonies were counted under an inverted microscope to determine growth in terms of cfu/ml. The same growth curve was then performed together with the complemented clone of the *pdhB* mutant. The latter growth curves were performed three times in duplicates.

2.8. *M. agalactiae* cell invasion *M. agalactiae's* invasion into HeLa-229 (ATCC CCL-2.1) cells was performed and analysed as described earlier (83) by the Double Immunofluorescence Assay (DIF). Invasion was quantified by randomly selecting and analyzing about hundred HeLa cells in random microscopic fields. The results were expressed as the average number of invaded mycoplasmas per 100 HeLa cells. The experiments were performed in duplicate for at least four different times.

2.9 Statistical analysis Results of growth curves performed at three different times in duplicates were expressed as mean ± standard deviation (SD) of *n* independent values. The invasion experiments were performed at least four times in duplicates. The significance of differences between means of experiments was calculated by Student's *t* test using GraphPad Prism 5 (Graphpad Software Inc, CA, USA). Differences with $P < 0.05$ were considered significant.

3 Results

3.1 *pdhB* transposon mutant exhibits slow growth during logarithmic phase and altered colony size and morphology Transposon mutants of *M. agalactiae* were checked for *in vitro* growth defects, and the results demonstrated slower growth of the *pdhB* mutant compared to the wild type strain PG2. A significantly slower growth rate ($P<0.05$) was observed for the *pdhB* mutant during the log phase (Fig. 2). A growth difference of nearly two-fold was observed at 6 h between mutant and wild type strain, which further increased to four-fold difference at 12 h (Fig. 2). At 24 h the growth difference was nearly 8 fold (Fig. 2) indicating the inability of the mutant to efficiently replicate during the prolific logarithmic growth phase. However, the mutant was able to recover its growth to that of wild type strain during stationary phase at 48 h (data not shown).

Transposon insertion in the *pdhB* gene also led to smaller colony size compared to the wild type counterpart, even when plated on rich SP4 agar

medium (Fig. 3). Not only this, the *pdhB* colonies failed to exhibit the typical "fried-egg" morphology with most colonies showing just the dark dense central "down growth" button without the peripheral surface growth when observed under an inverted microscope (87) (Fig. 3).

3.2 *pdhB* mutant is deficient in HeLa cell invasion Double immunofluorescence (DIF) assay was performed to evaluate the invasion capacity of *pdhB* mutant in comparison with the wild type PG2 strain. Extracellular mycoplasmas were stained with FITC conjugated antibody whereas Texas red was used to stain both intracellular and extracellular bacteria. After merging two images the intracellular mycoplasmas appear red in color where as the extracellular ones are yellow (Fig. 4). Observation of HeLa cells infected with *pdhB* mutant revealed few or no intracellular mycoplasmas for most tested microscopic fields (Fig. 4) whereas the wild type PG2 infected HeLa cells showed large numbers of intracellular bacteria (Fig. 4). The mutant showed significantly lower ($P<0.05$) number of invaded mycoplasmas as compared to wild type PG2 (Fig. 4 & 5). On an average, only about 120 mutant bacteria were observed per hundred HeLa cells, whereas the number of wild type PG2 cells was found to be nearly three fold higher (357 mycoplasma cells/100 HeLa cells) (Fig. 5). Hence, *pdhB* mutation led to a significant reduction in *M. agalactiae's* ability to invade non-phagocytic eukaryotic cells.

The inefficient invasiveness of *pdhB* mutant cannot be attributed to its slow growth phenotype because it showed slower growth only in the early logarithmic phase and reached wild type levels during the stationary phase.

Both the mutant and the wild type strains were grown for 48 hours and had reached stationary phase when used for infecting HeLa cells. This was also evident from the calculated MOIs, which were found to be comparable for both *pdhB* and wild type PG2. As controls, uninfected eukaryotic monolayers were also stained with FITC and Texas Red to rule out the possibility of any previous contamination with mycoplasmas and/or any cross-reactivity of antibodies (data not shown).

3.3 Complementation studies

3.3.1 Generation of reporter based complementation vector To confirm the role of *pdhB* in *M. agalactiae's* in vitro growth, altered colony morphology and ability to invade eukaryotic cells, wild type *pdhB* gene was cloned into a complementation vector pPlacZ that was constructed as described under Section 2.6. For proper expression of the cloned genes a strong promoter was desired in the complementation vector. It was decided to clone the putative *vpma* promoter as Vpmas are one of the most abundantly expressed proteins of *M. agalactiae* (Glew et al, 2000). The vector was generated by cloning a strong putative promoter upstream of *lacZ* gene. The putative promoter region upstream of *vpmaY* gene was analyzed for promoter prediction using two different softwares, namely BRPOM and NNPP. The software NNPP generated total ten fragments in the 400 bp region upstream of *vpmaY* gene when considering a cut-off score of 0.8. Among these fragments, one putative promoter was predicted within the 273-300 bp region upstream of *vpmaY* with the highest possible score of 1 (Table 2). The BPROM software also predicted possible -10 and -35 elements in the same promoter fragment that had scored

1 with NNPP analysis (Table. 2). So, this 400 bp upstream region of *vpmaY* gene was PCR amplified and cloned as a putative strong promoter in front of the *lacZ* gene in autonomously replicating vector placZ as described under Section 2.6.1. The cloned *vpma* promoter showed strong activity indicated by blue colored colonies when pPlacZ was transformed into *M. agalctiae* PG2 (Fig. S1 B). However, in absence of this promoter, the lacZ gene alone in the placZ failed to show blue colonies (Fig. S1 A). Hence, *pdhAB* was cloned in the complementation vector pPlacZ to generate pPlacpdhAB. Interestingly, pPlacZpdhAB also showed faint blue colored colonies when transformed into *pdhB* mutant (Fig. S1 C) indicating strong functional activity for the cloned *vpma* promoter. The pPlacZpdhAB was sequenced to confirm the orientation and correct sequence of *pdhAB*. The latter was then transformed into *pdhB* mutant to generate the complemented strain Δ*pdhB* :: pPlacpdhAB, in which the episomal presence of *pdhAB* was further verified by Southern blot analysis (data not shown).

3.3.2 Complementation of pdhB mutant phenotypes Results of the growth curve analysis clearly demonstrated the rescue of normal growth in the complemented strain Δ*pdhB* :: pP*pdhABlacZ*, which now exhibited log phase growth comparable to wild type PG2 strain (Fig. 2). Presence or absence of a functional *pdhB* gene led to a significant growth difference ($P<0.05$) between the complemented Δ*pdhB*::pP*pdhABlacZ* and the mutated *pdhB* strain, respectively (Fig. 2) clearly indicating the role of this gene in *M. agalactiae's* growth in axenic medium. Also, *pdhAB* complementation of the mutant

reverted its colony size and morphology back to the normal “fried-egg” phenotype (Fig. 3).

Similarly, when tested for HeLa cell invasion, the complemented Δ*pdhB* :: p*pdhABlacZ* strain showed significantly higher ($P<0.05$) number of intracellular mycoplasmas as compared to the *pdhB* mutant (Fig. 5). On an average, approximately 387 invaded mycoplasmas were counted per 100 HeLa cells infected with the complemented strain and observed randomly in different microscopic fields (Fig. 5). Although the counts were slightly higher than the wild type PG2 strain (357 mycoplasmas/100 HeLa cells), they are not significantly different ($P>0.05$) and quite comparable to it.

Altogether, these data clearly demonstrate that the growth and invasion deficits, as well as the altered colony morphology of *pdhB* mutant are indeed due to the absence of E1 beta subunit of the pyruvate dehydrogenase complex and are efficiently restored back to the normal wild type levels upon the episomal introduction of intact *pdhAB*.

4 Discussion

Transposon mutagenesis is a powerful tool to identify genetic factors involved in the virulence of pathogenic bacteria. It has also been widely used in mycoplasmas to understand the role of different genes involved in motility, cytadherence and restriction modification defence systems (29, 56, 57). After successful manipulation of the *M. agalactiae* genome using Tn*4001*mod, a library of mutants was created and all the disrupted genes were sequenced (43, 85). Several important loci belonging to different functional categories

were found disrupted, such as lipoproteins, transporters and metabolic enzymes. Among the several loci belonging to the latter category was the *pdhB* gene encoding E1β subunit of the PDH complex.

Here, we have studied the effect of *pdhB* disruption on *M. agalactiae's* growth and host cell invasion. This gene is part of the PDH multi-enzyme complex involved in the conversion of pyruvate to acetyl-CoA. It has been previously demonstrated that *M. agalactiae* growth shows a marked increase in presence of pyruvate (88) indicating it to be a major energy yielding pathway. Pyruvate is known to be metabolized to acetate in a series of reactions involving the PDH complex, EutD and AckA, with the generation of ATP. Although any defect in this pathway is likely to affect growth, it is interesting to note that unlike the *pdhB* mutant, transposon insertion in the *eutD* gene did not cause any growth retardation or altered colony morphology (data not shown). Our results with the *pdhB* mutant suggest that conversion of pyruvate to acetyl-CoA is critical and causes the slow log phase growth and altered colony morphology in *M. agalactiae.* The growth of the *pdhB* mutant recovers during the stationary phase, indicating the involvement of alternative pyruvate breakdown pathways operational at this stage (Fig. 6). It could be hypothesized that during the prolific log phase growth, *pdhB* mutant is unable to metabolize pyruvate to meet its energy needs and as a result pyruvate accumulates, which is then likely to slow down the other pyruvate metabolizing pathways, if operational, due to reverse kinetics. During the stationary phase, perhaps an alternative pathway becomes predominant (Fig. 6), namely conversion of pyruvate to lactate via lactate dehydrogenase, and hence the recovery of growth of the *pdhB* mutant. This study highlights the

fact that despite the many alternative pathways of (pyruvate) metabolism in *Mollicutes,* and despite the very rich growth media, the presence or absence of specific gene products can significantly influence the growth rate of these pathogens. Such variation in metabolism activity may have consequences with regard to pathogenesis, and also at a subtle level, to the survival and propagation of these pathogens.

It is also interesting to note that the *pdhB* mutation also has a negative effect on *M. agalactiae's* ability to invade HeLa cells. Involvement of mycoplasma glycolytic enzymes in host cell interactions has been reported earlier (19, 78, 89). Among them are α-enolase, GAPDH and PDHB proteins, which were shown to be involved in interactions with host extracellular matrix proteins (16-19, 78, 89). Since adhesion is a prerequisite for invasion into host cells it is likely that these proteins also play a role in internalization of bacteria. Although our results revealed inefficiency of the *pdhB* mutant to invade HeLa cells, we did not find any adverse effect on the cell adhesion phenotype of the mutant compared to the wild type strain. This indicates that pyruvate dehydrogenase likely acts at a stage secondary to the initial contact for successful internalization into eukaryotic cells. Confirmation of the role of pyruvate dehydrogenase in cell invasion is substantiated by complementation studies. To our knowledge this is the first report describing the role of pyruvate dehydrogenase in host cell invasion and further elaborate studies might help understand the exact details of this process

Even though PDH mutants show slow *in vitro* growth phenotypes, they have often been used in STM screens, and even accepted to play important roles in the pathogenicity of other bacterial pathogens (90, 91). Recently, PDHB of *M.*

bovis, showing 98% homology to *M. agalactiae* counterpart, was identified as a novel immunogenic target for a serodiagnostic ELISA test (92). Also, as *in vitro* cell invasiveness of pathogens is believed to play a significant role in their systemic spread and pathogenicity (83, 93-95) it is anticipated that pyruvate dehydrogenase might play a role in *M. agalactiae* ~~i~~nfections~~.~~

Acknowledgements

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5. References

1. **Peterson SN, Fraser CM.** 2001. The complexity of simplicity. Genome Biol **2:**COMMENT2002.
2. **Weisburg WG, Tully JG, Rose DL, Petzel JP, Oyaizu H, Yang D, Mandelco L, Sechrest J, Lawrence TG, Van Etten J, et al.** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. J Bacteriol **171:**6455-6467.
3. **Pollack JD, Williams MV, McElhaney RN.** 1997. The comparative metabolism of the mollicutes (Mycoplasmas): the utility for taxonomic classification and the relationship of putative gene annotation and phylogeny to enzymatic function in the smallest free-living cells. Crit Rev Microbiol **23:**269-354.
4. **Razin S, Yogev D, Naot Y.** 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiol Mol Biol Rev **62:**1094-1156.
5. **Chambaud I, Wroblewski H, Blanchard A.** 1999. Interactions between mycoplasma lipoproteins and the host immune system. Trends Microbiol **7:**493-499.
6. **Rosengarten R, Citti C, Glew M, Lischewski A, Droesse M, Much P, Winner F, Brank M, Spergser J.** 2000. Host-pathogen interactions in mycoplasma pathogenesis: virulence and survival strategies of minimalist prokaryotes. Int J Med Microbiol **290:**15-25.
7. **Dallo SF, Kannan TR, Blaylock MW, Baseman JB.** 2002. Elongation factor Tu and E1 beta subunit of pyruvate dehydrogenase complex act

as fibronectin binding proteins in *Mycoplasma pneumoniae*. Mol Microbiol **46:**1041-1051.

8. **Thomas C, Jacobs E, Dumke R.** 2013. Characterization of pyruvate dehydrogenase subunit B and enolase as plasminogen-binding proteins in *Mycoplasma pneumoniae*. Microbiology **159:**352-365.
9. **Alvarez RA, Blaylock MW, Baseman JB.** 2003. Surface localized glyceraldehyde-3-phosphate dehydrogenase of *Mycoplasma genitalium* binds mucin. Mol Microbiol **48:**1417-1425.
10. **Dumke R, Hausner M, Jacobs E.** 2011. Role of *Mycoplasma pneumoniae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mediating interactions with the human extracellular matrix. Microbiology **157:**2328-2338.
11. **Casadevall A, Pirofski LA.** 1999. Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. Infect Immun **67:**3703-3713.
12. **Pilo P, Vilei EM, Peterhans E, Bonvin-Klotz L, Stoffel MH, Dobbelaere D, Frey J.** 2005. A metabolic enzyme as a primary virulence factor of *Mycoplasma mycoides* subsp. *mycoides* small colony. J Bacteriol **187:**6824-6831.
13. **Rohmer L, Hocquet D, Miller SI.** 2011. Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. Trends Microbiol **19:**341-348.
14. **Halbedel S, Stulke J.** 2007. Tools for the genetic analysis of Mycoplasma. Int J Med Microbiol **297:**37-44.
15. **DaMassa AJ, Wakenell PS, Brooks DL.** 1992. Mycoplasmas of goats and sheep. J Vet Diagn Invest **4:**101-113.
16. **Corrales JC, Esnal A, De la Fe C, Sánchez A, Assunçao P, Poveda JB, Contreras A.** 2007. Contagious agalactia in small ruminants. Small Rumin Res **68:**154-166.
17. **Chopra-Dewasthaly R, Marenda M, Rosengarten R, Jechlinger W, Citti C.** 2005. Construction of the first shuttle vectors for gene cloning and homologous recombination in *Mycoplasma agalactiae*. FEMS Microbiol Lett **253:**89-94.
18. **Chopra-Dewasthaly R, Zimmermann M, Rosengarten R, Citti C.** 2005. First steps towards the genetic manipulation of *Mycoplasma agalactiae* and *Mycoplasma bovis* using the transposon Tn*4001*mod. Int J Med Microbiol **294:**447-453.
19. **Chopra-Dewasthaly R, Citti C, Glew MD, Zimmermann M, Rosengarten R, Jechlinger W.** 2008. Phase-locked mutants of *Mycoplasma agalactiae*: defining the molecular switch of high-frequency Vpma antigenic variation. Mol Microbiol **67:**1196-1210.
20. **Chopra-Dewasthaly R, Baumgartner M, Gamper E, Innerebner C, Zimmermann M, Schilcher F, Tichy A, Winter P, Jechlinger W, Rosengarten R, Spergser J.** 2012. Role of Vpma phase variation in *Mycoplasma agalactiae* pathogenesis. FEMS Immunol Med Microbiol **66:**307-322.
21. **Skapski A, Hygonenq MC, Sagne E, Guiral S, Citti C, Baranowski E.** 2011. Genome-scale analysis of M*ycoplasma agalactiae* loci involved in interaction with host cells. PLoS One **6:**e25291.

22. **Hegde S, Hegde S, Spergser J, Brunthaler R, Rosengarten R, Chopra-Dewasthaly R.** 2014. In vitro and in vivo cell invasion and systemic spreading of *Mycoplasma agalactiae* in the sheep infection model. Int J Med Microbiol.
23. **McAuliffe L, Ellis RJ, Miles K, Ayling RD, Nicholas RA.** 2006. Biofilm formation by *mycoplasma* species and its role in environmental persistence and survival. Microbiology **152:**913-922.
24. **Fleury B, Bergonier D, Berthelot X, Peterhans E, Frey J, Vilei EM.** 2002. Characterization of P40, a cytadhesin of *Mycoplasma agalactiae*. Infect Immun **70:**5612-5621.
25. **Sirand-Pugnet P, Lartigue C, Marenda M, Jacob D, Barre A, Barbe V, Schenowitz C, Mangenot S, Couloux A, Segurens B, de Daruvar A, Blanchard A, Citti C.** 2007. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. PLoS Genet **3:**e75.
26. **Solsona M, Lambert M, Poumarat F.** 1996. Genomic, protein homogeneity and antigenic variability of *Mycoplasma agalactiae*. Vet Microbiol **50:**45-58.
27. **Czurda S, Jechlinger W, Rosengarten R, Chopra-Dewasthaly R.** 2010. Xer1-mediated site-specific DNA inversions and excisions in *Mycoplasma agalactiae*. J Bacteriol **192:**4462-4473.
28. **Knudtson KL, Minion FC.** 1993. Construction of Tn*4001* lac derivatives to be used as promoter probe vectors in mycoplasmas. Gene **137:**217-222.
29. **Sharma VM, Chopra R, Ghosh I, Ganesan K.** 2001. Quantitative target display: a method to screen yeast mutants conferring quantitative phenotypes by 'mutant DNA fingerprints'. Nucleic Acids Res **29:**E86-86.
30. **Hegde S, Zimmermann M, Rosengarten R, Chopra-Dewasthaly R.** 2011, p 87. DGHM 2011, Essen, Germany.
31. **Sambrook J, Russell DW.** 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.
32. **Meloni GA, Bertoloni G, Busolo F, Conventi L.** 1980. Colony morphology, ultrastructure and morphogenesis in Mycoplasma hominis, Acholeplasma laidlawii and Ureaplasma urealyticum. J Gen Microbiol **116:**435-443.
33. **Dybvig K, Cao Z, French CT, Yu H.** 2007. Evidence for type III restriction and modification systems in *Mycoplasma pulmonis*. J Bacteriol **189:**2197-2202.
34. **Hasselbring BM, Page CA, Sheppard ES, Krause DC.** 2006. Transposon mutagenesis identifies genes associated with *Mycoplasma pneumoniae* gliding motility. J Bacteriol **188:**6335-6345.
35. **Mudahi-Orenstein S, Levisohn S, Geary SJ, Yogev D.** 2003. Cytadherence-deficient mutants of *Mycoplasma gallisepticum* generated by transposon mutagenesis. Infect Immun **71:**3812-3820.
36. **Miles RJ, Wadher, B.J., Henderson, C.L. and Mohan, K.** 2008. Increased growth yields of *Mycoplasma* spp. in the presence of pyruvate. Lett Appl Microbio **7:**149-151.
37. **Chen H, Yu S, Shen X, Chen D, Qiu X, Song C, Ding C.** 2011. The *Mycoplasma gallisepticum* alpha-enolase is cell surface-exposed and

mediates adherence by binding to chicken plasminogen. Microb Pathog **51:**285-290.
38. **Schreiner SA, Sokoli A, Felder KM, Wittenbrink MM, Schwarzenbach S, Guhl B, Hoelzle K, Hoelzle LE.** 2012. The surface-localised α-enolase of *Mycoplasma suis* is an adhesion protein. Vet Microbiol **156:**88-95.
39. **Song Z, Li Y, Liu Y, Xin J, Zou X, Sun W.** 2012. alpha-Enolase, an adhesion-related factor of *Mycoplasma bovis*. PLoS One **7:**e38836.
40. **Chang J, Pang E, He H, Kwang J.** 2008. Identification of novel attenuated *Salmonella Enteritidis* mutants. FEMS Immunol Med Microbiol **53:**26-34.
41. **Pang E, Tien-Lin C, Selvaraj M, Chang J, Kwang J.** 2011. Deletion of the *aceE* gene (encoding a component of pyruvate dehydrogenase) attenuates *Salmonella enterica* serovar Enteritidis. FEMS Immunol Med Microbiol **63:**108-118.
42. **Sun Z, Fu P, Wei K, Zhang H, Zhang Y, Xu J, Jiang F, Liu X, Xu W, Wu W.** 2014. Identification of novel immunogenic proteins from Mycoplasma bovis and establishment of an indirect ELISA based on recombinant E1 beta subunit of the pyruvate dehydrogenase complex. PLoS One **9:**e88328.
43. **Cieri MV, Mayer-Hamblett N, Griffith A, Burns JL.** 2002. Correlation between an in vitro invasion assay and a murine model of *Burkholderia cepacia* lung infection. Infect Immun **70:**1081-1086.
44. **Much P, Winner F, Stipkovits L, Rosengarten R, Citti C.** 2002. *Mycoplasma gallisepticum*: Influence of cell invasiveness on the outcome of experimental infection in chickens. FEMS Immunol Med Microbiol **34:**181-186.
45. **Winner F, Rosengarten R, Citti C.** 2000. In vitro cell invasion of *Mycoplasma gallisepticum*. Infect Immun **68:**4238-4244.

Tables and Figures

Table 1. List of oligonucleotides used in this study. Underlined sequences represent incorporated restriction sites.

Name	Sequence
PromfpB	AGT CAA GGA TCC TCT TCA CGA TTC TGT T
PromrpN	CTC TAT CCA TGG CGC TAC AAT CTT CTA CT
pdhABfpN	ACT TAT CCA TGG CAG CTA GTC CGC TTA
pdhABrfN	GTA CTT CCA TGG CTC CGT ACG GTT ATA

Table 2. Promoter regions upstream of the *vpmaY* gene predicted by two different promoter prediction softwares

Input sequence	Start	End	Score	Promoter sequence	Software
-400 bp of vpmaY	273	318	1.00	acaaaaaatttttgctatttttttttttttttatataatattgtcatctat	NNPP
	269	318	1.00	caaaaaatttttgctatttttttttttttttatataatattgtcatctatt -35 -10	BPROM

Figure Legends

Figure 1. **Schematic representation of the pyruvate dehydrogenase (PDH) complex in *M. agalactiae* type strain PG2**. Transposon (Tn) insertion in the *pdhB* gene is shown.

Figure 2. *pdhB* affects *M. agalactiae in vitro* growth. Growth curves of wild type strain PG2 (circles), Δ*pdhB* mutant (squares) and complemented strain *ΔpdhB* :: pP*pdhABlacZ* (triangles) in SP4 medium.

Figure 3. Effect of *pdhB* disruption on colony morphology of *M. agalactiae.* Colonies of wild type PG2, mutant (Δ*pdhB*) and complemented (Δ*pdhB* :: pP*pdhABlacZ*) strain observed microscopically after 7 days of growth on SP4 agar. The pictures were taken at the same resolution.

Figure 4. Micrographs depicting the interaction of *M. agalactiae* with HeLa-229 cells. Double immunofluorescence staining showing the invasion of the wild type PG2 strain, the *pdhB* mutant (*ΔpdhB*) and the complemented strain (Δ*pdhB* :: pP*pdhABlacZ)* of *pdhB* mutant into HeLa cells. The three panels of each strain correspond to the same area of the infected monolayer. FITC fluorescence showing green colored extracellular mycoplasmas and Texas Red fluorescence showing extracellular and intracellular mycoplasmas. Merged images indicating the localization of extracellular (yellow) and intracellular (red) mycoplasmas. Bars, 10 μm

Figure 5. Effect of *pdhB* mutation on HeLa cell invasion analyzed via the double immunofluorence assay. Cellular invasion was evaluated using the wild type *M. agalacatie* PG2 strain, the *pdhB* mutant (Δ*pdhB*) and the complemented strain (Δ*pdhB* :: p*pdhABlacZ*) by counting the internalized mycoplasmas using an Olympus AX-70 epifluorescence microscope. The average number of internalized *M. agalactiae* (MA) per 100 HeLa cells was determined using 100 randomly selected microscopic fields. The bars and error bars indicate the means and standard deviations of triplicate experiments. * P<0.05

Figure 6: **Schematic representation of alternative pathways of pyruvate metabolism in *M. agalactiae***.

Supplementary Figure S1. Schematic representation of the plasmids used in this study with the corresponding *M. agalactiae* transformant colonies depicted on the right. (A) pPlacZ with the *lacZ* gene alone failed to show expression (white colonies), (B) pPlacZ carrying the *vpma* promoter in front of the *lacZ* gene led to intense blue colored colonies, (C) p*lacZpdhAB* carrying the *pdhAB* cloned between the *vpma* promoter and the *lacZ* gene showed intermediate blue colored colonies thereby indicating strong promoter activity.

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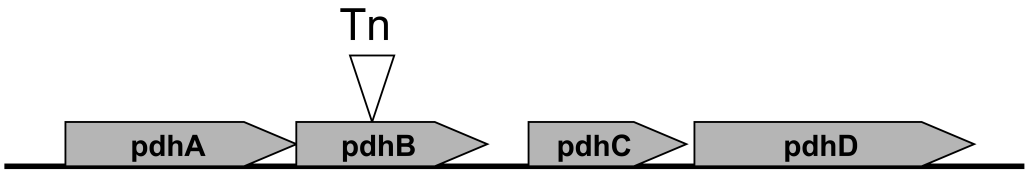

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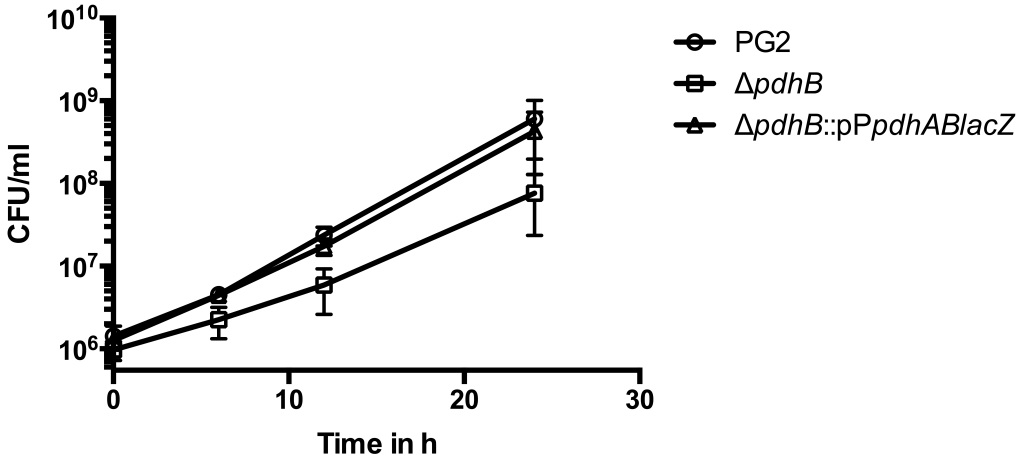

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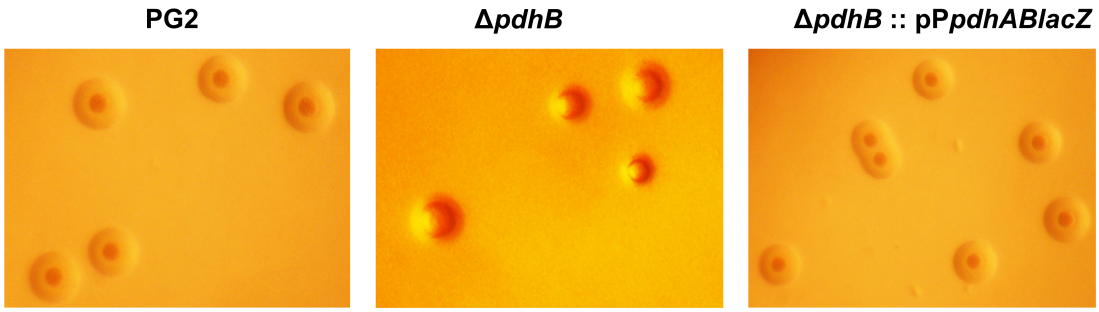

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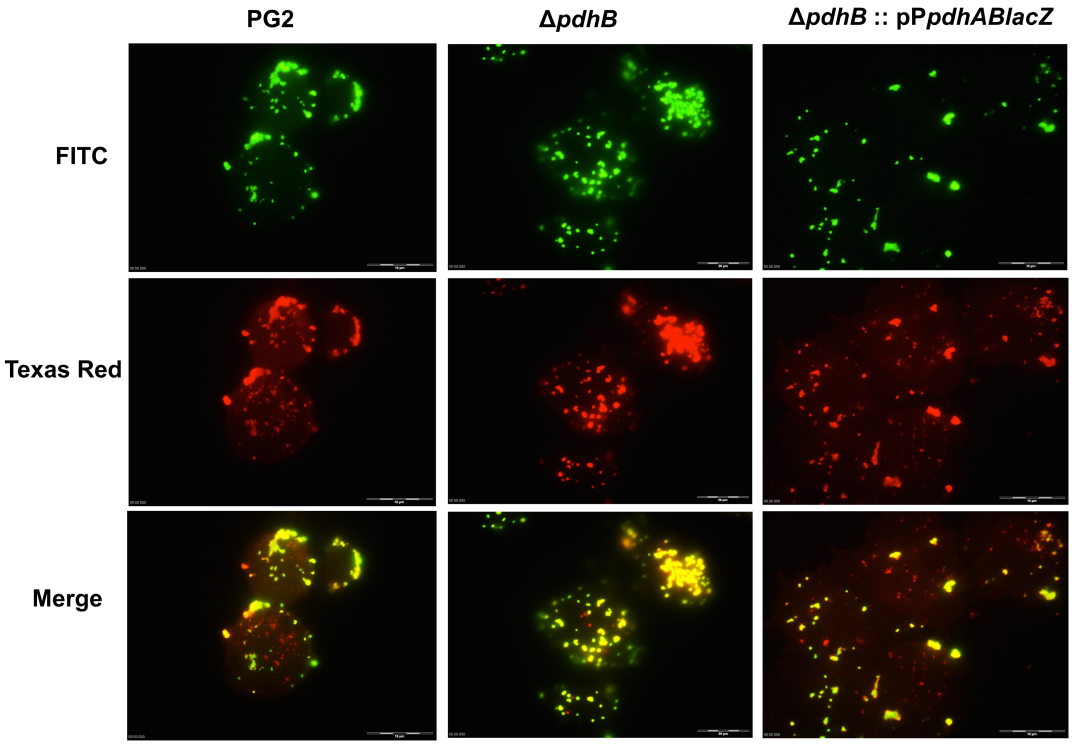

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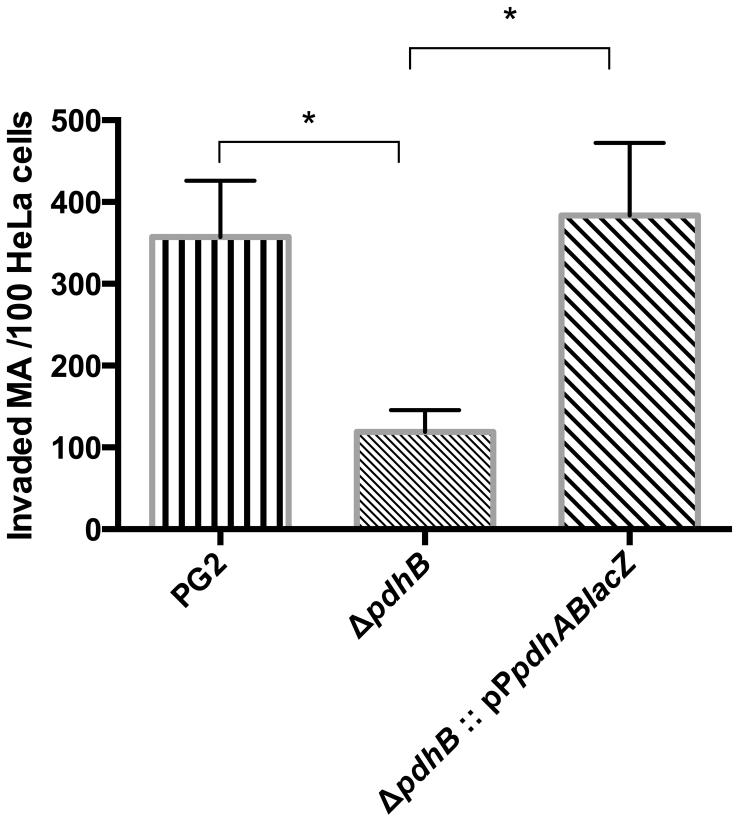
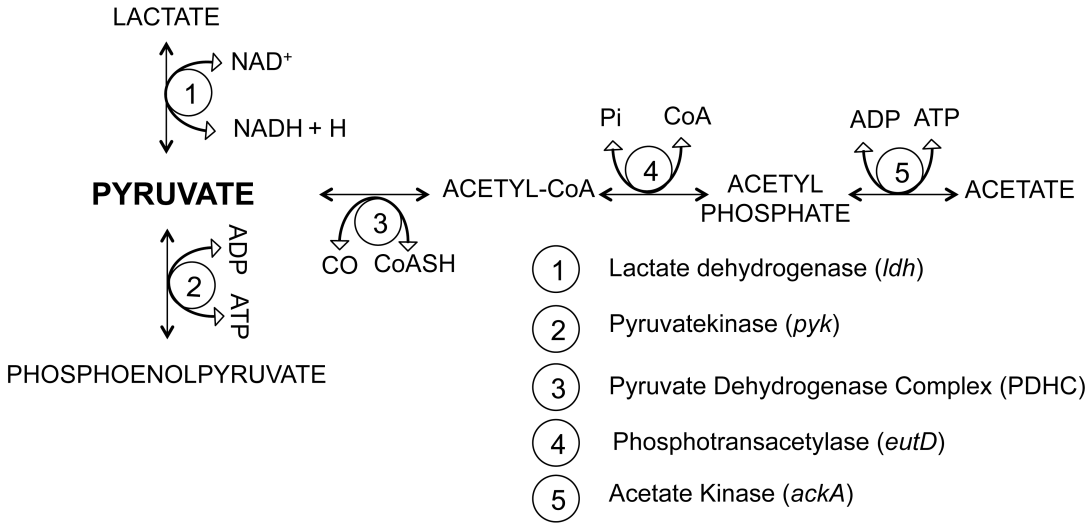


Figure 6: **Schematic representation of alternative pathways of pyruvate metabolism in *M. agalactiae*.**



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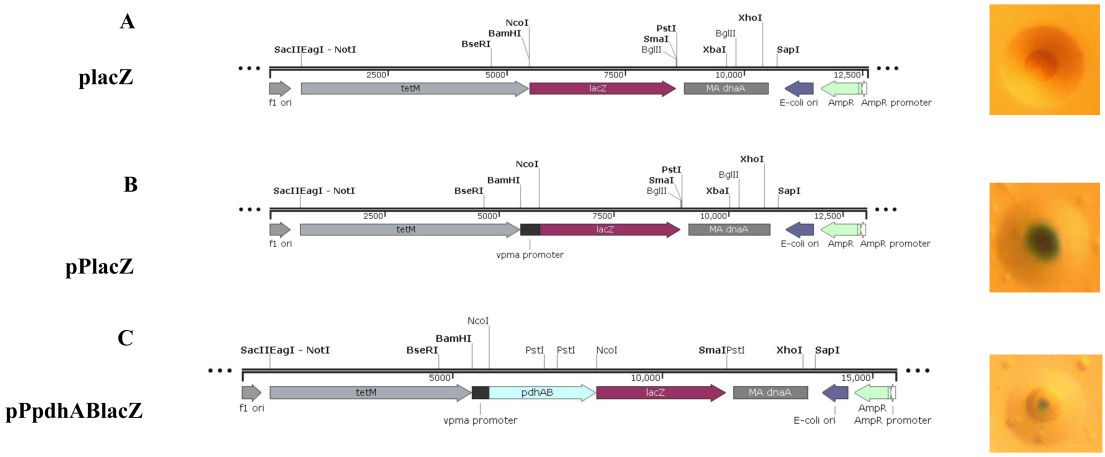

4.3 Manuscript II (under review Infection and Immunity)

Simultaneous identification of putative pathogenicity factors of *Mycoplasma agalactiae* in the natural host (sheep) using negative selection

Running title: Pathogenicity determinants of *M. agalactiae*

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Abstract

Mycoplasmas possess complex pathogenicity determinants that are largely unknown at the molecular level. *Mycoplasma agalactiae* serves as a useful model to study the molecular basis of mycoplasma pathogenicity. Generation and in vivo screening of a transposon mutant library of *M. agalactiae* was employed to unravel its pathogenicity factors. Tn*4001*mod mutants were sequenced using a novel sequencing method and functionally heterogeneous pools containing 15 to 19 selected mutants were simultaneously screened through two successive cycles of sheep intramammary infections. A PCR based negative selection method was employed to identify mutants that failed to colonize the udder and lymph nodes in the animals. A total of 14 different mutants found to be absent in ≥ 95% of samples were identified and subsequently verified via a second round of stringent confirmatory screening where 100% absence was considered as attenuation. Using the criterion seven mutants with insertions in genes MAG1050, MAG2540, MAG3390, *uhpT, eutD, adhT*, and MAG4460 were attenuated, as they were not recovered from any of the infected animals. Among the attenuated mutants, many contain disruptions in hypothetical genes implying their previously unknown role in *M. agalactiae* pathogenicity. These data indicate the putative role of functionally different genes, including hypothetical ones, in the pathogenesis of *M. agalactiae*. Defining the precise functions of the identified genes is anticipated to increase our understanding of *M. agalactiae* infections and to develop successful intervention strategies against it.

Introduction

Being the smallest and simplest self-replicating prokaryotes, mycoplasmas can serve as useful models to study the molecular basis of bacterial pathogenicity. Despite their small genomes, they possess unique biological properties and complex pathogenicity determinants, and apart from rare exceptions, their infection biology and virulence mechanisms at the molecular and cellular level are still largely unknown (1). This study concentrates on *Mycoplasma* (*M.*) *agalactiae*, an important pathogen of small ruminants that can act as a general model for mycoplasma molecular pathogenesis studies, especially the molecular pathogenesis of ruminant mycoplasmosis, as it has acquired about 18% of its small genome from other mycoplasma pathogens via horizontal gene transfer (2) and also shows very close phylogenetic and symptomatic proximity to *Mycoplasma bovis*, a worldwide economically significant bovine pathogen. Besides, it grows well in the laboratory, is amenable to genetic manipulation and its disease pathology is well studied.

M. agalactiae is the main etiological agent of the chronic contagious agalactia syndrome in sheep and goats, which is mostly characterized by mastitis, conjunctivitis, and arthritis (3, 4). Despite causing significant economic losses, its pathogenic mechanisms and attributes are largely unknown. Earlier studies in this regard were based on in vitro analysis of genes involved in host-cell interaction (5) and a recent report has focused on a single factor in an experimental infection study (6). In general, the previously reported in vivo studies either dealt with the characterization of field strains or deciphering events occurring in mammary epithelial cells during *M. agalactiae* infection (7-

10). However, there is a complete lack of studies on the systematic analysis of *M. agalactiae* genetic factors involved in the colonization of the udder and subsequent systemic spread and persistence leading to chronic infections. *M. agalactiae* genome sequencing, together with the development of genetic tools for its genetic manipulation has opened new dimensions in understanding its pathogenesis (2, 11-13).

Although genomics, transcriptomics and proteomics are post-genomic approaches to discover and understand virulence factors (14), pre-genomic in vivo methods, such as transposon (Tn) based Signature Tagged Mutagenesis (STM) and In Vivo Expression Technology (IVET) still play important roles in understanding bacterial pathogenicity (15, 16). As for many other bacteria, random mutagenesis through transposition has been previously used in understanding mycoplasma pathogenesis, but more frequently via in vitro studies. Transposon mutagenesis was initially applied in *M. genitalium* to study the essential minimal set of genes required for independent existence (17, 18). Transposition studies have also helped in deciphering the role of different genetic factors involved in motility, cytadherence and defence systems of mycoplasmas (19-21). Although transposon mutagenesis is attained in many mycoplasmas, it is only in *M. gallisepticum* that Tn mutant libraries have been screened in vivo for identifying pathogenicity determinants using the chicken infection model (22, 23). However, there is no report of any such study in ruminant mycoplasmas, which is imperative in understanding and preventing infections caused by this economically important group of mycoplasmas. Even though transposition in *M. agalactiae* was attained much

earlier (13), it was only recently that this approach was used to identify genes involved in host cell interactions during in vitro cell culture studies (5, 24).

A combination of Tn mutagenesis and negative selection methodology allows identification of in vivo attenuated genes from a complex pool of mutants (15, 25). Although Tn mutagenesis remains a major approach for disruption of genes in mycoplasmas, STM has been applied only in very few instances to identify genes defective in host colonization (22, 23). Studying a similar approach in *M. agalactiae* and other ruminant mycoplasmas has been hindered due to the lack of small animal models. However, general experimental infections of natural ruminant hosts have demonstrated adequate antibody titers, host colonization, systemic spreading and visible clinical signs (8, 26), as also observed during *M. agalactiae* experimental intramammary infections of sheep (10, 27). To understand *M. agalactiae* factors mediating colonization of the host udder, the current study exploited the use of a heterogenous pool of Tn mutants that were simultaneously screened in a sheep intramammary infection model. Colonization-defective mutants in the udder and draining lymph nodes (LNs) were identified by negative selection (15) using Signature Sequence Mutagenesis (SSM) PCR (22). The results demonstrate an attenuation of mutants harboring insertions in different functional genes, some of which have also been previously implicated in the pathogenesis of other bacteria, thus underlining their similar role in *M. agalactiae* infections. Furthermore, many attenuated mutants were identified to carry insertions in hypothetical genes, thus indicating a likely role of these in *M. agalactiae* host colonization. One such hypothetical gene was

previously also identified to play a role during in vitro cell interaction studies (5) thus confirming the in vivo screening protocol used here as a valid approach to identify the actual genes involved in mycoplasma-host interaction during pathogenesis.

Material and Methods

Mycoplasma cultures and growth condition

M. agalactiae pathogenic type strain PG2 (2, 28) was used in this study and was previously isolated from an infected goat in Spain . All the mycoplasma cultures were grown at 37° C in standard Aluotto medium (29) containing penicillin G, 10% horse serum, 25% sodium pyruvate and 0.5% phenol red as described before (13). Gentamicin sulphate (50µg/ml) was used both in broth and agar plates for the propagation of Tn mutants. *E. coli* strain DH10B (Invitrogen GmbH, Lofer, Austria) containing Tn vector pISM2062 was grown in Luria broth (1% tryptone, 0.5% yeast extract and 0.5 % NaCl) supplemented with ampicillin (100 µg/ml) and gentamicin sulphate (7µg/ml).

Transposon mutant library

Transposon mutants were generated in *M. agalactiae* strain PG2 using the suicidal plasmid pISM2062 containing transposon Tn*4001*mod (30) as described earlier (13). Briefly, the mycoplasmas grown until late log phase were electroporated with 1-3µg of plasmid DNA and transformants selected on gentamicin plates were grown in Aluotto medium. Genomic DNA was

isolated from 30ml culture using QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) and stored at -20° C until further use.

Identification of transposon insertion site

Transposon mutants were sequenced using a modified QTD based ligation mediated PCR (31) schematically presented in Fig.1. Mutant DNA was digested with *Apo*I (NEB Inc.) at 50° C for 4 h as per manufacturer's instructions and ligated to adaptors using T4 DNA ligase (Promega, Madison, WI, USA) at 16° C overnight. Adaptors were prepared by annealing an equimolar mixture of oligos Ad1 and Ad2 (Table 1). Primary PCR was performed with diluted ligation mixtures as template in PCR carried out in 2mM $MgCl_2$ in presence of 40 nM of primers IS-1 and EcoRI (Table 1), 1M Betaine and HOT FIREPol® (Solis Biodyne) in 1x PCR Buffer B. Initial denaturation was carried out at 94°C followed by annealing at 58°C for 1 min, reduced by 0.7°C every cycle for 11 cycles after which it was kept constant at 52°C, and an extension at 72° C for 1 min. Primary PCR product was used as a template in subsequent semi-nested PCR using 200 µM of each dNTP, 200 nM of primers IS-N and EcoRI, 2 mM $MgSO_4$ and 5U of HOT FIREPol® (Solis Biodyne) *Taq* Polymerase in presence of 1× PCR buffer B. The secondary PCR product was gel purified and sequenced at LGC Genomics GmbH, Berlin, Germany. The position of Tn in the genome was determined by nucleotide blast in Molligen 3.0 (32).

In vitro growth analysis and transposon stability

Growth rate of Tn mutants was analyzed in Aluotto medium for 72 h at 37°C by plating serial dilutions at regular time intervals to determine viable mycoplasma counts. For analyzing Tn stability, each mutant was separately inoculated in 1.5-ml microcentrifuge tube containing 1 ml Aluotto broth and incubated at 37 °C until growth was evident. These mutant cultures were then successively passaged 30 times in nonselective broth, and at every 10^{th} passage cultures were serially diluted and plated on Aluotto agar plates with or without 50 g/ml gentamicin. Colonies on both plates were compared after one week to check for the loss of Tn. Additionally, at every 10^{th} passage each culture was transferred to 30 ml Aluotto medium without gentamicin to isolate genomic DNA. The latter was used as a template to perform SSM PCR (see below) to evaluate the loss and movement of the Tn when grown in non-selective medium. Southern blot analysis using Tn specific probe was also performed to further check the Tn stability as described earlier (13).

Screening of the mutant population for attenuated virulence through negative selection in vivo

The mutants were screened using the Signature Sequence Mutagenesis (SSM) PCR, which is a modification of the original STM technique used to identify attenuated mutants in vivo (22). Briefly, each mutant in the pool was identified by a unique PCR product obtained by using a common primer IS-N

annealing at the end of the Tn and a mutant-specific primer that binds to the mutated gene (Table S1). Gene specific primers were designed such that the amplicons fall in the range of 200-1000 bp. Initially, SSM PCR was performed with the genomic DNA of each of the single mutants and then checked by mixing equal amounts of genomic DNA of selected mutants to validate the PCR in a mixture. Each 25 µl PCR reaction mixture contained 300 ng of template, 2mM $MgCl_2$, 100 µM dNTP, 1 µM of each primer and 1.2 U HOT FIREPol® DNA polymerase in 1x Buffer B (Solis Biodyne). Amplification conditions were 95 °C for 12 min; 94 °C for 1 min, 54 °C for 30 sec and 72 °C for 1 min (30 cycles); and 72 °C for 5 min. The PCR products were separated on 1% agarose gel before staining with ethidium bromide and visualizing using the Gel Doc™ XR+ System (Bio-Rad, Hercules, CA).

Inoculum preparation

Inoculum was prepared as described previously (27) with some modification. The mutants were grown in 30 ml Aluotto at 37°C for 72 hrs. The cultures were centrifuged at 10,000 x g for 15 min at 4°C and pellets were washed with 1x Dulbecco's Phosphate Buffered Saline pH 7.4 (DPBS, Invitrogen Corporation, NY, USA) before final suspension and storage at -80°C as 200 µl aliquots. Killing rate after one freeze thaw cycle was determined by comparing the cfu at the time of freezing and seven days after freezing by plating serial dilutions on Aluotto agar. Based on these calculations, mutants were accordingly mixed in equal proportions to get a total number of 10^9 cells to be inoculated per sheep. The residual inocula left after initial infection was grown in 30 ml Aluotto to isolate 'input pool' genomic DNA.

Ethical statement

All procedures related to the animal experiments were performed with the permission of the Ethics Committee of the University of Veterinary Medicine Vienna and Austrian Federal Ministry for Science and Research (field of engineering and animal testing) with approval number (BMWF-68.205/002-II/3b/2011 and BMWF-68.205/0104-II/3b/2012).

Intramammary sheep infection model

The intramammary sheep infection model was used in this study as described earlier (27). Screenings were done in 1-2 years old lactating ewes that were purchased from a local sheep flock after serological examination and housed in separate stables. Sheep were monitored for one week through clinical and serological examinations. Bacteriological tests were performed with swabs and milk samples to negate the presence of mycoplasma and other pathogenic bacteria before infection. Each sheep received 5 ml of 10^9 cfu of pooled mutants prepared in DPBS (see above). Swabs were collected from ears, eyes and vagina during the course of infection and grown in Aluotto medium supplemented with 50 µg/ml each of penicillin, gentamicin and thallium acetate. Milk yield, body temperature and other clinical data were recorded regularly. Milk samples from both udder halves at the time of necropsy were used for bacteriological and SSM PCR analysis. Blood samples for haematology and serology were collected once in 7 days and sera were stored at -80°C after centrifugation (27). Sheep were necropsied two weeks post infection and LNs and udder tissue samples were collected aseptically.

Sample collection and cultivation

Milk samples were grown in Aluotto medium as 10 fold serial dilutions for 7 days at 37°C and genomic DNA was isolated from these cultures for SSM PCR analysis. LNs and organs collected during necropsy were processed as described before (27). Briefly, samples were grown in Aluotto medium with antibiotics as 10 and 100 fold dilutions and incubated at 37°C for 7 days to check for the presence of mycoplasmas by plating on Aluotto agar. Genomic DNA isolated from these cultures, designated as 'output pool' DNA, was additionally subjected to *M. agalactiae* specific PCR (33). Once confirmed to be positive for *M. agalactiae*, this 'output pool' DNA was used as a template to generate output SSM PCR.

Identification of in vivo attenuated mutants

Output pool DNA was used as template to produce output SSM PCR using mutant specific primer and Tn specific primer (IS-N). Pools of 15-19 mutants were tested in 15-19 individual PCRs/sample, each corresponding to individual mutated genes, and this procedure was applied to all samples originating from all three sheep in a group. Each output SSM PCR was compared with that of input SSM PCR from that group and a mutant was considered absent if the output PCR was negative and input PCR was positive for it. PCR results from the udder and all the tested LNs from each sheep were compiled and percentage of absence was calculated for each mutant. Absence percentage was calculated by dividing the number of negative PCR results per sheep by total number of samples tested from it, including udder and LNs. Average percentage of absence for each mutant

was then calculated from all the three sheep. The results are plotted as a bar graph with each bar depicting average percentage of absence ± standard deviation. Mutants with an average percentage of absence of ≥ 95% were considered attenuated in the initial screening and were subsequently subjected to the second round of confirmatory screening where attenuation was considered only in case of 100% absence. Similarly, output SSM PCR was generated using output mutant pool DNA from milk samples from each sheep at the time of necropsy. Output SSM PCR results from necropsied samples and milk were compared.

In vivo screening of mutants

The screening was carried out as two successive rounds of screening in lactating ewes, namely initial screening and confirmatory screening

Initial screening was performed with nine 1-2 years old sheep allocated into three groups of three animals each. Group 1, 2 and 3 were injected with 10^9 cfu of a pool of 15, 17 and 19 mutants respectively, via the right teat canal. As five mutants were kept common between two or all three groups as 'watermark' controls to negate the possibility of individual animal specific attenuation, the actual number of different mutants tested was 45 (Table S1). Four mutants were kept common between two groups (*rnhB*, *lip*, *adhT* & *nox* (double mutant) and *oppB),* whereas *ptsL* mutant was kept common between all three infected groups. Double mutant 9-31 (*adhT* & *nox*) was included in the study to assess the role of individual and/or both mutated genes, especially in comparison with their single gene mutant counterparts. For this, *nox* gene single mutant was included in the group 3 along with double mutant

9-31. All animals were euthanized after 2 weeks of infection and samples from local infection sites, such as the udder and supramammary LN were obtained from left and right sides. Together with the sampled knee tissue (right side) and other LNs, such as iliacis, cervicalis superficialis, mandibularis, mediastinalis & mesenterialis, a total of 13 samples/sheep were analyzed via SSM PCR, thereby corresponding to 39 samples per group of 3 sheep each. Mutants exhibiting an average percentage of absence of ≥95% were considered attenuated.

Confirmatory screening was carried out to verify the attenuation of the mutants selected in the initial round of screening. Three separate sheep were infected with a pool of 14 mutants that were selected via the initial round of screening as described before. All procedures during the experiment were followed as described before. However, the stringency level was further increased in this round by considering a mutant attenuated only if it was totally absent, that is when it showed an average percentage absence of 100%. Samples were collected from euthanized sheep as described in the initial screening, except that this time three additional LNs, namely right and left parotideal and left popliteal were also checked. Hence, total number of samples analyzed via SSM PCR now corresponded to 16 per sheep and 48 for all three sheep together. Primers and conditions for SSM PCR were the same as used before.

In vitro competitive assay

In order to assess competition between mutants in a pool, an in vitro growth assay was carried out with them in axenic medium. Fourteen mutants used in

the confirmatory screening were grown individually for 48 h in 1ml SP4 medium as described earlier and then mixed in equal amounts based on the total cell protein concentration determined for each culture as described earlier (34). The mixed mutant culture was grown for 72 h after removing an aliquot for T0 h genomic DNA isolation for qPCR analysis. After 72 h of growth, the culture was removed and genomic DNA was isolated using QIAamp DNA Mini Kit (Qiagen). Quantitative real time PCR was performed in CFX96 Real-Time System (Biorad) in 10 µl reaction volume containing 5 pmol of respective SSM PCR primer set specific to each of the 7 mutants that were absent in the final round of screening (Table. S1). The reaction mixture also comprised of 1X SYBR green master mix (Qiagen) and 250 ng of T0 and T72 h genomic DNA as template. Cycling parameters consisted of initial denaturation at 95°C for 10 min followed by 40 cycles of 40 sec of denaturation at 94°C, 45 sec of annealing at 54°C and 1 min of extension at 72°C, each and this was followed by melting curve analysis at the end of the amplification. Primer efficiency was calculated for each mutant via the standard curve method using serially diluted genomic DNA of each mutant as template. Each reaction was carried out in triplicate and the whole experiment was performed three times. Mean Cq values as indicator of growth were compared for each mutant for T0 and T72 h samples considering 16s rRNA (33) as reference gene. The significance of differences between means of experiments was calculated by paired Student's t test using GraphPad Prism 5 (Graphpad Software Inc, CA, USA). Differences with $P < 0.05$ were considered significant.

Statistical analysis

For each tested mutant, the SSM PCR results were expressed as average percentage of absence ± standard deviation from each group of three sheep. Mutants demonstrating ≥95% and 100% average absence were considered attenuated in the initial and confirmatory round of screening, respectively. Reproducibility of results between and within the experiments was checked by performing the Mann-Whitney test (35). Differences with $P < 0.05$ were considered significant.

Results

Identification of Tn*4001*mod insertion sites using a novel QTD based PCR method

A Tn mutant library was constructed in the pathogenic type strain PG2 of *M. agalactiae* using Tn*4001*mod (30). Disruption sites were mapped using a PCR method based on the principles of Quantitative Target Display (QTD) technique (31) as described in the Materials and Methods section. This sequencing method is based on a ligation mediated PCR using a single primer pair for all the mutants irrespective of the site of Tn insertion in the genome as illustrated in Fig. 1. In contrast to previously available methods that are either laborious, time-consuming or costly (36), this specific and selective amplification of the Tn insertion sites based on the QTD strategy is quite simple, cost effective and less laborious as many mutants can be simultaneously sequenced at the same time using the same pair of primers.

Besides, this strategy also avoids the problems that are generally encountered while using the other available methods, such as inverse PCR. Most importantly, even double insertion sites can be sequenced without any problem unlike the other available methods. In addition, the possibility of obtaining two PCR fragments corresponding to the two regions flanking a single transposon insertion offers a further specificity check for identifying the mutated gene. Using this unique method, the Tn insertion sites were mapped in more than 150 mutants. The sequence results were analyzed by BLAST homology search (37) against the annotated genome of *M. agalactiae* type strain PG2 (2) using the MolliGen database (32).

Based on the Hutchison criteria (18), the Tn insertions were analyzed and classified as functionally disruptive, if they were located after the first 3 codons and before the 3'-most 20% of the coding sequence of a gene. However, we have included in our screening protocol a few important Tn mutants that do not meet these criteria, such as *vpmaX and oppB*, because these criteria are not always valid as recently shown by May et al (2012) (38) for a sialidase Tn mutant of *M. gallisepticum* (that did not meet Hutchison's criteria) that was successfully used in chicken experiments to prove that the sialidase gene is an important virulence-associated gene. Several mutants had insertions either in inter-genic regions or at the end of coding sequences (CDS) and were considered not disrupted. Tn insertions were also confirmed by Southern blot analysis using Tn specific probe (data not shown). Even though Tn insertion was mostly random, few mutations were repeated with the same gene disrupted in many different mutants such as MAG6200, MAG6690 and *lplA*.

The library also included mutants with double Tn insertions. In such cases the corresponding single insertion mutants serve as important controls in comparing the role of individual genes. For instance, mutant 9-31 has two Tn insertions (*adhT* and *nox*), and the corresponding single insertion mutant at the *nox* site when used in conjunction served as an important control to compare the role of individual genes, namely *nox* and *adhT*. Numerous mutants exhibited gene disruptions in previously characterized important genes of *M. agalactiae*, namely MAG2540, MAG1890, *vpmaX, vpmaY, mraZ*, whereas several others have been implicated in virulence of other bacterial pathogens, such as *eutD, uhpT*, *nox, gtsB, pdhB* and *rnhB*. The mutants were grouped into arbitrary functional categories such as transporters, surface proteins or lipoproteins, metabolism related, regulatory as well as hypothetical.

In vitro analysis of transposon mutants

The mutants to be used for in vivo screening were first analyzed in vitro for their growth compared to the wild type strain PG2. Most of the examined mutants did not show any defect in growth and were as good as wild type strain PG2. However, mutants 6-29 (*pdhB*) and 6-14 (*uhpT*) exhibited smaller colony size as compared to PG2 strain on Aluotto agar. Interestingly, in Aluotto broth, only 6-29 (*pdhB*) showed a slightly slower growth rate (data not shown). Nevertheless, this mutant was also included in the current in vivo study because pyruvate dehydrogenase mutants in other bacteria are also known to exhibit in vitro slow growth phenotypes and yet have been used in STM screens and accepted to play important roles in the pathogenicity of

these bacteria (39, 40). In vitro growth competition assay performed with the pool of 14 selected mutants used as inocula for confirmatory screening did not reveal any growth retardation (P>0.05) for any of the 7 mutants showing 100% absence in vivo, This was demonstrated via qPCR analyses as illustrated in Fig. S1. This rules out the role of competition or growth defects in attenuation (Fig S1). When checked for stability of the Tn insertions, all mutants were found to have stable insertions. Also, any movement of the Tn within the genome of each of the mutants was excluded via Southern blot analysis.

Model for screening *M. agalactiae* transposon mutants

An appropriate infection model is essential for the successful identification of attenuated genes using negative selection methods (41). The lack of a small animal model has hampered *M. agalactiae* in vivo infection studies to study its pathogenicity attributes. Previously, we have studied the colonization of *M. agalactiae* wild type strain PG2 and PLMs (Vpma phase-locked mutants) using the sheep infection model (27). Following intramammary inoculation of 10^9 cfu in the right udder of lactating ewes, the PLMs and PG2 were recovered in milk right from Day 1 till the end of the study and also, upon necropsy, from both udder halves (left and right) and and LN samples throughout the body (27). Furthermore, the model can discriminate between colonizing and non-colonizing mycoplasmas and has also been effectively used in comparing and understanding the pathogenicity of different *M. agalactiae* strains (10, 27). In the current study, a total of 45 different mutants

were screened in two successive experiments through the negative selection method. Attenuation of mutants was assessed by their absence at local infection sites and in milk samples using SSM PCR. The results indicated the involvement of 14 different genomic loci in host colonization (Table 2) in the initial screening, which corresponds to mutants that were not recovered from the infected udders and LNs. The latter constituted about 31% of the inoculated mutants, thereby implying that the remaining 69% of mutants replicated normally within the host. These non-attenuated mutants might navigate in the body to colonize distant sites. The absence of mutants in milk samples was also monitored, and the results obtained around the time of necropsy further confirmed the mutant attenuation results based on tissue colonization. Even though tissue tropism was not observed with the set of mutants used in the current study, correlating the absence of particular mutants to specific organs/LN and to particular stages of infection could help in evaluating tissue-specific and time-specific attenuation, respectively. Bilateral spreading of *M. agalactiae* was clearly observed as mycoplasmas were frequently isolated from the left udder half of the sheep although initially inoculated in the right udder half. Besides the spreading of the organism from one initial site throughout the body, this route of infection also induces clinical signs that would be an important added advantage for any model while performing a challenge infection experiment with single selected mutants to test their virulence attenuation and to investigate their potential use as a novel prophylactic tool. Confirmation of colonization defects for the attenuated mutants even during the second round of our confirmatory screening clearly indicates the reproducibility of results with this model of infection. Altogether,

this model allows a systematic identification of pathogenic determinants in *M. agalactiae*.

Attenuation of mutants in vivo

For the first time a negative selection approach is used to screen Tn mutants of *M. agalactiae* for attenuation in the natural host by using a sheep intrammamary infection model. The schematic representation of the study is depicted in Fig. 2. For initial screening, three different pools of 15-19 Tn mutants were administered per group of three sheep each via the intramammary route. Sheep were found to be free of mycoplasmas as analyzed by serological and bacteriological tests. Milk yield, body temperature and clinical examinations during the course of infection did not yield significant difference between the groups (data not shown). Differential blood counts did not show any difference in counts between the groups (data not shown). About two weeks post infection, the animals were necropsied and tissue samples obtained from both udder halves and various LNs were also examined via SSM PCR. Mutants attenuated in the initial screening were re-assessed in a second round of confirmatory screening as described below.

In order to estimate the reproducibility of results between and within the initial and confirmatory experiments, Mann-Whitney test (35) was performed. The test was performed for mutants kept common between the groups in the initial screening and for mutants identified as attenuated in both screenings. Results indicate no significant difference in average percentage of absence ($p>0.05$) of attenuated mutants that are common between the groups in initial screening. Similarly, the p-value of 0.99 for the mutants that are attenuated in

both screening rounds indicates that results are similar and further confirms the validity of the screening protocols.

Initial screening

A total of 45 different mutants, distributed in three pools, were screened in 9 lactating ewes for two weeks as depicted in Fig. 2. A pool size of 15-19 mutants with each mutant represented in equimolar proportions in a total challenge dose of 10^9 cfu/sheep was used for infection in triplicate. Although it was possible to screen a higher number of mutants, a more complex pool was avoided as this is known to cause random loss of unattenuated mutants and inhomogenous results from different animals (42). The mutants included in the study constituted a functionally heterogeneous population with mutated genes encompassing various arbitrary categories, such as transporters, surface proteins/lipoproteins, and metabolism related, regulatory as well as hypothetical proteins. Mutants found attenuated through SSM PCR analyses are depicted in Fig. 3 where the average percentage of absence ± standard deviation is shown for three sheep of one group. All these attenuated mutants showed an average absence of ≥ 95% in the tested samples. Data suggest that 14 mutants are defective in colonization of local sites and could not spread or survive in the LNs (Table 2). This corresponds to about 31 % of the total infection mutants (i.e. 45 mutants) with many of the identified genes encoding hypothetical products.

Attenuated loci (as shown in Table 2) include four hypothetical genes, namely MAG1890, MAG3390, MAG4460, and MAG3650, which had no known functions so far and with this study could be assigned a role in host

colonization and pathogenicity of *M. agalactiae.* The other identified mutants corresponded to genes encoding functional proteins, such as substrate transporters, peptidases and other metabolic enzymes. Among the attenuated mutants is a double mutant 9-31 (*adhT* and *nox*). However, another mutant (give name & also refer to list or table no.) bearing a single Tn insertion in the *nox* gene was not attenuated indicating that *adhT* mutation is likely responsible for the deficient colonization of 9-31 doublemutant. The latter was kept common between group 1 and 3, and was 100% attenuated in all three animals of group 3 (Fig. 3C), whereas in group 1 it showed 95% attenuation (Fig. 3A) as it was present in just one sample each from the two sheep of group 1. However, the mutant was absent in milk samples of these two sheep at the time of necropsy. Moreover, the percentage attenuation difference between these two groups is statistically insignificant confirming the role of *adhT* in host colonization. Other mutants with Tn insertions in *uhpT, oppB, oppC, eutD, pdhB, gtsB, aminopeptidase* (MAG5520) and lipoprotein genes such as MAG1050 and MAG2540 were also found deficient in colonization. Mutant *oppB* was included in two different groups (group 2 and 3) and was completely (100%) absent in all sheep of both of these groups indicating its importance in the infection potential of *M. agalactiae*. Milk sample results for most of the mutants were the same as obtained from the organs and LNs (data not shown).

Contrary to attenuated mutants, several 'non-attenuated' mutants were repeatedly found positive in local infection sites, LNs and milk samples. For example, mutant 3-1 (*ptsL*) that was included in all three groups as a common

'watermark' control of the study was found positive in nearly all three groups in about 27% of local infection site samples from all 9 animals (Fig. 3A, 3B and 3C). Similar results were also obtained with mutant 8+1-5 (*rnhB*) that showed even better replication inside the host and was present in 50% samples from 6 sheep of two groups (Fig 3A and 3B). Likewise several other mutants were also found to exhibit fairly good replication in vivo and hence were not considered attenuated. Altogether, the results demonstrate considerable reproducibility as the mutants, even when mixed in different combinations, indicated no in vivo growth competition between them and gave similar results.

Confirmatory screening

The number of attenuated mutants obtained in the initial round of screening was much higher (31% of infected mutants) than expected. To confirm the role of the mutated genes in pathogenicity, ideally each attenuated mutant needs to be assessed in sheep individually compared to the WT PG2 and complimented mutant infection studies. However, this was not possible due to two main interrelated reasons, Firstly, the number of attenuated mutants was far too high to make 14 different individual sheep infection trials with all the necessary controls. Secondly, the sheep infection model is rather a too large one to handle under limited resources, and since there is a complete lack of any small animal model existing for *M. agalactiae*, individual mutant analysis was not possible. This prompted us to perform a second round of confirmatory screening via sheep intramammary infections with the selected pool of 14

mutants to verify their attenuation and to narrow down the number of non-colonizing mutants.

The 14 attenuated mutants showing an average cut-off of ≥95% absence in the first screening were re-injected as a pool into three sheep. Samples from the infected udders and LNs were analyzed via SSM PCR to confirm the previous results. However, the stringency level was further increased in this round by considering a mutant to be attenuated only if it was totally absent, that is, when it showed 100% absence in all three sheep. This was done to select only the most important mutants that played a definite role in *M. agalactiae* pathogenicity. Using this criterion, 7 genes were identified to be essential for in vivo survival, out of which MAG3390, and MAG4460 are hypothetical, whereas the remaining five genes, namely *uhpT, eutD, adhT*. MAG1050 and MAG2540 have known functions (Fig. 4, Table 3). The remaining 7 mutants in the pool were not 100% attenuated but a majority of them were absent in >85% of the local infection sites and LNs of the three experimental sheep and might also play some role in pathogenesis (Fig.4). Except for mutant 9-31, all the other six attenuated mutants were also missing in the milk samples obtained one day before necropsy (Table 3).

Discussion

Transposon mutagenesis is a powerful tool to identify the genetic factors involved in bacterial pathogenesis. Different genomic loci were disrupted in the small ruminant pathogen *M. agalactiae* type strain PG2 using Gram-

positive transposon Tn*4001*mod (13, 30). Location of insertion sites throughout the genome was sequenced using a novel method based on a modified version of Quantitative Target Display (QTD) (31). The Tn insertions were mapped and the mutated genes corresponded to different categories, such as lipoproteins, transporters, peptidases and metabolic proteins. Apart from these genes with known functions, many disrupted loci are hypothetical genes of unknown functions. Along with some of these hypothetical mutants many important disrupted genes, that have a proven role in the virulence and disease progression of other pathogens, were selected for in vivo screening. Instead of using the original STM or other hybridization based strategies for identifying the missing mutants in local infection sites and LNs, SSM was employed (22) to avoid the associated problems of cross-hybridization, poor detection and false negatives (43). Moreover, SSM had been successfully employed in a similar in vivo screening of selected *M. gallisepticum* mutants to identify pathogenicity-associated genes.

An appropriate infection model is essential for the successful identification of attenuated genes using negative selection methods. Previously, we have studied the colonization of *M. agalactiae* wild type strain PG2 and PLMs (Vpma phase-locked mutants) using the sheep infection model (27). This study utilizes the natural host and the natural route of infection of *M. agalactiae* for in vivo screening of mutants, namely, the intramammary sheep infection model. Besides the colonization and multiplication of *M. agalactiae* in the udder and simultaneous shedding in milk, it also leads to clinical signs and systemic spreading to various body sites (10, 27, 44, 45). This study is first of

its kind that exploits a negative selection method for identifying pathogenicity determinants of *M. agalactiae,* which can act as a useful model for understanding ruminant mycoplasmosis. Two successive rounds of experimental infections were conducted in lactating ewes to screen a total of about 50 different mutants in a maximum pool size of 19 mutants. Similar studies with other pathogenic bacteria have been performed using different animal models and pool sizes (46-48). Here we used a small pool of known mutants to observe a better spreading and to reduce competition. Additionally, in vitro growth analysis of the mixture of mutants attenuated in the first round of screening revealed no competitive growth defects for the seven mutants that were totally absent in all animals during confirmatory screening.

The study identified 7 different *M. agalactiae* genes involved in colonization of local infection sites and survival in the natural host (Table 3). Importantly, two of these attenuated mutants carried insertions in hypothetical CDS, namely MAG3390 and MAG4460. As observed for many other sequenced bacteria, the genome of *M. agalactiae* contains a large proportion of hypothetical proteins with no known functions. These genes might provide subtle quantitative advantages to the pathogen in the host microenvironment, especially when competing with other mutants in the population. Considering the minimal genomes of mycoplasmas, the identified hypothetical proteins are likely to play important roles in infection and disease. In this context, MAG3390 is especially interesting as it has no homology with any other mycoplasma gene and is also devoid of any functional motifs. On the other hand MAG4460 shares about 83% identity with the *M. bovis* lipoprotein

MBOVPG45_0528 and both genes share a common acid phosphatase_hydrolase motif. *M. bovis* is a close phylogenetic relative of *M. agalactiae* that possesses a homologous lipoprotein antigenic variation system and induces similar clinical symptoms of mastitis and arthritis in cattle (11). This could suggest a similar function for both genes in their respective hosts during infection and disease progression.

The *M. agalactiae* genome encodes abundant surface lipoproteins that participate in important interactions with host cells, including the modulation and evasion of the host immune response (27, 49). The final confirmatory in vivo screen identified two lipoproteins, MAG1050 and MAG2540, the latter predicted to be a Vpma like lipoprotein. These lipoproteins seem to be essential for colonization and survival in the host as the corresponding mutants were totally absent in all samples analyzed from all sheep. Furthermore, gene MAG2540 was previously shown to be involved in cell interaction studies performed in vitro using a co-cultivation assay (5). Both in vitro and in vivo results indicate the importance of this gene in *M. agalactiae* host cell interactions and pathogenesis.

It was suggested that genes involved in basic metabolism should not be considered as virulence factors (50). However, pathogens use basic metabolic genes to survive and multiply in the host, and sometimes deficiencies of these genes become much more prominent in host microenvironments. For instance, genes involved in iron metabolism play a significant role in the pathogenesis of many bacteria (51). Thus metabolic genes are frequently identified as important pathogenicity determinants in bacteria and the

corresponding mutant strains exhibit much less or no virulence compared to the wild type strains (52). Since mycoplasmas have limited biosynthetic and metabolic capabilities (1), the role and significance of their minimal set of genes is further enhanced during their interactions with the host. A previous study with *M. mycoides* subsp. *mycoides* clearly points to the fact that metabolic enzymes can act as primary virulence factors in mycoplasmas (53). The *eutD* gene of *M. agalactiae* was found to be attenuated as it showed 100% absence in all samples examined. *M. agalactiae* exhibits enhanced growth in the presence of pyruvate (54) and in the context of the pyruvate metabolism pathway, *eutD* converts acetyl-coA to acetyl phosphate. This is an intermediate step in the energy yielding process involving the conversion of pyruvate to acetate. Although the role of *eutD* in mycoplasma pathogenicity has never been demonstrated so far, it has been shown to be an important host colonization factor for some other important pathogenic bacteria (42, 55, 56). Hence, this gene is likely to play a significant role in *M. agalactiae's* survival and multiplication in the host, especially because it controls the main energy yielding reaction in its metabolism.

Apart from glycerol and pyruvate, *M. agalactiae* can also metabolise alcohols, preferably secondary alcohols like isopropanol (57). The gene encoding alcohol dehydrogenase (*adhT*), is another gene that is implicated to play an important role in *M. agalactiae* survival and pathogenicity as the mutant carrying an insertion in it was consistently absent in all the local infection sites and LNs examined. In other pathogens, in vitro studies have illustrated the role of AdhT in adhesion and biofilm formation (58, 59) whereas a single in

vivo study has demonstrated its role in host colonization (60). Thus, the data of the present study confirm that an alcohol dehydrogenase might be involved in mycoplasma host colonization.

Mycoplasmas import considerable number of biosynthetic precursors and macromolecular compounds to compensate their limited biosynthetic capabilities. Hence, a substantial number of genes annotated in the *M. agalactiae* genome play a role in substrate transport (2). Sugars are important nutrients that the pathogens acquire from their respective hosts for propagation and survival. Hexose phosphate transporter (*uhpT*) plays an important role in this process and it has been shown in other bacteria, however not in mycoplasmas so far, that the deletion of this transporter causes attenuation (61). The *M. agalactiae uhpT* mutant is unable to survive in its natural host during experimental infection indicating its possible role in pathogenicity. Interestingly, this mutant showed slightly smaller colonies on agar media compared to the wild type strain, but did not show any growth deficits in broth cultures (data not shown).

Altogether, these results indicate the diversity of *M. agalactiae* genetic factors involved in its pathogenicity. The identified mutants could serve as promising candidates for detailed investigations for developing them into possible live vaccine candidates provided they are capable of eliciting a strong immune response (39). Efforts made so far in understanding the pathogenicity of mycoplasmas using Tn mutagenesis were based mostly on in vitro studies with very few exceptions (6, 22, 23). The study presented here describes reports an in vivo analysis of several different Tn mutants in the natural host.

Most importantly, instead of checking individual mutants in animal models, pools of many different *M. agalactiae* mutants were simultaneously screened in vivo to identify genes that likely play a significant role in *M. agalactiae* host colonization and pathogenesis. To the best of our knowledge, this is the first report of a systematic analysis of genes involved in ruminant mycoplasmosis using in vivo negative selection screens.

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References

1. **Rosengarten R, Citti C, Glew M, Lischewski A, Droesse M, Much P, Winner F, Brank M, Spergser J.** 2000. Host-pathogen interactions in mycoplasma pathogenesis: virulence and survival strategies of minimalist prokaryotes. Int J Med Microbiol **290:**15-25.
2. **Sirand-Pugnet P, Lartigue C, Marenda M, Jacob D, Barre A, Barbe V, Schenowitz C, Mangenot S, Couloux A, Segurens B, de Daruvar A, Blanchard A, Citti C.** 2007. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. PLoS Genet **3:**e75.
3. **DaMassa AJ, Wakenell PS, Brooks DL.** 1992. Mycoplasmas of goats and sheep. J Vet Diagn Invest **4:**101-113.
4. **Corrales JC, Esnal A, De la Fe C, Sánchez A, Assunçao P, Poveda JB, Contreras A.** 2007. Contagious agalactia in small ruminants. Small Rumin Res **68:**154-166.

5. **Skapski A, Hygonenq MC, Sagne E, Guiral S, Citti C, Baranowski E.** 2011. Genome-scale analysis of M*ycoplasma agalactiae* loci involved in interaction with host cells. PLoS One **6:**e25291.
6. **Baranowski E, Bergonier D, Sagne E, Hygonenq MC, Ronsin P, Berthelot X, Citti C.** 2014. Experimental Infections with *Mycoplasma agalactiae* Identify Key Factors Involved in Host-Colonization. PLoS One **9:**e93970.
7. **Addis MF, Pisanu S, Ghisaura S, Pagnozzi D, Marogna G, Tanca A, Biosa G, Cacciotto C, Alberti A, Pittau M, Roggio T, Uzzau S.** 2011. Proteomics and pathway analyses of the milk fat globule in sheep naturally infected by *Mycoplasma agalactiae* provide indications of the in vivo response of the mammary epithelium to bacterial infection. Infect Immun **79:**3833-3845.
8. **Buonavoglia D, Fasanella A, Greco G, Pratelli A.** 1999. A study of an experimental infection of sheep with *Mycoplasma agalactiae*. New Microbiol **22:**27-30.
9. **Castro-Alonso A, De la Fe C, Espinosa de los Monteros A, Rodriguez F, Andrada M, Poveda JB, Herraez P.** 2010. Chronological and immunohistochemical characterization of the mammary immunoinflammatory response in experimental caprine contagious agalactia. Vet Immunol Immunopathol **136:**43-54.
10. **Sanchis R, Abadie G, Lambert M, Cabasse E, Dufour P, Guibert JM, Pepin M.** 2000. Inoculation of lactating ewes by the intramammary route with *Mycoplasma agalactiae*: comparative pathogenicity of six field strains. Vet Res **31:**329-337.
11. **Chopra-Dewasthaly R, Citti C, Glew MD, Zimmermann M, Rosengarten R, Jechlinger W.** 2008. Phase-locked mutants of *Mycoplasma agalactiae*: defining the molecular switch of high-frequency Vpma antigenic variation. Mol Microbiol **67:**1196-1210.
12. **Chopra-Dewasthaly R, Marenda M, Rosengarten R, Jechlinger W, Citti C.** 2005. Construction of the first shuttle vectors for gene cloning and homologous recombination in *Mycoplasma agalactiae*. FEMS Microbiol Lett **253:**89-94.
13. **Chopra-Dewasthaly R, Zimmermann M, Rosengarten R, Citti C.** 2005. First steps towards the genetic manipulation of *Mycoplasma agalactiae* and *Mycoplasma bovis* using the transposon Tn*4001*mod. Int J Med Microbiol **294:**447-453.
14. **Wu HJ, Wang AH, Jennings MP.** 2008. Discovery of virulence factors of pathogenic bacteria. Curr Opin Chem Biol **12:**93-101.
15. **Hensel M, Shea JE, Gleeson C, Jones MD, Dalton E, Holden DW.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400-403.
16. **Mahan MJ, Slauch JM, Mekalanos JJ.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686-688.
17. **Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, Hutchison CA, 3rd, Smith HO, Venter JC.** 2006. Essential genes of a minimal bacterium. Proc Natl Acad Sci U S A **103:**425-430.

18. **Hutchison CA, Peterson SN, Gill SR, Cline RT, White O, Fraser CM, Smith HO, Venter JC.** 1999. Global transposon mutagenesis and a minimal Mycoplasma genome. Science **286:**2165-2169.
19. **Dybvig K, Cao Z, French CT, Yu H.** 2007. Evidence for type III restriction and modification systems in *Mycoplasma pulmonis*. J Bacteriol **189:**2197-2202.
20. **Hasselbring BM, Page CA, Sheppard ES, Krause DC.** 2006. Transposon mutagenesis identifies genes associated with *Mycoplasma pneumoniae* gliding motility. J Bacteriol **188:**6335-6345.
21. **Mudahi-Orenstein S, Levisohn S, Geary SJ, Yogev D.** 2003. Cytadherence-deficient mutants of *Mycoplasma gallisepticum* generated by transposon mutagenesis. Infect Immun **71:**3812-3820.
22. **Hudson P, Gorton TS, Papazisi L, Cecchini K, Frasca S, Jr., Geary SJ.** 2006. Identification of a virulence-associated determinant, dihydrolipoamide dehydrogenase (*lpd*), in *Mycoplasma gallisepticum* through in vivo screening of transposon mutants. Infect Immun **74:**931-939.
23. **Tseng CW, Kanci A, Citti C, Rosengarten R, Chiu CJ, Chen ZH, Geary SJ, Browning GF, Markham PF.** 2013. MalF is essential for persistence of *Mycoplasma gallisepticum* in vivo. Microbiology **159:**1459-1470.
24. **Baranowski E, Guiral S, Sagne E, Skapski A, Citti C.** 2010. Critical role of dispensable genes in *Mycoplasma agalactiae* interaction with mammalian cells. Infect Immun **78:**1542-1551.
25. **Saenz HL, Dehio C.** 2005. Signature-tagged mutagenesis: technical advances in a negative selection method for virulence gene identification. Curr Opin Microbiol **8:**612-619.
26. **de la Fe C, Castro-Alonso A, Herraez P, Poveda JB.** 2011. Recovery of *Mycoplasma agalactiae* from the ears of goats experimentally infected by the intramammary route. Vet J **190:**94-97.
27. **Chopra-Dewasthaly R, Baumgartner M, Gamper E, Innerebner C, Zimmermann M, Schilcher F, Tichy A, Winter P, Jechlinger W, Rosengarten R, Spergser J.** 2012. Role of Vpma phase variation in *Mycoplasma agalactiae* pathogenesis. FEMS Immunol Med Microbiol **66:**307-322.
28. **Solsona M, Lambert M, Poumarat F.** 1996. Genomic, protein homogeneity and antigenic variability of *Mycoplasma agalactiae*. Vet Microbiol **50:**45-58.
29. **Aluotto BB, Wittler RG, Williams CO, Faber JE.** 1970. Standardized bacteriologic techniques for the characterization of *Mycoplasma* species. Int J Syst Bacteriol **20:**35-58.
30. **Knudtson KL, Minion FC.** 1993. Construction of Tn*4001* lac derivatives to be used as promoter probe vectors in mycoplasmas. Gene **137:**217-222.
31. **Sharma VM, Chopra R, Ghosh I, Ganesan K.** 2001. Quantitative target display: a method to screen yeast mutants conferring quantitative phenotypes by 'mutant DNA fingerprints'. Nucleic Acids Res **29:**E86-86.

32. **Barre A, de Daruvar A, Blanchard A.** 2004. MolliGen, a database dedicated to the comparative genomics of *Mollicutes*. Nucleic Acids Res **32:**D307-310.
33. **Chavez Gonzalez YR, Ros Bascunana C, Bolske G, Mattsson JG, Fernandez Molina C, Johansson KE.** 1995. In vitro amplification of the 16S rRNA genes from *Mycoplasma bovis* and *Mycoplasma agalactiae* by PCR. Vet Microbiol **47:**183-190.
34. **Yavlovich A, Tarshis M, Rottem S.** 2004. Internalization and intracellular survival of *Mycoplasma pneumoniae* by non-phagocytic cells. FEMS Microbiol Lett **233:**241-246.
35. **Mann HB, Whitney DR.** 1947. On a Test of Whether One of 2 Random Variables Is Stochastically Larger Than the Other. Ann Mathematical Stat **18:**50-60.
36. **van Opijnen T, Camilli A.** 2013. Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. Nat Rev Microbiol **11:**435-442.
37. **Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389-3402.
38. **May M, Szczepanek SM, Frasca S, Jr., Gates AE, Demcovitz DL, Moneypenny CG, Brown DR, Geary SJ.** 2012. Effects of sialidase knockout and complementation on virulence of *Mycoplasma gallisepticum*. Vet Microbiol **157:**91-95.
39. **Chang J, Pang E, He H, Kwang J.** 2008. Identification of novel attenuated *Salmonella Enteritidis* mutants. FEMS Immunol Med Microbiol **53:**26-34.
40. **Pang E, Tien-Lin C, Selvaraj M, Chang J, Kwang J.** 2011. Deletion of the *aceE* gene (encoding a component of pyruvate dehydrogenase) attenuates *Salmonella enterica* serovar Enteritidis. FEMS Immunol Med Microbiol **63:**108-118.
41. **Chiang SL, Mekalanos JJ, Holden DW.** 1999. In vivo genetic analysis of bacterial virulence. Annu Rev Microbiol **53:**129-154.
42. **Chiang SL, Mekalanos JJ.** 1998. Use of signature-tagged transposon mutagenesis to identify *Vibrio cholerae* genes critical for colonization. Mol Microbiol **27:**797-805.
43. **Mazurkiewicz P, Tang CM, Boone C, Holden DW.** 2006. Signature-tagged mutagenesis: barcoding mutants for genome-wide screens. Nat Rev Genet **7:**929-939.
44. **Gomez-Martin A, De la Fe C, Amores J, Sanchez A, Contreras A, Paterna A, Buendia AJ, Corrales JC.** 2012. Anatomic location of *Mycoplasma mycoides subsp. capri* and *Mycoplasma agalactiae* in naturally infected goat male auricular carriers. Vet Microbiol **157:**355-362.
45. **Hegde S, Hegde S, Spergser J, Brunthaler R, Rosengarten R, Chopra-Dewasthaly R.** 2014. In vitro and in vivo cell invasion and systemic spreading of *Mycoplasma agalactiae* in the sheep infection model. Int J Med Microbiol.

46. **Flashner Y, Mamroud E, Tidhar A, Ber R, Aftalion M, Gur D, Lazar S, Zvi A, Bino T, Ariel N, Velan B, Shafferman A, Cohen S.** 2004. Generation of *Yersinia pestis* attenuated strains by signature-tagged mutagenesis in search of novel vaccine candidates. Infect Immun **72:**908-915.
47. **Hendrixson DR, DiRita VJ.** 2004. Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. Mol Microbiol **52:**471-484.
48. **Lawlor MS, Hsu J, Rick PD, Miller VL.** 2005. Identification of *Klebsiella pneumoniae* virulence determinants using an intranasal infection model. Mol Microbiol **58:**1054-1073.
49. **Chambaud I, Wroblewski H, Blanchard A.** 1999. Interactions between mycoplasma lipoproteins and the host immune system. Trends Microbiol **7:**493-499.
50. **Casadevall A, Pirofski LA.** 1999. Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. Infect Immun **67:**3703-3713.
51. **Ratledge C, Dover LG.** 2000. Iron metabolism in pathogenic bacteria. Annu Rev Microbiol **54:**881-941.
52. **Rohmer L, Hocquet D, Miller SI.** 2011. Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. Trends Microbiol **19:**341-348.
53. **Pilo P, Vilei EM, Peterhans E, Bonvin-Klotz L, Stoffel MH, Dobbelaere D, Frey J.** 2005. A metabolic enzyme as a primary virulence factor of *Mycoplasma mycoides* subsp. *mycoides* small colony. J Bacteriol **187:**6824-6831.
54. **Miles RJ, Wadher, B.J., Henderson, C.L. and Mohan, K.** 2008. Increased growth yields of *Mycoplasma* spp. in the presence of pyruvate. Lett Appl Microbio **7:**149-151.
55. **Anfora AT, Halladin DK, Haugen BJ, Welch RA.** 2008. Uropathogenic *Escherichia coli* CFT073 is adapted to acetatogenic growth but does not require acetate during murine urinary tract infection. Infect Immun **76:**5760-5767.
56. **Kim YR, Brinsmade SR, Yang Z, Escalante-Semerena J, Fierer J.** 2006. Mutation of phosphotransacetylase but not isocitrate lyase reduces the virulence of *Salmonella enterica* serovar *Typhimurium* in mice. Infect Immun **74:**2498-2502.
57. **Abu-Amero KK, Abu-Groun EA, Halablab MA, Miles RJ.** 2000. Kinetics and distribution of alcohol oxidising activity in *Acholeplasma* and *Mycoplasma* species. FEMS Microbiol Lett **183:**147-151.
58. **Mukherjee PK, Mohamed S, Chandra J, Kuhn D, Liu S, Antar OS, Munyon R, Mitchell AP, Andes D, Chance MR, Rouabhia M, Ghannoum MA.** 2006. Alcohol dehydrogenase restricts the ability of the pathogen *Candida albicans* to form a biofilm on catheter surfaces through an ethanol-based mechanism. Infect Immun **74:**3804-3816.
59. **Jagadeesan B, Koo OK, Kim KP, Burkholder KM, Mishra KK, Aroonnual A, Bhunia AK.** 2010. LAP, an alcohol acetaldehyde dehydrogenase enzyme in *Listeria*, promotes bacterial adhesion to

enterocyte-like Caco-2 cells only in pathogenic species. Microbiology **156:**2782-2795.

60. **Polissi A, Pontiggia A, Feger G, Altieri M, Mottl H, Ferrari L, Simon D.** 1998. Large-scale identification of virulence genes from *Streptococcus pneumoniae*. Infect Immun **66:**5620-5629.

61. **Chico-Calero I, Suarez M, Gonzalez-Zorn B, Scortti M, Slaghuis J, Goebel W, Vazquez-Boland JA, European Listeria Genome C.** 2002. Hpt, a bacterial homolog of the microsomal glucose- 6-phosphate translocase, mediates rapid intracellular proliferation in *Listeria*. Proc Natl Acad Sci U S A **99:**431-436.

Tables and Figures

TABLE 1. Primer and adaptor sequences used in QTD-based transposon sequencing and in Signature Sequence Mutagenesis PCR

Primer	Sequence
Ad1	CTC GTA GAC TGC GTA CC
Ad2	AAT TGG TAC GCA GTC TAC
IS-N	AAA GGA CTG TTA TAT GGC
IS-1	TGT ACC GTA AAA GGA CTG
EcoRI	TAG ACT GCG TAC CAA TTC

TABLE 2. *M. agalactiae* mutants attenuated in the initial round of sheep intramammary screening

Mutant	Gene	Insertion site/ gene size (bp)	Percentage absence
Apo7	MAG1890	283/975	100
Apo2-12	MAG1050	343/987	100
3-4-0	MAG3390	788/2355	100
3-29	MAG2540	628/1089	100
6-14	MAG4970_uhpT	432/1497	97
6-29	MAG0940_pdhB	982/987	100
6-32	MAG1390_eutD	125/957	100
6-9-2	MAG0360_oppC	71/1014	95
7+1-3	MAG3650	156/720	100
9-31	MAG4340_adhT& MAG2630_nox	367/1041 &193/1365	97
9-40	MAG2320_gtsB	332/987	95
23	MAG5520_aminopeptidase	353/1086	100
81-1	MAG0370_oppB	1014/1086	98
137	MAG4460	1000/1389	100

TABLE 3. *M. agalactiae* genes required for host colonization. Corresponding mutants showed 100% absence in all tissue samples tested from all three sheep during the second round of confirmatory screening in the intramammary sheep infection model. Presence/absence in milk samples one day before necropsy is also shown.

Mutant	Gene	ORF identity	Milk
6-32	MAG1390_eutD	phosphotransacetylase	Absent
9-31	MAG4340_adhT and MAG2630 _nox	alcohol dehydrogenase and NADH oxidase	Present
6-14	MAG4970_uhpT	hexosephosphate transport protein	Absent
3-4-0	MAG3390	hypothetical protein	Absent
137	MAG4460	hypothetical protein	Absent
Apo2-12	MAG1050	lipoprotein	Absent
3-29	MAG2540	Vpma like lipoprotein	Absent

Figure Legends

FIG. 1. Flow diagram of the selective and specific amplification of Tn insertion sites using the Quantitative Target Display based PCR methodology (Sharma et al, 2001).

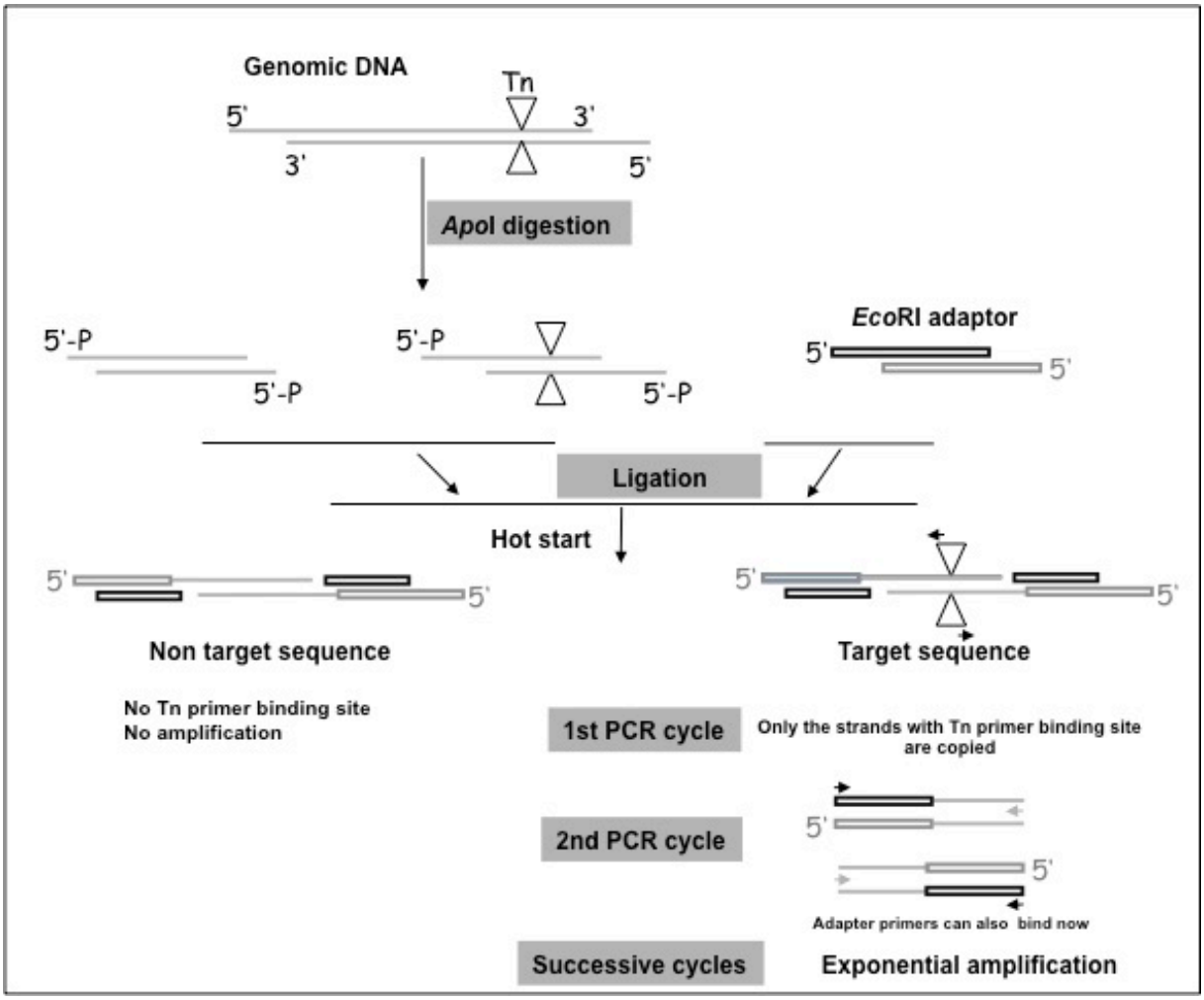
FIG. 2. Flow diagram of the *in vivo* screening of *M. agalactiae* Tn mutants. Three different pools of 15-19 mutants were administered into each group of three sheep each via the right teat canal. Genomic DNA isolated from the input pool served as template for input SSM PCR, which was compared with output SSM PCR results corresponding to the mycoplasma populations recovered from udder halves, LNs and milk samples. Mutants found absent in output SSM PCR (depicted here as mutants 2 and 4) are considered attenuated, as they cannot survive in the host due to mutations in the specific genes.

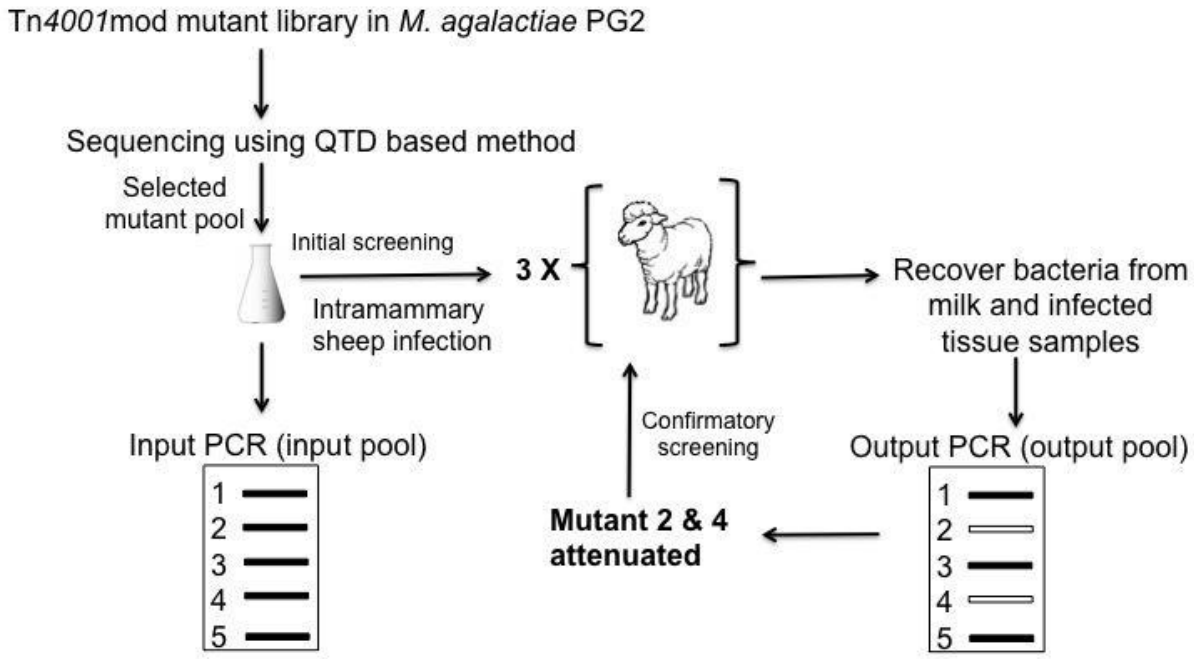
FIG. 3. Attenuation of Tn mutants in the initial round of intramammary sheep screening. A total of 46 mutants were screened in three groups of three sheep each. Group 1 (**A**), group 2 (**B**) and group 3 (**C**) were injected with a pool of 15, 17 and 19 mutants each. Bars represent the average percentage of absence (± standard deviation) of individual mutants as calculated from the SSM PCR results obtained from all the examined local infection sites and LNs from all three sheep of each group.

FIG. 4. Average percentage of absence of mutants in the confirmatory round of intramammary sheep screening. Each bar represents the average percentage of absence ± standard deviation of individual mutants as

calculated from the SSM PCR results of udder and LNs obtained from all three sheep.

FIG. 1. Flow diagram of the selective and specific amplification of Tn insertion sites using the Quantitative Target Display based PCR methodology (Sharma et al, 2001)





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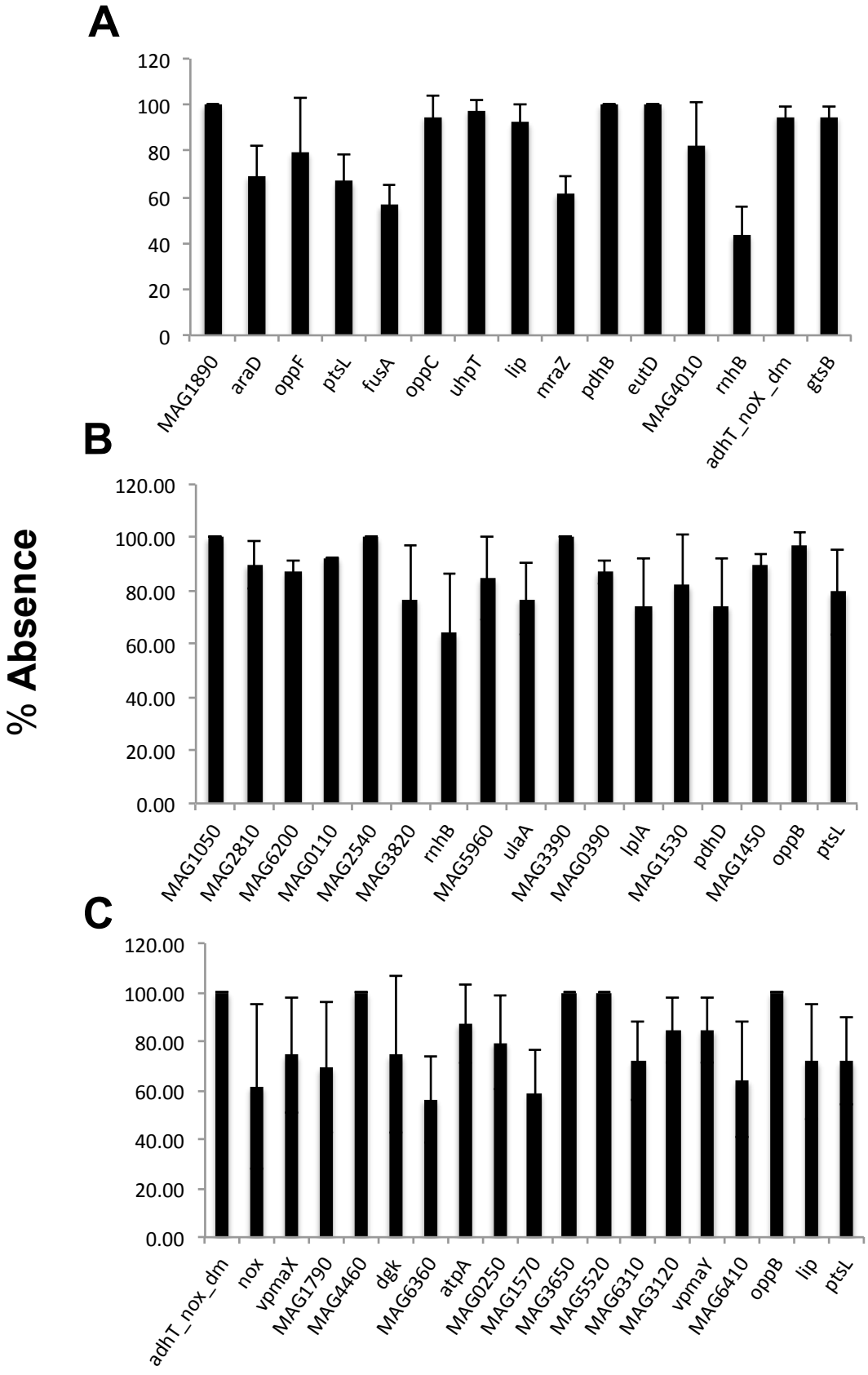
A
120
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MAG1890
araD
oppF
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oppC
uhpT
lip
mraZ
pdhB
eutD
MAG4010
rnhB
adhT_noX_dm
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120.00
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60.00
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20.00
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MAG1050
MAG2810
MAG6200
MAG0110
MAG2540
MAG3820
rnhB
MAG5960
ulaA
MAG3390
MAG0390
lplA
MAG1530
pdhD
MAG1450
oppB
ptsL
C
120.00
100.00
80.00
60.00
40.00
20.00
0.00
adhT_nox_dm
nox
vpmaX
MAG1790
MAG4460
dgk
MAG6360
atpA
MAG0250
MAG1570
MAG3650
MAG5520
MAG6310
MAG3120
vpmaY
MAG6410
oppB
lip
ptsL

FIG. 3. Attenuation of Tn mutants in the initial round of intramammary sheep screening. A total of 46 mutants were screened in three groups of three sheep each. Group 1 (**A**), group 2 (**B**) and group 3 (**C**) were injected with a pool of 15, 17 and 19 mutants each. Bars represent the average percentage of absence (± standard deviation) of individual mutants as calculated from the SSM PCR results obtained from all the examined local infection sites and LNs from all three sheep of each group.

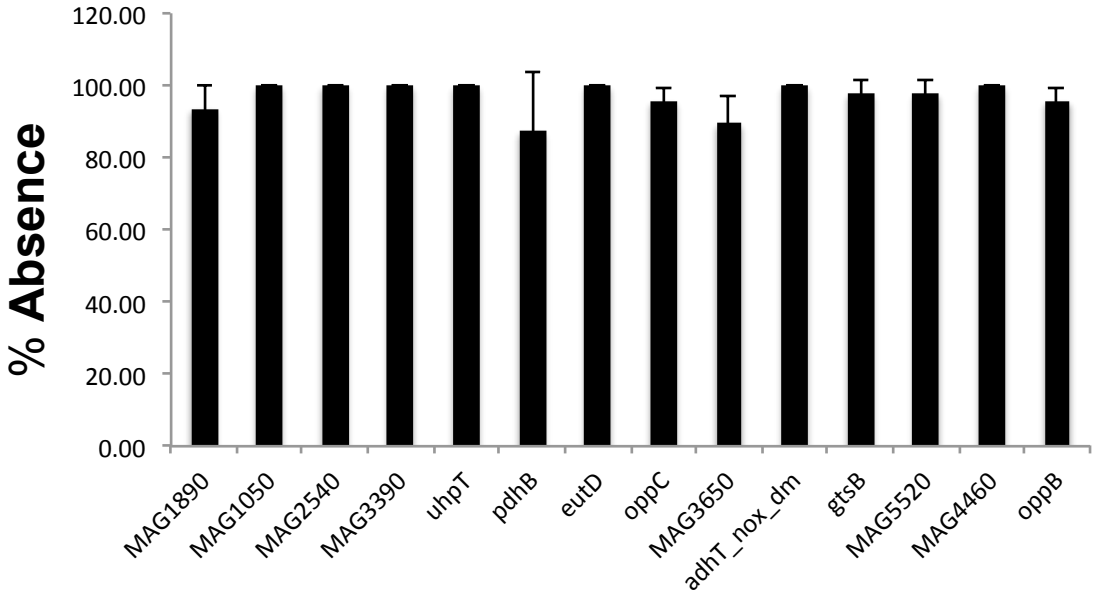


FIG. 4. Average percentage of absence of mutants in the confirmatory round of intramammary sheep screening. Each bar represents the average percentage of absence ± standard deviation of individual mutants as calculated from the SSM PCR results of udder and LNs obtained from all three sheep.

Supplementary tables and figures

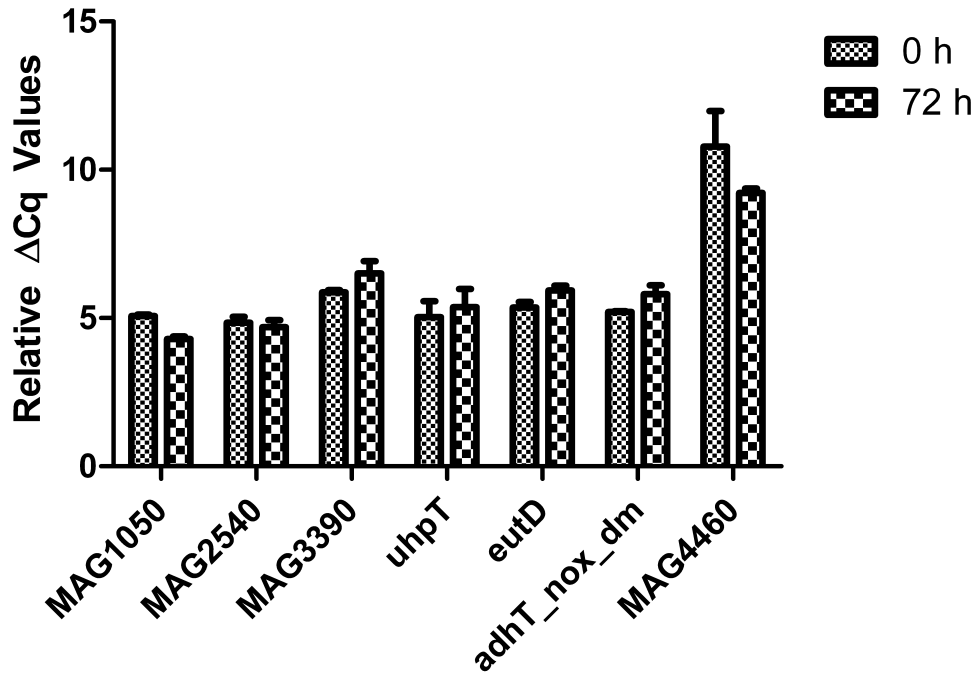
TABLE S1. Gene specific primer sequences used in SSM PCR

Mutant	Primer designation	Gene	Primer sequence
Apo7	MAG1890 R	MAG_1890	CTA ATG TTG GAG CAC TAG TGT G
Apo 6	sgae F	MAG_6330	GCG ACA CTT AAG CCT GAA G
26	oppF F	MAG_0340	CCT AGA CAT TGT CTA TGG CTC
3-1 PtsL	ptsL R	MAG_6300	CAG ATA ACA CAG CTG CTG CAG
3-15 fusA	FusA F	MAG_5920	GCA ATC AGG TGG TAA AGG TC
6-9-2	oppC F	MAG_0360	GTA GCA ACG TTT GGG ATA G
6-14	uhpT F	MAG_4970	TAG GTG GTA AGG GTG TTG TG
6-15	Lip R	MAG_0040	GTG TCC TGT TTT AGG CAT TC
6-21	MrzA R	MAG_3740	AAC TCG TCC TCA CTA TAA G
6-29	pdhBfpN	MAG_0940	ACT TGC CCA TGG AAT AAT TGT TCC C
6-32	Pta R	MAG_1390	ACA TCG TCA GTT AAG CTT CC
7+1-8	Zinc R	MAG_4010	CTT CTT TAT TTC ATC ACT AGG
8+1-5	Rnh R	MAG_6730	TAA TCT GTG TTC AGG AGA GG
9-31	AdhT_Nox R	MAG_2630 &MAG_4340	CAA TAA CAG CTC TTC CTA AG (primer for MAG_2630)
9-40	gtsBfpN	MAG_2320	ACT TAT CCA TGG CAA GCA TTG AAG TTG
Apo2-12	MAG1050 F	MAG_1050	ATG TTA TCA ATG CCG GTA GC
1-3-0	MAG2810 R	MAG_2810	GTA ATT TAG CTG CAA TGC CCG
15-2	MAG6200 R	MAG_6200	GGC ATA TGA GGC AAT TGA AG
170	MAG0110 F	MAG_0110	GCT TCT GTT GGT TGG TTC TC
3-29	MAG2540 F	MAG_2540	TAA CCC TGG TGA CAC AAC TG
4-1-0	GTP R	MAG_3820	GTG GCA AAT ATC TCC ATC TTG C
8+1-4	ABC ATP F	MAG_5960	GCC AAG ACC ATA ATG CTT ATG

Contd…

Mutant	Primer designation	Gene	Primer sequence
8+1-8	SGAT R	MAG_6380	AGC TGT GCG CCT AAT GAC AC
3-4-0	TonB R	MAG_3390	AGC CTA TGT AAT CCG GAT G
9-28	MAG0390 F	MAG_0390	GTGCTC TTA CAC CAA ACT CAA C
62	LplA F	MAG_0070	CAG CAC AAC TTG GAA GTT GTT C
212	T3RM R	MAG_1530	CAT GAC CAG TTG TTC CTG AAC C
9-8	pdhD F	MAG_0960	CAT TGC CTG CAT CAA TGG TC
9-16	MAG1450 F	MAG_1450	ATT GTT GCT TTC ATA CCG CC
81-1	oppB F	MAG_0370	CTC GTC AAT TCC ATC ACT AG
3-20	Nox F	MAG_4340	GAA TTT AGT CAA CCA GAT GGT C
13	VpmaX F	MAG_7070	TAC TTG GAT CGG TTG CTT C
14	Meth F	MAG_1790	ATG ATA AGA GTT ATT TCA GGC
137	Acidph F	MAG_4460	AGC TGC TAC ATG TCA AGA TCC
8-1	Dgk R	MAG_0180	GCG GAA TGA TAA GTA GTG G
1-2-0	PenA F	MAG_6360	CTG GAA TGG GAA CCA GCA TG
96-2	AtpA_2930 R	MAG_2930	TCT CCT AAT TTA AGT GCA AC
5-1	Indi F	MAG_0250	GTA AGG AAG TTG AAA GCG C
6-13	MAG1570 R	MAG_1570	TCC ACG TCT GTA ATT TCA CC
7+1-3	MAG3650 F	MAG_3650	GGC TTG GAA TGA TTG ATG G
23	Amino pep R	MAG_5520	AGT AGG AAC ACC ACC AAT ACC
6-12	Transreg F	MAG_6310	GCC AAT TCA TCA GAA GCA GC
San5	MAG3120 R	MAG_3120	GAT GTA CCA TTT GAG CCT G
Vpma Y 6-15	VpmaY F	MAG_7080	ATC AGT TGC TTC AAT GGC CTC
3-23	MAG6410 lipo F	MAG_6410	ATG ACT TTG GCA AGG TTC AGG

FIGURE S1. Graph depicting the mean ΔCq (Mean Cq of target gene - Mean Cq of reference gene) values of input pool (0 h) and output pool (72 h) obtained after 72 h of mixed culture competitive growth of the 14 mutants used as inocula for confirmatory screening. No significant decrease in the respective gene abundance was observed at 72 h compared to 0 h ($P>0.05$) for all the 7 mutants found absent in the final round of confirmatory screening. The experiment was performed 3 times in triplicates.



4.4 Manuscript III

Identification of *Mycoplasma agalactiae* genes involved in systemic spreading during experimental intramammary infection of sheep

Abstract

Mycoplasma agalactiae causes chronic infection involving distant body sites following systemic spreading. The study presented here made an attempt to identify genetic factors playing a role in the systemic infection process using a combination of transposon mutagenesis and negative selection. Two successive rounds of screening in the sheep intramammary infection model were able to show the attenuation of a set of different mutants at local and systemic sites with disrupted genes belonging to diverse functional categories. The results of signature sequence mutagenesis (SSM) PCR obtained from analysis of mutants recovered from distant body sites revealed a putative role of *oppC*, *oppB*, *gtsB*, *pdhB*, MAG5520, MAG1890, and MAG3650 in dissemination of *M. agalactiae*. Additionally, mutants *pdhB, oppC* and MAG4460 exhibited significantly less growth in the presence of HeLa cells in MEM medium. In addition to the *in vivo* findings*, in vitro* results indicate the role of different genomic loci in *M. agalactiae* infection. Future studies involving individual mutants are planned in order to reveal the detailed mechanism of each genetic locus in the pathogenesis of *M. agalactiae*.

1 Introduction

The term “mycoplasmas” designates a group of bacteria belonging to the class *Mollicutes* that have evolved from gram-positive ancestors through genome reduction (1). Members of this group include the smallest self-replicating organism, namely *Mycoplasma genitalium* with the minimum set of essential genes (2, 3). During reductive evolution mycoplasmas have lost important genomic components, and this explains in part their parasitic lifestyle (4). They are mainly pathogens infecting humans, animals and plants. Mycoplasma diseases are mainly chronic in nature due to persistent infections of the respective human and animal hosts. Even though our knowledge in mycoplasma genomics has considerably improved over the years, the mechanisms by which mycoplasmas cause infection and disease and persist inside their hosts are still largely unknown.

Of the several pathogenic mycoplasmas of ruminants, *M. agalactiae* is an important agent of small ruminants causing contagious agalactia (CA). As an economically important disease, CA is listed as notifiable disease of the World Organization for Animal Health (OIE) (http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online). Shedding of the pathogen by diseased or asymptomatic carriers continues for months or years after the initial infection with a potential risk of infecting other susceptible animals (5-7). Antibiotic therapy tends to reduce the clinical signs but promotes the carrier state (8). Due to the well-known clinical symptoms and infectious process, *M. agalactiae* represents a very good general model organism to study mycoplasma pathogenesis in its natural host.

Genome sequencing of the *M. agalactiae* PG2 type strain revealed that several of its gene loci were horizontally transferred from the mycoides cluster (9). However, its virulence mechanisms and factors involved in pathogenesis are not have still to be explored at the molecular and cellular level. Our mycoplasma research laboratory has initiated such studies to understand pathogenesis of *M. agalactiae* by gene disruption through homologous recombination and transposon mutagenesis (10, 11). These studies have also shown the role of the *vpma* locus in *M. agalactiae* pathogenesis (12). Other independent studies using transposon mutagenesis were able to show the role of different genomic loci involved in interaction with host cells (13) and the results obtained were then further evaluated *in vivo* to conclude that the NIF locus is important for the pathogenicity of *M. agalactiae* (14). Recently the ability of *M. agalactie* to invade eukaryotic cells both in *in vivo* and *in vitro* was discovered along with its ability to systemically disseminate throughout the body of the infected host (15). However, a comprehensive analysis of the genetic factors involved in this systemic spreading of *M. agalactiae* in its natural host is unavailable.

To study systemic spreading, clinical manifestations and serological response during *M. agalactiae* infection, intramammary sheep infection is a suitable experimental infection model(15). Intramammary infection of lactating ewes with pools of transposon mutants was able to detect attenuated mutants in local sites and lymph nodes (Hegde et al., unpublished results). Further analysis of inner organs revealed non-colonizing mutants that were different

from those recovered from the local sites of infection. Some of the attenuated mutants also showed defects in co-cultivation assays in the presence of HeLa cells and in killing of murine macrophages *in vitro*. These results depict clearly the role of different sets of genes involved in systemic and local infection of *M. agalactiae*.

2 Material and Methods

2.1 Bacterial cultures and growth conditions

The *M. agalactiae* type strain PG2 (9, 16) was used in this study. All mycoplasma cultures were grown at 37°C in standard Aluotto medium (17) as described before (11). Gentamicin sulphate (50µg/ml) was used both in broth and agar plates for the propagation of transposon mutants. *E-coli* strain DH10B (Invitrogen GmbH, Lofer, Austria) containing the transposon vector was grown in Luria broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) supplemented with ampicillin (100 µg/ml) and gentamicin sulphate (7µg/ml).

2.2 Transposon mutant library and identification of insertion sites

Transposon mutants were generated in *M. agalactiae* strain PG2 using the suicidal plasmid pISM2062 containing the transposon Tn*4001*mod (18) as described earlier (11). Transposon mutants were sequenced using ligation-mediated QTD based PCR (19) as described earlier (Hegde *et al*., unpublished results). The position of the transposon in the genome was determined by nucleotide blast in Molligen 3.0 (20).

2.3 Growth analysis and stability of transposon mutants

Growth rate and insertion stability of each of the transposon mutants were assessed in Aluotto medium. Mycoplasma cultures were grown up to 72 h and then plated on Aluotto agar plates at regular intervals to assess the growth. The transposon mutant stability was assessed as described earlier (21) except for the loss or movement of the transposon that was determined by SSM PCR (see below).

2.4 Signature Sequence Mutagenesis (SSM) PCR

SSM PCR is based on genetic foot printing for identification of attenuated mutants *in vivo* (21). Gene-specific primers were designed in such a way that amplicons had a size between 200-1000 bp (Supplementary Table 1). The PCR conditions were validated for individual mutants and mutant pools. Each 25 µl PCR reaction mixture contained 300 ng of template, 2mM $MgCl_2$, 100 µM dNTP, 1 µM of each primer and 1.2 U HOT FIREPol® DNA polymerase in 1x Buffer B (Medibena Life Science and Diagnostic Solution, Vienna, Austria). Amplification conditions were 95°C for 12 min; 94°C for 1 min, 54°C for 30 sec and 72°C for 1 min (30 cycles); and 72°C for 5 min. The PCR products were separated on 1% agarose gel before staining with ethidium bromide and visualizing using the Gel Doc™ XR+ System (Bio-Rad, Hercules, CA, USA).

2.5 Animals and ethical statement

Screening was done in lactating ewes of 1-2 year age that were purchased from a local sheep flock after serological examination and housed in separate stables. All procedures related to the animal experiments were performed with

the permission of the Ethics Committee of the University of Veterinary Medicine Vienna and the Austrian Federal Ministry for Science and Research (field of engineering and animal testing) based on the approval numbers BMWF-68.205/002- II/3b/2011 and BMWF-68.205/0104-II/3b/2012.

2.6 Initial screening

A total number of 9 sheep was used in the initial screening to assess the attenuation of 45 different transposon mutants. They were randomly divided into three groups of three sheep each. The viability of the inoculum following one freeze-thaw cycle was calculated as described previously (12). Based on the viability, pools of 15-19 mutants were prepared by mixing equal proportions of each mutant to get a total number of 10^9 viable mycoplasma cells to be inoculated per sheep. Group I infected with 15 mutants, group II infected with 17 mutants and group III infected with 19 mutants were screened for potential virulence factor. Five mutants kept common between the groups as 'watermarks'. Each sheep received in the right teat canal 5 ml of inoculum containing 10^9 cfu of the mutant pool prepared in PBS. One portion of the inoculum was grown in 30 ml Aluotto medium to isolate the genomic DNA from the 'input pool' to be analyzed by SSM PCR.

Two weeks post infection (p.i.), the sheep were necropsied, and samples of the spleen, liver, lung (left and right), uterus, kidney (left and right) and synovial fluid from the left and right knee were aseptically removed. The tissue samples were inoculated in 3 ml of Aluotto medium containing 50 µg/ml of gentamicin, penicillin and thallium acetate, respectively, and incubated at 37°C for 7 days or till the growth was evident by metabolic color change.

Simultaneously, 10- and 100-fold diluted tissue samples in Aluotto medium were also incubated to reduce the inhibitory effect of tissue components. The genomic DNA ('output pool' DNA) from each grown culture was extracted, and the presence of *M. agalactiae* in these samples was checked by 16S rDNA specific PCR (22) and by plating on Aluotto agar plates. *M. agalactiae*-positive output pool DNA was used to perform SSM PCR to determine the presence or absence of individual mutants in the output population by using primer sets containing a transposon-specific primer (IS-N) and a primer specific for each transposon mutant (Supplementary Table 1). The amplicons were visualized in an ethidium bromide-stained 1% agarose gel. The PCR profile from this output population was compared to the input population profile, and those mutants not present in the output population but present in the input pool were considered as absent. The PCR analysis was repeated with *M. agalactiae*-positive samples from each sheep using primers specific for each mutant in the mutant pool. The results were evaluated individually for each sheep in terms of the totally absent mutants, and then the average based on three sheeps was calculated. The final results are expressed as ratio of average SSM PCR positive samples to the total samples analyzed for each mutant.

2.7 Confirmatory screening

All mutants found to be attenuated in the initial screening experiment were re-screened to confirm their possible role in *M. agalactiae* pathogenesis. A total of 14 mutants that were in the initial screening identified as attenuated were pooled and infected into three sheep via the intramammary route for confirmatory screening using the same set up and procedures as described in

the previous section (Section 2.6). Apart from the tissue samples also included in the initial screening experiment, additional samples such as brain, heart, and carpal joint tissues were also examined. The presence or absence of *M. agalactiae* in isolated tissue samples from the mutant-infected animal group was compared with that of the wild type PG2-infected group (15).

2.8 Cell culture

The HeLa-229 (ATCC CCL-2.1) and J744A.1 (ATCC TIB-67) cell lines used in this study were purchased from the American Type Culture Collection (ATCC; Manassas, Va. USA) and certified to be free of mycoplasmas. HeLa-229 cells were maintained in MEM with 10% heat-inactivated FBS, and J744A.1 cells were propagated in DMEM with 10% FBS and 2mM L-Glutamine (Sigma). Trypsin and 1x PBS were purchased from PAA Laboratories GmbH (Pasching, Austria) or Sigma-Aldrich. All cell cultures were regularly checked for mycoplasma contamination by culture and PCR.

2.9 Quantitative growth analysis in mammalian cells

The ability of the mutants and the wild type PG2 strain to grow in the presence of HeLa cells was examined quantitatively by co-cultivation. The mutants and wild type strain PG2 were grown as described before (see section 2.1). PG2 and mutant cultures were diluted in MEM and co-infected with $4x10^4$ HeLa cells/well to get an MOI of 10-50 in 24- well tissue culture plates (CELLSTAR® Greiner Bio-One GmbH, Germany). The infected cells were incubated at 37°C with 5% CO_2 for 48 h. The supernatant along with the trypsinized mycoplasmas containing HeLa cell suspension were serially

diluted in SP4 medium and plated on SP4 agar plates with appropriate antibiotics. All the experiments were carried out at least three times in duplicates. The results were represented as doubling time that was calculated by using the following formula:

Doubling time = (t2-t1) X (log (2)/ log (cfu at t1/ cfu at t1)

t1= Starting time of assay; t2= End time of assay

2.10 Macrophage killing assay

The ability of *M. agalactiae* to kill infected murine macrophages J744.A1 was assessed quantitatively by a non-radioactive colorimetric assay. The release of LDH was quantified using the CytoTox 96® Non-Radioactive cytotoxicity assay kit (Promega). Initially, pilot experiments with uninfected J744A.1 cells were performed to determine the optimum number of target cells to be used with the CytoTox 96® assay according to the manufacturer's instructions. A 96-well V-bottom shaped microtitre plate containing $5x10^3$ cells/well of J744.A1 was infected with *M. agalactiae* wild type strain and mutants to get an MOI of about 100. The plates were centrifuged at 250xg for 4 min at RT and incubated at 37°C with 5% CO_2 for 24 h. The released LDH as an indicator of macrophage cell lysis expressed in percentage of cytotoxicity was calculated using the formula given in the kit protocol. The assay was done in triplicate and repeated at least twice.

2.11 Statistical analysis

The SSM PCR results were expressed as percentage of attenuation ± standard deviation from each group of three sheep. Mutants with 100%

absence were considered attenuated, and results from both screening experiments were compared using the Mann-Whitney test (23). The results of the *in vitro* assay were statistically analyzed by the unpaired parametric Student's t test using the GraphPad Prism 5 software (Graphpad Software Inc, CA, USA), and a value of $P<0.05$ was considered as statistically significant.

3 Results

3.1 *M. agalactiae* genes required for systemic spreading and colonization

The study was carried out in two parts, of which the first one was an initial screening experiment while the aim of the second one was to confirm the initial data. A total of 45 mutants were tested in the initial screening by intramammary infection of lactating ewes. Before using the mutants in the *in vivo* infection study, *in vitro* growth curve analysis was performed. The results showed no defect in growth except a *pdhB* mutant that was growing one log slower in logarithmic phase of growth (Hegde *et al.,* unpublished). The slowly growing *pdhB* mutant was included in the study to assess its replication and survival inside the host. Earlier such studies have also included mutants that were growing more slowly *in vitro* to assess their *in vivo* fitness (24, 25). Additionally, transposon stability analysis of mutants revealed that all the insertions were stable even after 30 i*n vitro* passages in non-selective medium (Fig. S1). Two weeks p.i., the tissue samples collected at necropsy were transferred into Aluotto medium, and DNA was extracted from the cultures

before checking by *M. agalactiae*-specific PCR (22). Of the tissue samples, the left lung, liver and spleen were negative in all nine sheep (Table 1). Additionally, these negative samples did not show any amplification in SSM PCR (Fig. S2). Hence, it was considered that these mutants were attenuated, at least they were unable to colonize these three inner organs. The uterus and the synovial fluid from the left knee were positive for *M. agalactiae* in four and 7 sheep, respectively (Table 1). The SSM PCR results revealed mutants with disrupted *rnhII*, *ptsL*, *fusA*, *araD*, *lplA* or other genes that were detected in the uterus and synovial knee fluid demonstrating that these gene defects did not affect their ability to colonize these organ-specific sites. Other organs such as the left and the right kidney, the right lung, the joint of the right knee were colonized in one to three sheep.

SSM PCR was performed with all *M. agalactiae*-positive and a few negative samples to identify the attenuated mutants. A mutant that was absent in ≥95% of tissue samples from each group was considered as attenuated. After analyzing the complete set of tissue samples from all 9 sheep, 21 mutants were identified that were absent at a frequency of ≥95% in the distant sites (Table 2). When comparing the SSM PCR results, there were 14 mutants absent altogether. Genes such as *lip*, MAG6200, MAG0110, MAG1790, MAG0390, MAG0250 and *atpA* were absent only in systemic locations (Table 2). Thus, 14 mutants that were not present at a percentage of ≥95% in the udder, LNs and systemic sites were considered as attenuated in this initial screening experiment and therefore used in an additional screening experiment to confirm these data (Table 2). The total number of attenuated

mutants identified by initial screening corresponds to about 31%, and this is higher than previously reported (26, 27). Earlier studies have analysed attenuated bacterial mutants individually in *in vivo* competition experiments with the wild type strain to determine the competitive index (CI) (27). In this study using the sheep infection model it was difficult to analyse such a high number of individual mutants through *in vivo* challenge or competitive experiments due to the unavailability of a larger number of animals and the required resources. Instead, we decided to test these mutants by pooling them and using them again for infection of sheep in a confirmatory screening experiment. After two week of infection, tissue samples from heart, brain, carpal joint (left and right) in addition to lung (left and right), kidney (left and right), spleen, liver, synovial fluid of the knee (left and right), and uterus were taken and incubated in Aluotto medium. As no growth was detected after enrichment in Aluotto medium and direct plating on Aluotto agar plates, all mutants re-screened in the confirmatory screening experiment were defined as attenuated with respect to the different host sites investigated. In an independent study where spreading of the wild type PG2 strain into distant body sites was assessed through intramammary inoculation, dissemination into different body sites such as uterus, kidney, lung and popliteal LN was confirmed by PCR and immunohistochemical analysis (15). Hence, the present results indicate that transposon insertions into specific genes are responsible for the inability of these mutants to spread systemically and colonize distant body sites.

In order to estimate the reproducibility of results obtained with common 'watermark' mutants between different groups in the initial screening, the Mann-Whitney test (Mann & Whitney, 1947) was applied. The results indicate no significant difference in average percentage of absence ($P>0.05$) of attenuated mutants that are common between the groups.

3.2 Phenotypic analysis of attenuated mutants

The results obtained from the *in vivo* screening studies showed attenuation of *M. agalactiae* in different body sites, and this refers to about 31% of the mutants included in this investigation. Our anticipation was to identify a single locus involved in *M. agalactiae* pathogenesis, however, the results indicate that more than one genetic locus seems to play a pathogenetic role, although it is difficult to predict the function of these genes in virulence without studying their role as individual genes. Whereas it is difficult to study a single mutant in a challenge study along with the wild type PG2 strain, all these attenuated mutants can be instead used in *in vitro* assays to assess their interaction with host cells in cell culture. The ability of these mutants to grow in the presence of HeLa cells and their involvement in killing of murine macrophages was assessed as described above in the Materials and Methods section. The results showed that mutant *pdhB*, *oppC* and MAG4460 were growing more slowly in the presence of HeLa cells as compared to the PG2 strain. Growth of mutant *pdhB* was significantly ($P<0.05$) less than of other mutants, and this corresponds to an about two-fold higher growth index compared to the wild type strain (Fig. 1). Similarly, mutants *oppC* ($P<0.005$) and MAG4460 ($P<0.05$) were growing considerably slower compared to the wild type (Fig.1),

even though their multiplication was normal in axenic medium (data not shown). In contrast, mutants MAG1890 and MAG2540, which were showing in an independent study reduced growth in the presence of bovine cell lines (28), did not show any defect in growth (Fig.1).

The ability of the mutants and of the wild type strain to kill murine macrophages was assessed using a non-radioactive based assay. None of the mutants showed significantly altered killing compared to the wild type PG2. However, the mutant MAG5520 was nearly two-fold deficient ($P>0.05$) in killing (Fig. 2). This mutant showed only 13% of macrophage killing at 24 h p.i. whereas the wild type exhibited a cytotoxicity rate of about 27%. All other attenuated mutants showed a similar killing rate of macrophages as the wild type (Fig. 2). Hence, these results suggest that transposon insertions into specific genes did not alter the killing ability of these mutants.

4 Discussion

Bacteria belonging to the genus *Mycoplasma* are capable of causing chronic and persistent infections in variety of hosts. As a pathogen of small ruminants, *M. agalactiae* is capable of subverting the host immune response through its sophisticated Vpma antigenic variation system (12). Besides this, the factors involved in the pathogenesis of *M. agalactiae* are largely unknown. The aim of the study presented here was to identify in the sheep intramammary infection model genetic factors of *M. agalactiae* that play a role in colonization of systemic sites in the natural host. Successive screening of mutants enabled the identification of 14 defective genes in total that are obviously required for

systemic spreading and colonizing of different inner organs (Fig. 3). In particular, whereas disruption of 7 genes resulted in attenuation only with respect to systemic spreading and colonization, other attenuated mutants did not colonize the udder and local lymph nodes (Fig. 3). These results therefore suggest a different role of genes in causing local and systemic infection. Among the 7 attenuated mutants unable to cause systemic infection and to colonize inner organs, genes such as *oppB*, *oppC* and *gtsB* are acquired from the mycoides cluster through horizontal gene transfer (9). Genetic factors identified in *M. agalactiae* as being relevant for pathogenicity could therefore also be important in understanding the infection biology of other mycoplasma species belonging to the mycoides cluster.

Metabolic proteins and transporters are known to play a crucial role in mycoplasma pathogenesis (29-32). This study identified two genes coding for the permease component of an oligopeptide transporter, namely *oppB* and *oppC*, and it is the first time that these genes are being implicated to play a role in mycoplasma pathogenesis. The *oppA* gene, coding for an ATP binding domain of an oligopeptide transporter in *M. hominis*, was shown to be important for the interaction with host cells and was associated with apoptotic cell death (33). The involvement of permease components in virulence has been so far reported only for pathogenic bacteria other than mycoplasmas (34-37). In addition, the mutant *oppC* is also growth-deficient in the presence of HeLa cells indicating that the transport of peptides is important for both *in vitro* and *in vivo* survival of *M. agalactiae*. Based on these *in vitro* and *in vivo* data nutrient acquisition such as the uptake of small peptides seems to play an important role in survival of *M. agalactiae* in the host. Likewise, glycerol

plays a crucial role in mycoplasma pathogenesis as a substrate for toxic metabolic products like H_2O_2 (32, 38). In this study, a mutant defective in the permease component of a glycerol ABC transporter (*gtsB*) is unable to survive in systemic sites. The genes involved in transport and metabolism of glycerol are considered as virulence factors (29-31, 38). The glycerol transport locus is well-characterised in mycoplasmas of the *mycoides* group (39) and has been shown to play a role in virulence (32). As *M. agalactiae* acquired its *gtsABC* from the mycoides cluster through horizontal gene transfer (4), it is likely that this locus also plays a role in its pathogenesis. Production of H_2O_2 has been reported in *M. agalactiae* (40), but there is no study showing its link to glycerol transport and metabolism. Taking into account that *gtsB* mutant was found to be attenuated in this study; further analyses could prove the role of the glycerol uptake system and the catabolism of glycerol in pathogenesis of *M. agalactiae*. At least it is clear from these findings that transport of metabolic substrates is important for the survival of *M. agalactiae* within its natural host.

Apart from oligopeptide transporter genes, disruption of genes coding for the beta subunit of the pyruvate dehydrogenase complex was also found to result in attenuation. This gene is responsible for the metabolism of pyruvate, which is the major energy yielding process in *M. agalactiae* (41). The *pdhB* mutant was showing reduced growth in axenic medium during logarithmic phase, but was recovered *in vivo* from the udder and lymph nodes of infected animals in the confirmatory screening experiment (Hegde *et al*., unpublished data) indicating its survival in local sites of infection. But the attenuation of this mutant with respect to systemic sites shows its inability to disseminate throughout the body to cause systemic infection. As systemic spreading and

cell invasion are related (15, 42), it is possible that the mutant's inability to cross the epithelial barrier may be responsible for its deficient spreading capacity. Additionally, the *pdhB* mutant also showed a growth defect in the presence of HeLa cells *in vitro* and this may be due to its inability to interact with host cell proteins. Earlier studies with *M. pneumoniae* have shown that PdhB is binding to extracellular matrix (ECM) proteins such as fibronectin, plasminogen (43, 44). Thus, like many other mycoplasmas (45, 46), *M. agalactiae* might also be utilising a single gene product to carry out multiple functions.

Aminopeptidases play important roles in pathogenesis of bacteria by providing a source of amino acids from exogenous proteins by destroying host immunological effector peptides. An *M. agalactiae* mutant with disruption of the gene coding for glutamyl aminopeptidase (MAG5520) was unable to survive inside the infected sheep. This mutant also showed reduced killing of murine macrophages as measured in the cytotoxic assay. A similar protein in *M. hyopneumoniae* was able to bind and cleave plasminogen (46) and, importantly, it exhibits more than 50% identity to the *M. agalactiae* aminopeptidase. Hence, it is possible that disruption of this gene results in the inability or reduced the ability of the organism to generate amino acids for its survival inside the host. As shown for *M. hyopneumoniae*, the aminopeptidase is surface-located (46) so that a transposon insertion as in the *M. agalactiae* mutant may affect the ability to induce macrophage cell death.

In addition to mutants of annotated genes, this study was able to identify loci coding for hypothetical or conserved hypothetical proteins. Mutants with

disrupted genes MAG1890 and MAG3650 both coding for proteins of unknown functions are attenuated only in systemic sites but they were recovered from the udder and LNs. An independent study has shown that transposon insertion in MAG1890 resulted in reduced interaction with HeLa (13) and TIGMEC or TIGEF cells (28). However, our studies with HeLa cells did not show significant difference in growth ($P>0.05$). This may be due to the difference in the cell lines used. Mutation of MAG3650, which codes for a hypothetical protein, resulted in attenuation only in terms of systemic dissemination and colonization. Sequence analysis showed 86% identity to a putative membrane protein of *M. bovis* (*MBOVPG45_0454*). Since both of these ruminant mycoplasmas cause similar infections in their respective hosts, they also might share their protein functions. Thus, deciphering the role of unknown protein in one species might be applied for the other one. These results indicate that apart from known lipoproteins, transporters and metabolic proteins acting as virulence factors, hypothetical proteins seem to play a crucial role in *M. agalactiae* pathogenesis.

Altogether, the study presented here has shown the possible role of several genomic loci involved in spreading of *M. agalactiae* during experimental infection of lactating ewes. The results provide a better understanding how *M. agalactiae* utilizes its genomic machinery to establish systemic infection in its natural host. To our knowledge, this is the first report describing the potential role of a set of different genetic factors involved in local and systemic *M. agalactiae* infection of small ruminants. Further studies of these factors in individual infection experiments could pave the way to identify a suitable mutant that can be developed further as good vaccine candidate.

References

1. **Razin S.** 1997. The minimal cellular genome of mycoplasma. Indian J Biochem Biophys **34:**124-130.
2. **Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, Hutchison CA, 3rd, Smith HO, Venter JC.** 2006. Essential genes of a minimal bacterium. Proc Natl Acad Sci U S A **103:**425-430.
3. **Hutchison CA, Peterson SN, Gill SR, Cline RT, White O, Fraser CM, Smith HO, Venter JC.** 1999. Global transposon mutagenesis and a minimal Mycoplasma genome. Science **286:**2165-2169.
4. **Sirand-Pugnet P, Citti C, Barre A, Blanchard A.** 2007. Evolution of *Mollicutes*: down a bumpy road with twists and turns. Res Microbiol **158:**754-766.
5. **Bergonier D, Berthelot X, Poumarat F.** 1997. Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control. Rev Sci Tech **16:**848-873.
6. **Bergonier D, Poumarat F.** 1996. [Contagious agalactia of small ruminants: epidemiology, diagnosis and control]. Rev Sci Tech **15:**1431-1475.
7. **Corrales JC, Esnal A, De la Fe C, Sánchez A, Assunçao P, Poveda JB, Contreras A.** 2007. Contagious agalactia in small ruminants. Small Rumin Res **68:**154-166.
8. **Nicholas RA.** 2002. Improvements in the diagnosis and control of diseases of small ruminants caused by mycoplasmas. Small Rumin Res **45:**145-149.
9. **Sirand-Pugnet P, Lartigue C, Marenda M, Jacob D, Barre A, Barbe V, Schenowitz C, Mangenot S, Couloux A, Segurens B, de Daruvar A, Blanchard A, Citti C.** 2007. Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. PLoS Genet **3:**e75.
10. **Chopra-Dewasthaly R, Citti C, Glew MD, Zimmermann M, Rosengarten R, Jechlinger W.** 2008. Phase-locked mutants of *Mycoplasma agalactiae*: defining the molecular switch of high-frequency Vpma antigenic variation. Mol Microbiol **67:**1196-1210.
11. **Chopra-Dewasthaly R, Zimmermann M, Rosengarten R, Citti C.** 2005. First steps towards the genetic manipulation of *Mycoplasma agalactiae* and *Mycoplasma bovis* using the transposon Tn*4001*mod. Int J Med Microbiol **294:**447-453.
12. **Chopra-Dewasthaly R, Baumgartner M, Gamper E, Innerebner C, Zimmermann M, Schilcher F, Tichy A, Winter P, Jechlinger W, Rosengarten R, Spergser J.** 2012. Role of Vpma phase variation in *Mycoplasma agalactiae* pathogenesis. FEMS Immunol Med Microbiol **66:**307-322.
13. **Baranowski E, Guiral S, Sagne E, Skapski A, Citti C.** 2010. Critical role of dispensable genes in *Mycoplasma agalactiae* interaction with mammalian cells. Infect Immun **78:**1542-1551.
14. **Baranowski E, Bergonier D, Sagne E, Hygonenq MC, Ronsin P, Berthelot X, Citti C.** 2014. Experimental infections with *Mycoplasma*

agalactiae identify key factors involved in host-colonization. PLoS One **9:**e93970.

15. **Hegde S, Hegde S, Spergser J, Brunthaler R, Rosengarten R, Chopra-Dewasthaly R.** 2014. In vitro and in vivo cell invasion and systemic spreading of *Mycoplasma agalactiae* in the sheep infection model. Int J Med Microbiol (http://dx.doi.org/10.1016/j.ijmm.2014.07.011).
16. **Solsona M, Lambert M, Poumarat F.** 1996. Genomic, protein homogeneity and antigenic variability of *Mycoplasma agalactiae*. Vet Microbiol **50:**45-58.
17. **Aluotto BB, Wittler RG, Williams CO, Faber JE.** 1970. Standardized bacteriologic techniques for the characterization of *Mycoplasma* species. Int J Syst Bacteriol **20:**35-58.
18. **Knudtson KL, Minion FC.** 1993. Construction of Tn*4001* lac derivatives to be used as promoter probe vectors in mycoplasmas. Gene **137:**217-222.
19. **Sharma VM, Chopra R, Ghosh I, Ganesan K.** 2001. Quantitative target display: a method to screen yeast mutants conferring quantitative phenotypes by 'mutant DNA fingerprints'. Nucleic Acids Res **29:**E86-86.
20. **Barre A, de Daruvar A, Blanchard A.** 2004. MolliGen, a database dedicated to the comparative genomics of *Mollicutes*. Nucleic Acids Res **32:**D307-310.
21. **Hudson P, Gorton TS, Papazisi L, Cecchini K, Frasca S, Jr., Geary SJ.** 2006. Identification of a virulence-associated determinant, dihydrolipoamide dehydrogenase (*lpd*), in *Mycoplasma gallisepticum* through in vivo screening of transposon mutants. Infect Immun **74:**931-939.
22. **Chavez Gonzalez YR, Ros Bascunana C, Bolske G, Mattsson JG, Fernandez Molina C, Johansson KE.** 1995. In vitro amplification of the 16S rRNA genes from *Mycoplasma bovis* and *Mycoplasma agalactiae* by PCR. Vet Microbiol **47:**183-190.
23. **Mann HB, Whitney DR.** 1947. On a Test of Whether One of 2 Random Variables Is Stochastically Larger Than the Other. Ann Mathematical Stat **18:**50-60.
24. **Chang J, Pang E, He H, Kwang J.** 2008. Identification of novel attenuated *Salmonella Enteritidis* mutants. FEMS Immunol Med Microbiol **53:**26-34.
25. **Pang E, Tien-Lin C, Selvaraj M, Chang J, Kwang J.** 2011. Deletion of the *aceE* gene (encoding a component of pyruvate dehydrogenase) attenuates *Salmonella enterica* serovar Enteritidis. FEMS Immunol Med Microbiol **63:**108-118.
26. **Autret N, Charbit A.** 2005. Lessons from signature-tagged mutagenesis on the infectious mechanisms of pathogenic bacteria. FEMS Microbiol Rev **29:**703-717.
27. **Saenz HL, Dehio C.** 2005. Signature-tagged mutagenesis: technical advances in a negative selection method for virulence gene identification. Curr Opin Microbiol **8:**612-619.

28. **Skapski A, Hygonenq MC, Sagne E, Guiral S, Citti C, Baranowski E.** 2011. Genome-scale analysis of M*ycoplasma agalactiae* loci involved in interaction with host cells. PLoS One **6:**e25291.
29. **Pilo P, Vilei EM, Peterhans E, Bonvin-Klotz L, Stoffel MH, Dobbelaere D, Frey J.** 2005. A metabolic enzyme as a primary virulence factor of *Mycoplasma mycoides* subsp. *mycoides* small colony. J Bacteriol **187:**6824-6831.
30. **Grosshennig S, Schmidl SR, Schmeisky G, Busse J, Stulke J.** 2013. Implication of glycerol and phospholipid transporters in *Mycoplasma pneumoniae* growth and virulence. Infect Immun **81:**896-904.
31. **Schmidl SR, Otto A, Lluch-Senar M, Pinol J, Busse J, Becher D, Stulke J.** 2011. A trigger enzyme in *Mycoplasma pneumoniae*: impact of the glycerophosphodiesterase GlpQ on virulence and gene expression. PLoS Pathog **7:**e1002263.
32. **Vilei EM, Frey J.** 2001. Genetic and biochemical characterization of glycerol uptake in *Mycoplasma mycoides* subsp. *mycoides SC*: its impact on H(2)O(2) production and virulence. Clin Diagn Lab Immunol **8:**85-92.
33. **Hopfe M, Dahlmanns T, Henrich B.** 2011. In *Mycoplasma hominis* the OppA-mediated cytoadhesion depends on its ATPase activity. BMC Microbiol **11:**185.
34. **Flores-Valdez MA, Morris RP, Laval F, Daffe M, Schoolnik GK.** 2009. Mycobacterium tuberculosis modulates its cell surface via an oligopeptide permease (Opp) transport system. FASEB J **23:**4091-4104.
35. **Kerr AR, Adrian PV, Estevao S, de Groot R, Alloing G, Claverys JP, Mitchell TJ, Hermans PW.** 2004. The Ami-AliA/AliB permease of Streptococcus pneumoniae is involved in nasopharyngeal colonization but not in invasive disease. Infect Immun **72:**3902-3906.
36. **Wang CH, Lin CY, Luo YH, Tsai PJ, Lin YS, Lin MT, Chuang WJ, Liu CC, Wu JJ.** 2005. Effects of oligopeptide permease in group a streptococcal infection. Infect Immun **73:**2881-2890.
37. **Wu TK, Wang YK, Chen YC, Feng JM, Liu YH, Wang TY.** 2007. Identification of a *Vibrio furnissii* oligopeptide permease and characterization of its in vitro hemolytic activity. J Bacteriol **189:**8215-8223.
38. **Hames C, Halbedel S, Hoppert M, Frey J, Stulke J.** 2009. Glycerol metabolism is important for cytotoxicity of *Mycoplasma pneumoniae*. J Bacteriol **191:**747-753.
39. **Djordjevic SP, Vilei EM, Frey J.** 2003. Characterization of a chromosomal region of *Mycoplasma* sp. bovine group 7 strain PG50 encoding a glycerol transport locus (*gtsABC*). Microbiology **149:**195-204.
40. **Khan LA, Miles RJ, Nicholas RA.** 2005. Hydrogen peroxide production by *Mycoplasma bovis* and *Mycoplasma agalactiae* and effect of in vitro passage on a *Mycoplasma bovis* strain producing high levels of H_2O_2. Vet Res Commun **29:**181-188.

41. **Miles RJ, Wadher, B.J., Henderson, C.L. and Mohan, K.** 2008. Increased growth yields of *Mycoplasma* spp. in the presence of pyruvate. Lett Appl Microbio **7:**149-151.
42. **Much P, Winner F, Stipkovits L, Rosengarten R, Citti C.** 2002. *Mycoplasma gallisepticum*: Influence of cell invasiveness on the outcome of experimental infection in chickens. FEMS Immunol Med Microbiol **34:**181-186.
43. **Thomas C, Jacobs E, Dumke R.** 2013. Characterization of pyruvate dehydrogenase subunit B and enolase as plasminogen-binding proteins in *Mycoplasma pneumoniae*. Microbiology **159:**352-365.
44. **Dallo SF, Kannan TR, Blaylock MW, Baseman JB.** 2002. Elongation factor Tu and E1 beta subunit of pyruvate dehydrogenase complex act as fibronectin binding proteins in *Mycoplasma pneumoniae*. Mol Microbiol **46:**1041-1051.
45. **Dumke R, Hausner M, Jacobs E.** 2011. Role of *Mycoplasma pneumoniae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mediating interactions with the human extracellular matrix. Microbiology **157:**2328-2338.
46. **Robinson MW, Buchtmann KA, Jenkins C, Tacchi JL, Raymond BB, To J, Roy Chowdhury P, Woolley LK, Labbate M, Turnbull L, Whitchurch CB, Padula MP, Djordjevic SP.** 2013. MHJ_0125 is an M42 glutamyl aminopeptidase that moonlights as a multifunctional adhesin on the surface of *Mycoplasma hyopneumoniae*. Open Biol **3:**130017.

Tables and Figures

Table 1. Detection of *M. agalactiae* in tissue samples 2 weeks p.i. by *M. agalactiae*-specific PCR. NA- Not applicable

	Positive samples/total number of samples examined	
Organ/Site	**Expt. I**	**Expt. II**
Liver	0/9	0/13
Spleen	0/9	0/13
Kidney L	1/9	0/13
Kidney R	2/9	0/13
Lung L	0/9	0/13
Lung R	1/9	0/13
Uterus	4/9	0/13
Knee Fluid L-Synovial Fluid	3/9	0/13
Knee Fluid L-Synivial Fluid	7/9	0/13
Heart	NA	0/13
Brain	NA	0/13
Carpal joint fluid L	NA	0/13
Carpal joint fluid R	NA	0/13

Table 2. Absence of mutants in systemic sites (inner organs), LNs and udder, systemic sites LNs and udder combined based on SSM PCR primary screening results

		Percentage absence		
Mutant	**Gene**	**Systemic sites**	**LNs and udder**	**Systemic sites, LNs and udder**
Apo7	MAG1890	100	100	100
6-9-2	MAG0360_oppC	100	95	97
6-15	lip	96	92	94
6-14	MAG4970_uhpT	96	97	96
6-29	MAG0940_pdhB	96	100	98
6-32	MAG1390_eutD	96	100	98
9-31	MAG4340_adhT& MAG2630_nox	100	97	98
9-40	MAG2320_gtsB	100	95	97
Apo2-12	MAG1050	100	100	100
15-2	MAG6200	96	87	91
170	MAG0110	92	92	92
3-29	MAG2540	100	100	100
3-4-0	MAG3390	100	100	100
9-28	MAG0390	96	87	91
81-1	MAG0370_oppB	100	98	99
14	MAG1790	96	69	82
137	MAG4460	100	100	100
96-2	MAG2930_atpA	96	87	91
5-1	MAG0250	96	79	87
7+1-3	MAG3650	100	100	100
23	MAG5520_aminopeptidase	96	100	98

Table 3. Categorization of disrupted genes in mutants confirmed to be attenuated in systemic sites by confirmatory screening experiments

Mutants	Gene	ORF identity
Apo7	MAG1890	Hypothetical protein
6-29	MAG0940_pdhB	Pyruvate dehydrogenase beta subunit
6-9-2	MAG0360_oppC	Oligopeptide ABC transporter permease proteins
7+1-3	MAG3650	Hypothetical protein
9-40	MAG2320_gtsB	Glycerol ABC transporter permease
23	MAG5520_aminopep	Aminopeptidase
81-1	MAG0370_oppB	Oligopeptide ABC transporter permease component

Figure 1. Doubling time of the wild type strain PG2 and mutants in the presence of HeLa cells 48 h p.i. Each bar represents the average doubling time ± standard deviation of three independent experiments done in duplicates. *P<0.05; ** is P<0.005

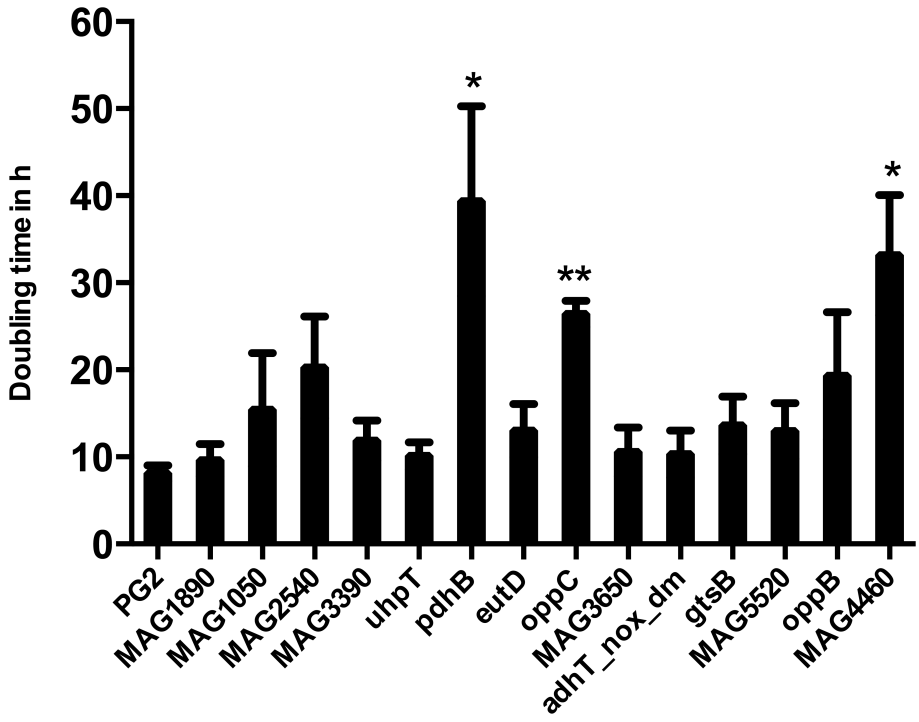


Figure 2. Percentage of cytotoxicity of the wild type strain PG2 and mutants in murine macrophages J744.A1 at 48 h p.i. Each bar represents the average cytotoxicity ± standard deviation of three independent experiments done in triplicates.

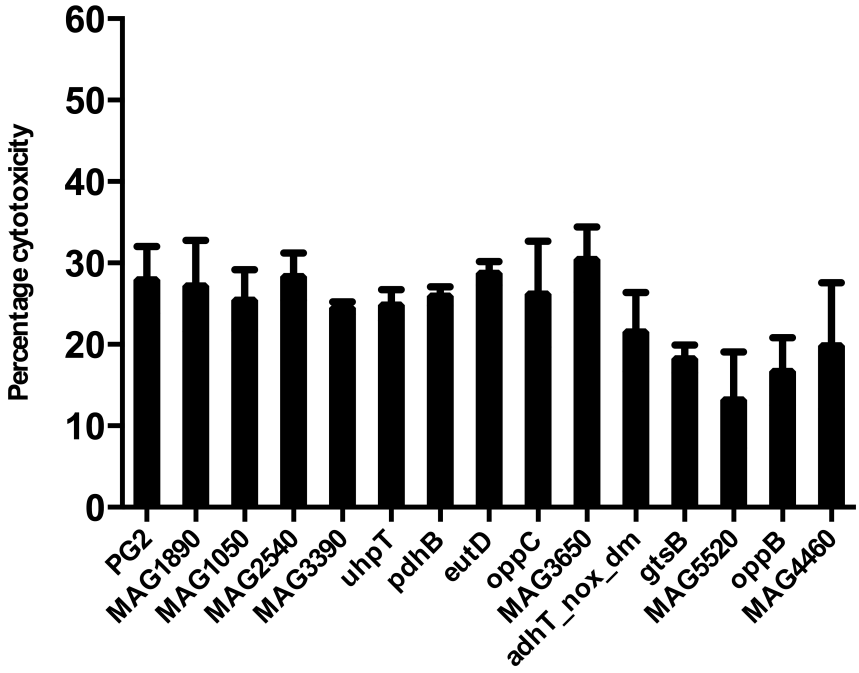
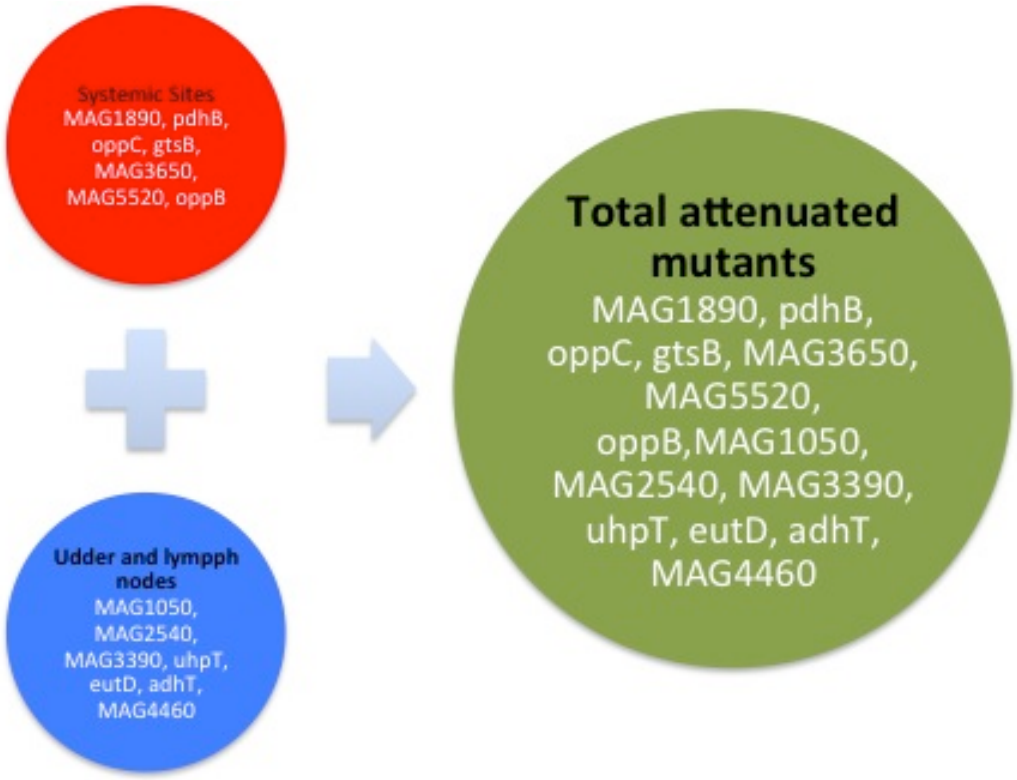


Figure 3. Colored circles depict disrupted genes of attenuated mutants in systemic sites (red) and in the udder and lymph nodes (blue). The total attenuated mutants are represented in the green circle combining systemic sites, lymph nodes and udder.



Supplementary Tables

Table S1. Gene-specific primer sequences used in SSM PCR

Mutant	Primer designation	Gene	Primer sequence
Apo7	MAG1890 R	MAG_1890	CTA ATG TTG GAG CAC TAG TGT G
Apo 6	sgae F	MAG_6330	GCG ACA CTT AAG CCT GAA G
26	oppF F	MAG_0340	CCT AGA CAT TGT CTA TGG CTC
3-1 PtsL	ptsL R	MAG_6300	CAG ATA ACA CAG CTG CTG CAG
3-15 fusA	FusA F	MAG_5920	GCA ATC AGG TGG TAA AGG TC
6-9-2	oppC F	MAG_0360	GTA GCA ACG TTT GGG ATA G
6-14	uhpT F	MAG_4970	TAG GTG GTA AGG GTG TTG TG
6-15	Lip R	MAG_0040	GTG TCC TGT TTT AGG CAT TC
6-21	MrzA R	MAG_3740	AAC TCG TCC TCA CTA TAA G
6-29	pdhBfpN	MAG_0940	ACT TGC CCA TGG AAT AAT TGT TCC C
6-32	Pta R	MAG_1390	ACA TCG TCA GTT AAG CTT CC
7+1-8	Zinc R	MAG_4010	CTT CTT TAT TTC ATC ACT AGG
8+1-5	Rnh R	MAG_6730	TAA TCT GTG TTC AGG AGA GG
9-31	AdhT_Nox R	MAG_2630 &MAG_4340	CAA TAA CAG CTC TTC CTA AG (primer for MAG_2630)
9-40	gtsBfpN	MAG_2320	ACT TAT CCA TGG CAA GCA TTG AAG TTG
Apo2-12	MAG1050 F	MAG_1050	ATG TTA TCA ATG CCG GTA GC
1-3-0	MAG2810 R	MAG_2810	GTA ATT TAG CTG CAA TGC CCG
15-2	MAG6200 R	MAG_6200	GGC ATA TGA GGC AAT TGA AG
170	MAG0110 F	MAG_0110	GCT TCT GTT GGT TGG TTC TC
3-29	MAG2540 F	MAG_2540	TAA CCC TGG TGA CAC AAC TG
4-1-0	GTP R	MAG_3820	GTG GCA AAT ATC TCC ATC TTG C
8+1-4	ABC ATP F	MAG_5960	GCC AAG ACC ATA ATG CTT ATG

Table S1 contd. Gene-specific primer sequences used in SSM PCR

Mutant	Primer designation	Gene	Primer sequence
8+1-8	SGAT R	MAG_6380	AGC TGT GCG CCT AAT GAC AC
3-4-0	TonB R	MAG_3390	AGC CTA TGT AAT CCG GAT G
9-28	MAG0390 F	MAG_0390	GTGCTC TTA CAC CAA ACT CAA C
62	LplA F	MAG_0070	CAG CAC AAC TTG GAA GTT GTT C
212	T3RM R	MAG_1530	CAT GAC CAG TTG TTC CTG AAC C
9-8	pdhD F	MAG_0960	CAT TGC CTG CAT CAA TGG TC
9-16	MAG1450 F	MAG_1450	ATT GTT GCT TTC ATA CCG CC
81-1	oppB F	MAG_0370	CTC GTC AAT TCC ATC ACT AG
3-20	Nox F	MAG_4340	GAA TTT AGT CAA CCA GAT GGT C
13	VpmaX F	MAG_7070	TAC TTG GAT CGG TTG CTT C
14	Meth F	MAG_1790	ATG ATA AGA GTT ATT TCA GGC
137	Acidph F	MAG_4460	AGC TGC TAC ATG TCA AGA TCC
8-1	Dgk R	MAG_0180	GCG GAA TGA TAA GTA GTG G
1-2-0	PenA F	MAG_6360	CTG GAA TGG GAA CCA GCA TG
96-2	AtpA_2930 R	MAG_2930	TCT CCT AAT TTA AGT GCA AC
5-1	Indi F	MAG_0250	GTA AGG AAG TTG AAA GCG C
6-13	MAG1570 R	MAG_1570	TCC ACG TCT GTA ATT TCA CC
7+1-3	MAG3650 F	MAG_3650	GGC TTG GAA TGA TTG ATG G
23	Amino pep R	MAG_5520	AGT AGG AAC ACC ACC AAT ACC
6-12	Transreg F	MAG_6310	GCC AAT TCA TCA GAA GCA GC
San5	MAG3120 R	MAG_3120	GAT GTA CCA TTT GAG CCT G
Vpma Y 6-15	VpmaY F	MAG_7080	ATC AGT TGC TTC AAT GGC CTC
3-23	MAG6410 lipo F	MAG_6410	ATG ACT TTG GCA AGG TTC AGG

Figure. S1. Transposon stability analysis (A) Comparison of cfu/ml of each mutant passaged 30 times in non-selective medium and plated separately on SP4 agar plates with (+) or without (-) gentamicin. This procedure was repeated with all the mutants used in the sheep infection. (B) SSM PCR performed with genomic DNA isolated from mutants 8-1 and 13 before (P0) the first passage and after the 30th passage (P30). All other passaged mutant used in the sheep infection experiments were similarly analyzed by PCR.

M, 1 kb marker; C, PCR control

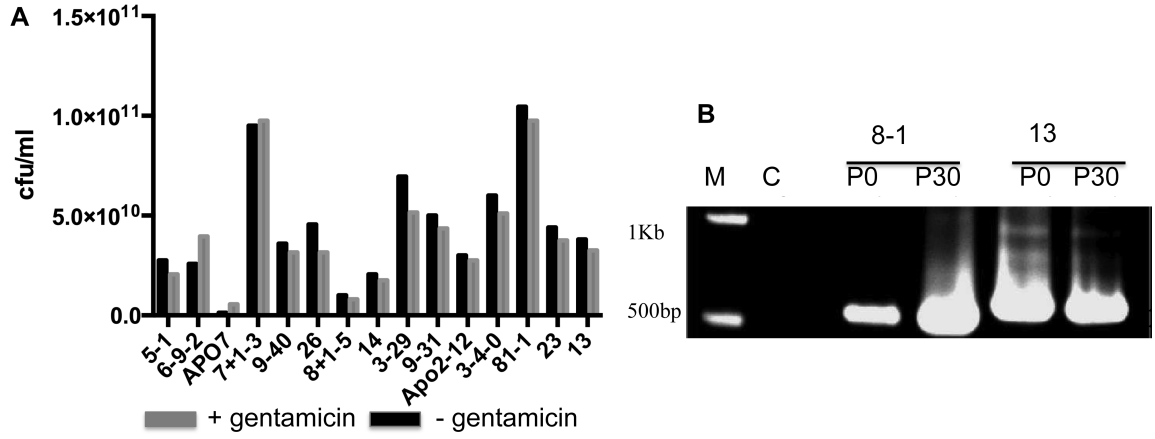
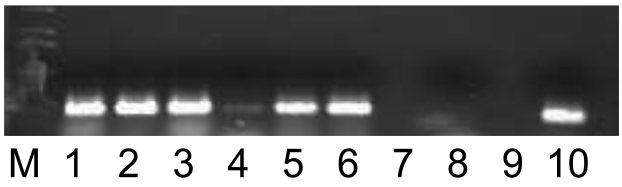


Figure S2. *M. agalactiae*-specific 16S rDNA PCR of samples from infected sheep after necropsy.

M, 1Kb ladder; 1, uterus; 2, synovial fluid, knee right; 3, suprammammary LN right; 4, LN mesenterialis; 5, LN mediastinalis; 6, udder left; 7, ung left; 8, spleen; 9-, iver; 10, M. *agalactiae* type strain PG2.



5 Summary

Mycoplasma agalactiae is the main causative agent of the Contagious Agalactia (CA) syndrome in sheep and goats, which is mainly characterized by mastitis, conjunctivitis and arthritis. Despite causing significant economic losses, the pathogenic mechanisms and attributes of this important pathogen are still largely unknown, a fact that can be mostly attributed to its long resistance to genetic manipulation. However, our mycoplasma research laboratory reported the first successful genetic manipulation of *M. agalactiae* using Tn*4001*mod, and this paved the way for a better understanding of its pathogenesis. This dissertation project exploited the use of a heterogenous pool of Tn mutants that were simultaneously screened in a sheep intramammary infection model. Screening of 45 different mutants through two successive cycles using a negative selection method identified 14 disrupted genes that are likely to play an important role in *M. agalactae* pathogenesis. The results demonstrated attenuation of mutants harboring insertions in different functional genes, some of which have also been previously implicated in the pathogenesis of other bacteria, thus underlining their potential similar role in *M. agalactiae* infections. Furthermore, many attenuated mutants were identified to carry insertions in hypothetical genes, thus assigning an important function to these genes in *M. agalactiae* host colonization. Some of the mutants are attenuated only in the udder and lymph nodes indicating the importance of the corresponding mutated genes in local infection and lymphogenic spreading. *In vitro* studies with attenuated mutants revealed that some of them are significantly deficient in their ability to

grow with HeLa cells during co-cultivation assays, and also in killing murine macrophages, when compared with the wild type PG2 strain. Results of *in vitro* analysis of a *pdhB* mutant revealed its growth deficit in axenic medium during log phase. Additionally, this mutant also exhibited smaller colony size and was deficient in invading HeLa cells. Genetic and functional complementation was achieved by cloning the full-length *pdhB* gene in a newly developed expression vector that was transformed into the mutant. As expected the complemented clones regained their normal *in vitro* growth profile, colony size and HeLa cell invasion capacity that were comparable to the wild type strain. Hence, results from *in vitro* and *in vivo* studies with transposon mutants identified new genetic factors involved in *M. agalactiae* infection.

This project also investigated whether *M. agalactiae* has the capability to enter, survive and exit the eukaryotic host cells in a viable state, as this could explain the chronic, persistent and difficult-to-eradicate nature of its infections in spite of long antibiotic therapies. The data obtained provide evidence for the first time that *M. agalactiae* is able to invade eukaryotic host cells whereby quantitative data are supported by the corresponding results of the qualitative double immunofluorescence assay. Intracellular mycoplasmas were detected not only after *in vitro* infection but also *in vivo* in various tissue samples from experimentally infected sheep using immunohistochemistry. Also, by the isolation of mycoplasmas from various inner organs of sheep experimentally infected via the intramammary route we have formally demonstrated that *M. agalactiae* has the capability to cross local epithelial barriers and to

disseminate to distant body sites. Overall, the findings of these *in vitro* and *in vivo* studies indicate that *M. agalactiae* is capable of entering host cells. This might be the strategy that *M. agalactiae* employs at the population level to evade the host immune response and antibiotic action, and to disseminate to new and safer niches where it again may proliferate upon the return of favorable conditions, with the outcome of a persistent chronic infection.

Altogether, the results presented in this dissertation show different facets of *M. agalactiae* pathogenesis. Each of the identified attenuated mutants has the potential to be developed into a good candidate vaccine after thorough further analysis. Demonstrating the ability of *M. agalactiae* to invade host cells for the first time is also an important step in understanding the persistent and chronic nature of *M. agalactiae* infections and diseases. The results are anticipated to advance our understanding of the complex mechanisms involved in the overall success of this small ruminant mycoplasma species as a pathogen.

6 Appendix

6.1 Solutions and Media

6.1.1 Media, media additives and antibiotics

SP4 Broth	0.35% mycoplasma broth base (Oxoid) 1% tryptone (Oxoid) 0.53% peptone (Merck) pH 7.8 with NaOH (Sigma) autoclaved at 120°C for 20 min 17 ml/100 ml heat inactivated fetal calf serum (Gibco) 5 ml/100 ml CMRL (Invitrogen) 5 ml/100 ml 4% aqueous yeastolate solution (Difco) 2.5 ml/100 ml 15% filter sterile yeast extract solution (GIbco) 1 ml/100 ml 50% filter sterile glucose solution (Roth) 1 ml/100 ml 0.5% filter sterile phenol red solution (ICN Biomedicals) 2 ml/100 ml 25% filter sterile sodium pyruvate (Sigma) solution

SP4 agar plates	SP4 broth with 1% noble agar (Difco) before autoclaving add ddH2O instead of phenol red and sodium pyruvate solutions
Aluotto Broth	2.1% Heart infusion Broth (Difco) pH 7.8 with NaOH and autoclaved 10 ml/100 ml heat inactivated horse serum (Gibco) 3.7 ml/100 ml 15% yeast extract solution 1 ml/100 ml 0.5% phenol red solution 2 ml/100 ml 25% sodium pyruvate solution
Aluotto agar plates	Aluotto broth with 1% noble agar (Difco) before autoclaving ddH2O instead of phenol red and sodium pyruvate solutions
LB Broth	5% yeast extract (Merck) 10% NaCl (Roth) 10% tryptone (Oxoid)
LB agar plates	LB broth with 1.5% agar (Oxoid)

Ampicillin Stock (Roth)	100 mg/ml in ddH2O
Tetracycline Stock (Sigma)	10 mg/ml in ddH2O
Gentamicin Stock (Sigma)	10 mg/ml in ddH2O
Thallium acetate stock (Roth)	10 mg/ml in ddH_20

6.1.2 Southern Blot analysis

Depurination Solution	250 mM NaCl (Roth)
Denaturation Solution	500 mM NaOH (Roth) 1.5 M NaCl (Roth)
Neutralization Solution	500 mM Tris (Roth) 3 M NaCl (Roth) pH 7.5 adjusted with HCl (Roth)
20 x Sodium Chloride	3 M NaCl (Roth)
Sodium Citrate Buffer (SSC)	300 mM sodium citrate (Roth)
Maleic Acid Buffer	100 mM maleic acid (Roth) 150 mM NaCl (Roth)

	pH 7.5 adjusted with NaOH (Roth)
Washing Solution	maleic acid buffer with 0.3% Tween 20
Blocking Solution	maleic acid buffer 1% blocking reagent (Roche)
Detection Buffer	100 mM Tris (Roth) 100 mM NaCl (Roth) pH 9.5 adjusted with NaOH (Roth)

6.1.3 Agarose gel electrophoresis

6 x DNA Loading Buffer	40% glycerol (Roth) 0.1% bromophenol blue (Sigma)
50 x TAE	2 M Tris (Roth) 1 M acetic acid (Roth) 500 mM EDTA (Merck)
Agarose gel	0.8 - 1% agarose (Roth) boiled in 1X TAE

6.1.4 Cell culture

Minimum Essential Medium	500 ml MEM (Gibco) 10 ml/100 ml heat inactivated fetal calf serum 3 ml/ 100 ml Non-Essential Amino acid (NEA) (PAA laboratories)
Trypsin-EDTA	1X trypsin-EDTA (PAA laboratories)

6.1.5 DNA isolation

Washing Solution	250 mM NaCl (Roth) 100 mM EDTA (Merck)
Suspension Solution	50 mM Tris pH 8.0 (Roth) 10 mM EDTA (Merck)
Phenol / Chloroform / Isoamyl Alcohol Solution (25:24:1)	(Sigma)
TE Buffer	10 mM Tris pH 8.0 (Roth) 1 mM EDTA (Merck)
RNase Stock (Roth)	10 mg/ml in ddH_2O
Proteinase K Stock (Roth)	10 mg/ml in 10 mM Tris pH 8.0 (Roth)

Potassium Acetate	5 M potassium acetate (Roth) in ddH2O
Sodium Acetate	3 M sodium acetate (Roth) pH 5.2 adjusted with HCl (Roth)

6.1.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

APS Solution	10% ammonium peroxide sulfate (Roth) in ddH2O
40% Acrylamide Solution	(Roth)
Urea Stock	30% urea (Roth) in ddH2O
SDS Stock	10% SDS (Roth) in ddH2O
TEMED Solution	(Roth)
Resolving Gel Buffer	1.5 M Tris (Roth) pH 8.8 adjusted with HCl (Roth)
Stacking Gel Buffer	250 mM Tris-HCl pH 6.8 (Roth) 50% glycerol (Roth)

	10% SDS (Roth) 7.5% DTT (Roth) 0.1% bromophenol blue (Sigma)
10 x Laemmli Electrode Buffer (LEB)	250 mM Tris (Roth) 1.9 M glycine (Roth)

6.1.6 Western blot and colony blot analysis

10 x Tris Buffered Saline (TBS)	1.5 M NaCl (Roth) 100 mM Tris (Roth) pH 7.4 adjusted with HCl (Roth)
TBS Tween	1 x TBS 0.05% Tween 20 (Roth)
Ponceau Stain	0.25% Ponceau S (Roth) 5% acetic acid (Roth)
Blocking Solution	3% milk powder (Bio-Rad) in 1x TBS
CN Stock	0.3% 1-chloro-4-naphthol (Bio-Rad) in 100% methanol (Roth)
CN Working Solution	2 ml CN stock 10 ml 10 x TBS 10 µl H2O2

Blotting Buffer	1 x LEB 20% methanol (Roth)

6.1.7 Double Immunofluorescence

PBS+ 2% BSA	1x PBS (Gibco) + 2% BSA (Roth)
Mowiol Solution I	20 g MOWIOL 4-88 (Hoechst) 80 ml PBS pH 7.3 stir over night 40 ml Glycerin (Merck) stir over night Centrifuge 15,000 RPM for 1 hr remove supernatant, add 0.1% NaN_3 Store at 4 °C
Mowiol Solution II	2.5 g n-propyl-Gallat (Sigma) in 50 ml PBS pH 7.0 50 ml Glycerin (Merck) stir over night Store at 4 °C in dark
Mounting Medium	Mix 3 vol. of sol. I and 1 vol. of sol. II before use

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CURRICULUM VITAE

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EDUCATION

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2005 — Master of Science (M.Sc) in Biochemistry
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2003 — Bachelor of Science (M.Sc) in Chemistry, Physics, Mathematics
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RESEARCH & PROFESSIONAL EXPERIENCE

2010-present — Ph.D. Student, Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine Vienna
Dissertation project with focus on the screening of a *Mycoplasma agalactiae* transposon mutant library in the sheep intramammary infection model using a negative selection method. Several genetic factors were identified that were confirmed to play a role in host colonization. In addition, *Mycplasma agalactiae* cell invasion was shown for the first time both in *in vivo* and *in vitro*.
Supervisory Experience: One bachelor student, two undergraduate research trainees, two short-term internship students.

2007-2010 — Research Assistant, Department of Microbiology and Cell Biology, Indian Institute of Science (IISc), Bangalore, India.
Research Experience: Characterization of DNA topoisomerase of *Mycobacterium smegmatis*. Cloning, purification and characterization of metal ion binding motif mutants of topoisomerase I.

2006-2007 — Lab Instructor, Sir M Visvesvaraya Institute of Technology (Sir. MVIT), Bangalore, Karnataka, India.
Supervision of bachelor students in practical training courses in Biochemistry.

PUBLICATIONS

1. Hegde, S, S. Hegde, J. Spergser, R. Brunthaler, R. Rosengarten and R. Chopra-Dewasthaly. 2014. *In vitro* and *in vivo* cell invasion and systemic spreading of *Mycoplasma agalactiae* in the sheep infection model. *Int. J. Med. Microbiol.* (DOI10.1016/j.ijmm.2014.07.011).

2. Hegde, S., M. Zimmermann, M. Floeck, J. Spergser, R. Rosengarten and R. Chopra-Dewasthaly. 2014. Simultaneous identification of novel pathogenicity factors of *Mycoplasma agalactiae* in the sheep infection model using negative selection". *Infect. Immun.* (under review).

3. Hegde, S., R. Rosengarten and R. Chopra-Dewasthaly. 2014. Disruption of the *pdhB* pyruvate dehyrogenase gene affects colony morphology, *in vitro* growth and cell invasiveness of *Mycoplasma agalactiae*". *PLoS One* (under review).

4. Leelaram, M.N., A.G. Bhat, S.M. Hegde, R. Manjunath and V. Nagaraja. 2012. Inhibition of type A topoisomerase by a monoclonal antibody through perturbation of DNA cleavage-religation equilibrium". *FEBS J.* 279, 55-65.

5. Bhat, A.G., M.N. Leelaram, S.M. Hegde, V. Nagaraja. 2009. Deciphering the distinct role for the metal coordination motif in the catalytic activity of *Mycobacterium smegmatis* topoisomerase I. *J. Mol. Biol.* 393, 788-802.

MANUSCRIPTS

1. Hegde, S., M. Zimmermann, M. Floeck, J. Spergser, R. Brunthaler, R. Rosengarten and R. Chopra-Dewasthaly. Identification of *Mycoplasma agalactiae* genes involved in systemic spreading during experimental intramammary infection of sheep. Manuscript in preparation.

2. Czurda, S., S. Hegde, W. Jechlinger, R. Rosengarten and R. Chopra-Dewasthaly. Xer1-independent mechanisms of Vpma phase variation are triggered by the presence of Vpma-specific antibodies. Manuscript in preparation.

CONFERENCE PRESENTATIONS (*presenting author)

1. **Hegde, S.***, M. Zimmermann, M. Kargl, M. Floeck, J. Spergser, R. Rosengarten and R. Chopra-Dewasthaly. 2013. Genetic factors associated with *Mycoplasma agalactiae* host colonization and survival: a window into mycoplasma pathogenicity. Poster presentation at the 5th FEMS Congress, Leipzig, Germany, 21-25 July 2013.

2. Hegde, S.*, **S. Hegde**, M. Zimmermann, R. Rosengarten and R. Chopra-Dewasthaly. 2012. Intracellular invasion as a window into understanding *Mycoplasma agalactiae's* mechanisms of infection and persistence. Poster presentation at the 5th FEMS congress, Leipzig, Germany, 21-25 July 2013.

3. **Hegde, S.***, M. Zimmerman, R. Rosengarten and R. Chopra Dewasthaly. 2012. Identification of *Mycoplasma agalactiae* pathogenicity determinants through *in vivo* screening of transposon mutants in a sheep intramammary infection model. Poster presentation at the 19th IOM Congress, Toulouse, France, 15-19 July 2012.

4. **Hegde**, **S.,** M. Zimmerman, R. Rosengarten and R. Chopra-Dewasthaly*. 2011. Transposon mutants of *Mycoplasma agalactiae*: exploiting a modified QTD PCR methodology as a novel strategy for sequencing Tn insertion sites and its optimization for selective quantitative screening. Abstract in: *Int. J. Med. Microbiol.* 301S1, September 2011. Oral presentation at the 63. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM) e. V. Essen, Germany, 25- 28 September 2011.

5. **Hegde, S.***, R. Rosengarten and R. Chopra-Dewasthaly. 2011. Critical role of PdhB in contrast to Pta during log phase growth of *Mycoplasma agalactiae.* Oral presentation at the Mycoplasma 2011 Conference, Göttingen, Germany, 4-5 July 2011.