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Molecular Characterisation of *Mycoplasma mycoides* subsp. *mycoides* SC

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Abstract

Mycoplasma mycoides subsp. *mycoides* SC (*M. mycoides* SC), is the causative agent of contagious bovine pleuropneumonia (CBPP), a severe respiratory disease in cattle. The disease has spread alarmingly across the African continent during the last decade and the problems with eradication are many. Existent diagnostic systems lack specificity and sensitivity, and the available vaccines are not efficient. Hitherto, very little is known about the pathogenicity of this organism and new information is sorely needed to control the disease.

The first study in the thesis comprises the development of two diagnostic PCR systems that detect *M. mycoides* SC in various specimens. One system is designed for automated analysis of the PCR products in which the amplicons are detected by laser induced fluorescence to give a high sensitivity and accuracy. The second method can be performed with standard equipment for PCR and agarose gel electrophoresis and the CBPP agent can be distinguished after restriction of the amplicon.

Insertion sequences are transposable DNA elements that have been shown to be efficient markers for epidemiology. In the second study, a new IS element of *M. mycoides* SC was discovered and characterised, ISMmy*1*. IS-typing with an ISMmy*1*-specific probe indicates that this insertion sequence can be a useful marker to differentiate naturally infected CBPP cases and vaccinated animals. ISMmy*1* was also found in another bovine pathogen, *Mycoplasma bovis* and our data suggests that horizontal transfer has occurred at some stage.

In the third investigation, a putative virulence factor, Vmm, was identified and characterised. Vmm is a small lipoprotein located at the surface of the bacterial membrane, which undergoes reversible phase variation. By comparing the *vmm* gene regions from subcloned phenotypic variants, it was shown that expression of Vmm is regulated at the transcriptional level by insertions and deletions in the promoter spacer.

The whole genome of *M. mycoides* SC was sequenced in the fourth project. It is a bacterial genome of 1.14 Mbp with very low G+C content (24%). Altogether, 1,067 open reading frames were identified and the putative genes were annotated and correlated to a function or metabolic process, wherever possible.

Keywords: *Mycoplasma mycoides* subsp. *mycoides* SC, mycoplasma, CBPP, PCR, insertion sequence, variable surface protein, Vmm, genome sequencing

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Appendices

This thesis is based on the following articles, which will be referred to by their Roman numerals:

I. Persson A., Pettersson, B., Bölske, G., and Johansson, K.-E. 1999. Diagnosis of contagious bovine pleuropneumonia by PCR-laser induced fluorescence and PCR-restriction endonuclease analysis based on the 16S rRNA genes of *Mycoplasma mycoides* subsp. *mycoides* SC. Journal of Clinical Microbiology 37: 3815-3821.

II. Westberg J., Persson, A., Pettersson, B., Uhlén, M., and Johansson, K.-E. 2002. ISMmy*1*, a novel insertion sequence of *Mycoplasma mycoides* subsp. *mycoides* small colony type. FEMS Microbiology Letters 208: 209-215.

III. Persson A., Jacobsson, K., Frykberg, L., Johansson, K.-E., and Poumarat, F. Variable surface protein Vmm of *Mycoplasma mycoides* subsp. *mycoides* SC. Submitted.

IV. Westberg, J., Persson, A., Holmberg, A., Johansson, K.-E., Pettersson, B., and Uhlén, M. Genome sequencing of *Mycoplasma mycoides* subsp. *mycoides* SC, the causative agent of contagious bovine pleuropneumonia (CBPP). Manuscript.

Abbreviations

A	adenine
aa	amino acid
AFLP	amplified fragment length polymorphism
AP	arbitrarily primed
bp	base pair
C	cytosine
CBPP	contagious bovine pleuropneumonia
CFT	complement fixation test
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
G	guanine
GP	glycerophosphate
IS	insertion sequence
LIF	laser induced fluorescence
M. capri	*Mycoplasma mycoides* subsp. *capri*
M. capricolum	*Mycoplasma capricolum* subsp. *capricolum*
M. capripneumoniae	*Mycoplasma capricolum* subsp. *capripneumoniae*
M. mycoides LC	*Mycoplasma mycoides* subsp. *mycoides* large colony type
M. mycoides SC	*Mycoplasma mycoides* subsp. *mycoides* small colony type
mAb	monoclonal antibody
Mycoplasma bg7	*Mycoplasma* sp. bovine group 7
OIE	Office International des Epizooties
ORF	open reading frame
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
PTS	phosphotransferase system
REA	restriction endonuclease analysis
RNA	ribonucleic acid
rRNA	ribosomal RNA
T	thymine
tRNA	transfer RNA
U	uracil
Vmm	variable protein of *Mycoplasma mycoides*

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Background

Mycoplasma mycoides subsp. *mycoides* SC *(M. mycoides* SC) is the causative agent of a severe respiratory disease of cattle, the contagious bovine pleuropneumonia (CBPP). CBPP is a vast problem for many African countries and the losses are severe, not only from an animal health perspective and economical point of view, but also for the public health. A study in Botswana showed that malnutrition in children tripled during an eradication program for CBPP, and that 54% of the increase in malnutrition was directly correlated to the slaughter of the affected cattle (Boonstra et al., 2001).

Also southern Europe has recently experienced a number of outbreaks of CBPP. The epizootics prompted the authorities and animal health organisations to alert the surveillance and to give high priority to research that could lead to improved diagnostics and vaccines, and facilitate epidemiological investigations. Joint collaborations between different laboratories in Europe were initiated and as a part of this process, the work presented in this thesis commenced.

Introduction

Contagious bovine pleuropneumonia

History

Contagious bovine pleuropneumonia is not a new disease and there are clinical descriptions of CBPP that date back to 1550 (Provost et al., 1987). According to an historical overwiew by Hutyra and his collegues (Hutyra et al., 1938), the disease was carefully described in the 18th century by Bourgelat (1769) and Haller (1773). At this time CBPP was regarded either as a typhoid affection or as paralysis of the lung, and it was assumed that the disease arose spontaneously. Chabert proclaimed in 1794 the contagious character of the disease, which was accepted by the majority of observers and confirmed by commissions in France, Germany and other countries that were appointed to study the disease, some 50 years later. Meanwhile, Willems demonstrated that cattle could be infected with the pulmonary exudates from diseased animals (1850-1852). Subsequently, the infectious agent was isolated by Nocard and Roux in 1898, and immunisation with pure cultures could be performed (Nocard and Roux, 1898). Dujardin-Beaumetz, a co-worker of Nocard and Roux, wrote his thesis on "Bacteriological study of a microorganism at the limit of visability; the microbe of pleuropneumonia and its culture" in which he introduced the use of filters to separate this slow growing microbe from the bigger, fast growing contaminants

(Bové et al., 1994). He was also the first to cultivate the pleuropneumonia microbe on solid medium and he described the characteristic morphology of mycoplasma colonies with an opaque nipple in the center. Interestingly, he cites a German scientist Löffler, who stated in 1898 that the foot and mouth agent could be filtered but not cultivated while the pleuropneumonia microbe could. Despite this clear and fundamental distinction between viruses and bacteria, mycoplasmas can still be mistaken as virus-like organisms.

Clinical signs and pathology

CBPP affects cattle and buffalo but the causative agent has occasionally been isolated from sheep and goats (Kusiluka et al., 2001a, Brandao, 1995). The incidence for disease in these ruminants is not known, neither their potential of being a transmitting reservoir. CBPP is the only bacterial disease in the A-list of communicable animal diseases (Anonymous, 2000a), which means it is globally classified as one of the 15 most serious animal diseases.

The rates of morbidity and mortality from CBPP are very variable. In Africa, mortality rates are typically 10-70% (Egwu et al., 1996), while the epizootics in Europe after 1980, was characterised by low morbidity and the mortality was nearly nil although the pathological lesions were identical to those seen in Africa (Nicholas et al., 2000, ter Laak, 1992). Most cases in Portugal, Spain and Italy were chronic, which is associated with an endemic situation. The differences in morbidity and mortality depend on many factors such as the virulence of the *M. mycoides* SC strains, the infective dose, the immune status and breed of cattle, and physical stress (ter Laak, 1992, Nicholas and Palmer, 1994, Provost et al., 1987). Antibiotics and anti-inflammatory drugs suppress the clinical symptoms and promote the formation of chronic lesions. This is true also for the vaccinated animals that failed to obtain full immunity upon vaccination.

Cattle of all ages can be affected by CBPP. The severity of symptoms range from hyperacute, through acute, subacute and chronic forms of pleuropneumonia, while calves up to six months normally develop arthritis and show lameness from swollen, hot, painful limb joints. The incubation period varies from five to more than 200 days, although mostly CBPP develops within 20 to 40 days.

The clinical picture of acute CBPP at an early stage follows the general signs of disease with dullness, anorexia, irregular rumination, fall in milk yield, a temperature of up to 40°C and only slight signs of respiratory problems can be noticed unless the animals are forced to exercise. As the disease progresses and lung lesions develop, the symptoms become more pronounced. Painful, irregular and rapid respiration, moaning during expiration, cough, hyperthermia up to 42°C and cessation of rumination. The stance is typically an extended head and neck, protruding tongue and forelegs spread apart. Much mucous is present around the

nostrils and foam around the mouth. Percussion of the chest wall reveals pleuritic pain and areas of dullness due to massive pleural effusion. The typical acute case has a course of ten days to three weeks.

Hyperacute CBPP affects up to 10% of the infected animals. It is characterised by an accelerated progress of the disease, sometimes without defined clinical symptoms. Death is sudden and occurs within one to seven days, often caused by asphyxia from the pleural exudate or from pericarditis.

Subacute CBPP occurs frequently and the syndrome is normally transiently converted to a chronic form of CBPP. The symptoms resemble those of the acute form but are less severe and the fever is intermittent.

Chronic form evolves from both acute and subacute CBPP, however it can also be developed directly. The so-called lungers have a healthy appearance although sequestered lung lesions are present and these animals do transmit the infectious agent. Diagnosis is often possible after slaughter only.

The pathological lesions of CBPP are essentially located in the thoracic cavity and lungs. Pathogonomic signs are acute inflammation of the pleura accompanied by large amounts of sero-fibrinous fluid (up to 30 litres) that leaves omelette like deposits. The pleura are thickened and the fibrin deposits cause adhesion between the lung and the chest wall. Usually, lesions are found in only one lung and its pleura.

The lungs are oedemic and have a marbled appearance due to thickened interlobular septa, infiltration of a serous fluid from dilated lymphatics, and lung lobules with different stages of hepatisation that gives different colours (Fig. 1, top). Encapsulated necrotic tissue, sequestra, are found in late stage and chronic cases. Normally, the sequestra are localized to the regions of adhesion, and the size can vary from one to 30 cm (Fig. 1, bottom). A sequestrum may remain infected for years and infectious material can be drained into a bronchus which results in discharge of the causal agent. Bronchial lymph nodes will be enlarged in acute cases.

Other lesions may be exudative pericarditis, exudative peritonitis, synovitis and renal infarctions. The isolation of *M. mycoides* SC from lymph and lymph nodes (Scanziani et al., 1997), serum and plasma (Gourlay, 1964), kidneys (Grieco et al., 2001), semen (Stradaioli et al., 1999), and urine (Masiga et al., 1972), indicates that *M. mycoides* SC forms more or less systemic infections.

All clinical and pathological descriptions except Fig. 1, derived from (ter Laak, 1992, Scudamore, 1995, Provost et al., 1987, Anonymous, 1997).

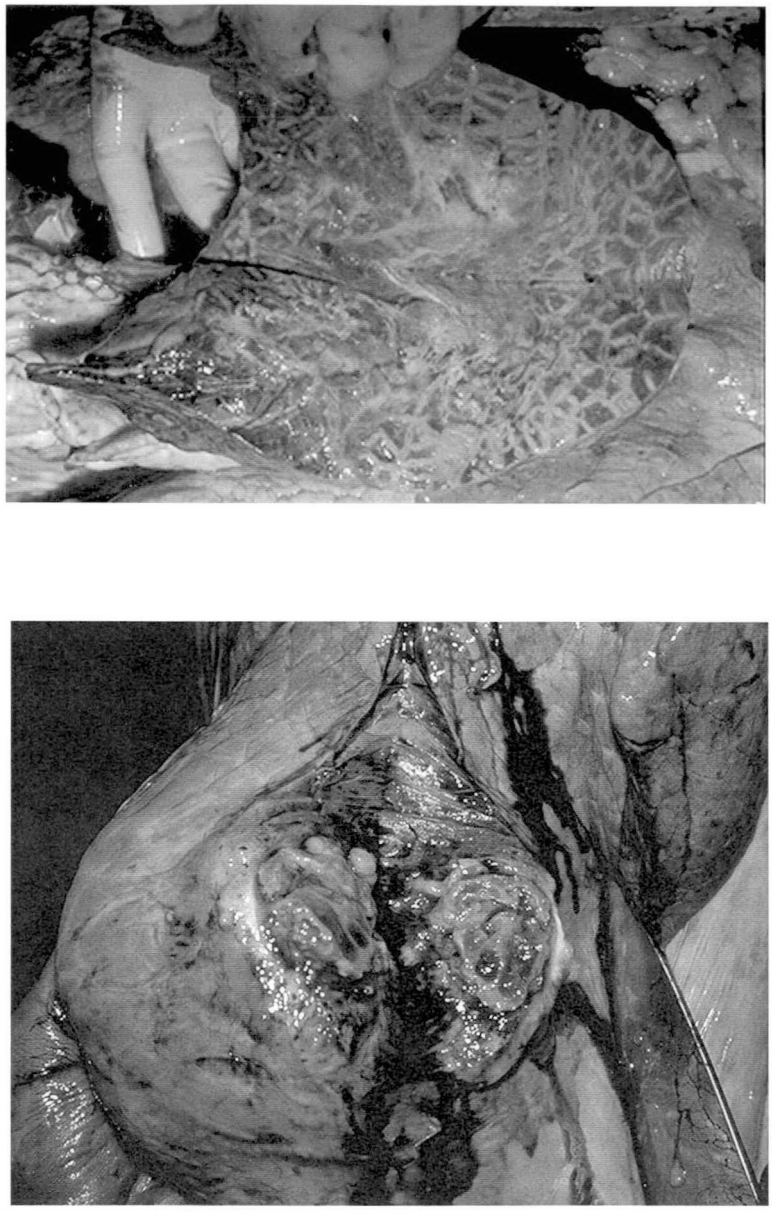


Figure 1. Typical lung lesions of CBPP. Marbled lung with thickened interlobular septa and different stages of hepatisation (top). Sequestered infection in the lung with necrotic tissue (bottom). The photos were kindly provided by John Bashiruddin.

Occurrence

CBPP has long been endemic in western Africa and the sub-Saharan areas but it was clinically suppressed by routine vaccinations during the rinderpest control programs that started in the 1960s. Since the late 1980s there has been a dramatic increase and spread into the central, eastern and southern countries of Africa and CBPP has become the major infectious disease in livestock. Today huge regions of the continent are endemic and CBPP is considered an emerging disease of Africa due to the rapid changes in incidence and the notably higher morbidity of the new outbreaks (Rweyemamu et al., 2000). During year 2000, the world organisation for animal health (OIE) received reports of outbreaks from 18 countries, while other organisations state that at least 27 African countries reported CBPP the same year (Fig. 1) (Anonymous, 2000b, OIE, 2002a, Rweyemamu et al., 2000). The official reports are not necessarily in accordance with the actual situation as some regions have poor surveillance facilities. Nonetheless, it is clear that the situation of CBPP in Africa is devastating.

Figure 2. Occurrence of CBPP in Africa. Areas in dark grey represent countries that have reported outbreaks of CBPP in year 2000 or 2001 according to the World organisation for animal health, OIE. Regions in light grey represent areas with unclear CBPP status.

In Europe, outbreaks of CBPP have occurred in France, Italy, Portugal and Spain in the last two decades (OIE, 2002a). Eradication programs were alerted and the concerned herds were stamped out by slaughtering. Portugal suffered from an endemic situation in the north western provinces until 1999, but no cases of CBPP have been documented since.

Surveillance of CBPP in Middle East and Asia is partly lacking and it is therefore difficult to summarise where CBPP exists and to what extent. Bangladesh, Pakistan and Qatar reported outbreaks in 1997. Also Burma, Kuwait and the United Arab Emirates have documented CBPP during the 1990's and China in 1985 (OIE, 2002a).

America and Australia successfully eradicated CBPP a long time ago. The last known cases were 1892 in the United States and 1967 in Australia (OIE, 2002a).

Control

Several circumstances make the control of CBPP an extremely difficult task. The clinical symptoms can be vague and difficult to diagnose and the incubation period may be as long as 200 days. Thus the disease can become established and spread before it is detected and tracing the source of the infectious agent becomes difficult. It needs to be mentioned in this context that a nomadic lifestyle is common in many countries where CBPP is endemic. Severe droughts and civil unrest are conditions that promote uncontrolled cattle movements and make CBPP control even more complicated. (Masiga et al., 1996).

Diagnostic systems based on serology are not sufficiently sensitive (Bellini et al., 1998). Cross-reactions with closely related species are frequent with several methods and it is a major problem to diagnose chronic carriers of the CBPP agent. Molecular techniques that provide sensitivity and specificity have recently been developed, (se below) but the sampling procedure per se is critical and there is still an urgent need for simple field tests at low cost.

Antibiotic therapy such as tylosin and the tetracyclins can efficiently give an apparent cure, but it also causes an increased number of chronic carries that continue to transmit the disease. Hence, antibiotic is not a means to eradicate the disease, it is rather a problem for diagnosis and control.

Although it seems that CBPP was recently eradicated from Europe by stamping out all infected herds, history has shown that the disease has re-emerged every decade during the 20th century (Nicholas et al., 2000). Furthermore, no country in Africa in which CBPP is endemic, can afford to eradicate the disease by mass slaughter (Windsor, 2000).

This leaves one option only, extensive vaccination (Windsor, 2000). The problem is that there are no reliable vaccines available today. Recent, well managed vaccination campaigns have failed due to poor efficacy of the vaccines (Thiaucourt et al., 2000, Thiaucourt et al., 1998, Wesonga and Thiaucourt, 2000, Masiga et al., 1996). Vaccinated animals without immunity do generally have less

pronounced symptoms and lesions which again disables diagnosis and control. Some of the problems with the current vaccines are due to the fact that live vaccines are used, normally of strain T1 or its streptomycin resistant derivative T1Sr as recommended by OIE (Anonymous, 2000a). The titre and viability of the vaccine will affect its efficacy and these parameters will in turn depend on the composition of the growth media and the time and temperature for storage and transport (lyophilised vials need to be reconstituted before injection) (Waite and March, 2001, Miles, 1983, Garba, 1980, Windsor, 1978). The incidence for severe post-vaccinal reactions is sometimes high (Lorenzon et al., 2000). It takes a long time for the vaccinated animals to aquire immunity, normally one to three months for primo-vaccination (Thiaucourt et al., 1998) and the duration of immunity is short. Annual re-vaccinations are therefore required for successful vaccination. It was estimated that a vaccination campaign must persist for a minimum of 10 years to be successful, preferably extended to 15 years (Windsor, 2000).

Naturally, there is also an economical problem in eradicating CBPP. The cost to control CBPP in central and southern Africa has been analysed and described by Windsor and Wood (Windsor and Wood, 1998). Basically, the analysis involved governmental costs of surveillance, diagnostics, vaccines, and compensation for slaughter, as well as income losses from reduced trade and restricted livestock movements. It was furthermore described in the article how the local people are affected. Briefly, the local costs include loss of livelihood and the costs associated with quarantine restrictions and international movement control.

The CBPP agent, *Mycoplasma mycoides* subsp. *mycoides* SC

Taxonomy and phylogeny

More than 100 years ago, the French scientists Nocard and Roux managed to isolate the infectious agent of CBPP. Their observations of "Le microbe de la péripneumonie" was published two years after the first experiments, in 1898 (Nocard and Roux, 1898). It would thereafter take a long time before the organism was phylogenetically classified. Any related species that were discovered, were simply referred to as "pleuropneumonialike organisms". Then, in 1956, Edward and Freundt suggested the classification system that is still in use, if though somewhat extended (Edward and Freundt, 1956). *M. mycoides* SC belongs to the genus *Mycoplasma*, family *Mycoplasmataceae*, order *Mycoplasmatales*, and more recently class *Mollicutes* (Edward et al., 1967). The trivial name mycoplasma often refers to all of the species within the class *Mollicutes*, including all of the eight genera *Acholeplasma, Anaeroplasma, Asteroleplasma, Entomoplasma Mesoplasma, Mycoplasma, Spiroplasma,* and

Ureaplasma, and the phytoplasmas whose taxonomic status is unclear because they cannot be cultivated (Johansson and Pettersson, 2002).

Mycoplasmas are known as the smallest self-replicating organisms. These prokaryotes are phylogenetically close to gram-positive bacteria. In contrast to most other eubacteria, they lack a rigid cell wall, which is also reflected in the nomenclature where *Mollicutes* originates from the Latin *mollis* meaning soft and *cutis*, skin (Razin, 1992). Also the name mycoplasma is based on the morphological characteristics and it is derived from the Greek *mykes* for fungus and *plasma* for formed or moulded and it refers to the mycelioid appearance of the pleuropneumonia agent (*M. mycoides* SC) in culture. The species name *mycoides* is a description of the mucoid sheath (Provost et al., 1987) and SC means small colony, as opposed to the close relative *Mycoplasma mycoides* subsp. *mycoides* LC (*M. mycoides* LC) that forms large colonies.

The mycoplasmas have been classified into 5 phylogenetic groups (Fig 3) (Weisburg et al., 1989). *M. mycoides* SC is one of six closely related species that group into the classical *Mycoplasma mycoides* cluster of the spiroplasma group (Pettersson et al., 1996, Cottew et al., 1987). The *M. mycoides* cluster comprises *Mycoplasma capricolum* subsp. *capricolum (M. capricolum)*, *Mycoplasma capricolum* subsp. *capripneumoniae (M. capripneumoniae)*, *Mycoplasma mycoides* subsp. *capri (M. capri)*, *M. mycoides* LC, *M. mycoides* SC, and *Mycoplasma* sp. bovine group 7 (*Mycoplasma* bg7). All the members of the classical *M. mycoides* cluster are highly pathogenic and they cause severe diseases in ruminants (Table 1). Serological cross-reactions between these species are often observed and it is a major problem for diagnostics. The species *Mycoplasma cottewii*, *Mycoplasma yeatsii*, and *Mycoplasma putrefaciens* are sometimes included in the phylogenetic *M. mycoides* cluster that is based on analysis of the 16S rRNA genes (Heldtander et al., 1998).

Evolution

The mycoplasma ancestor is believed to have evolved from the *Clostridia-Bacilli*-branch of the phylum *Firmicutes* about 600 million years ago. Its progeny divided into two major branches that independently underwent degenerate evolution, which meant substantial reduction of the genetic material from 2000 kb to 580-1700 kb (Maniloff, 1996). As a consequence, the mycoplasmas have limited metabolic capacity (Pollack et al., 1997) and they depend on external supply of metabolites from their animal or plant host, such as nucleic acid precursors and amino acids.

Table 1. Diseases caused by mycoplasmas of the classical *M. mycoides* cluster (top 6 strains) and the phylogenetic cluster (all). The table was adapted from (Bölske, 1995, Nicholas and Bashiruddin, 1995)

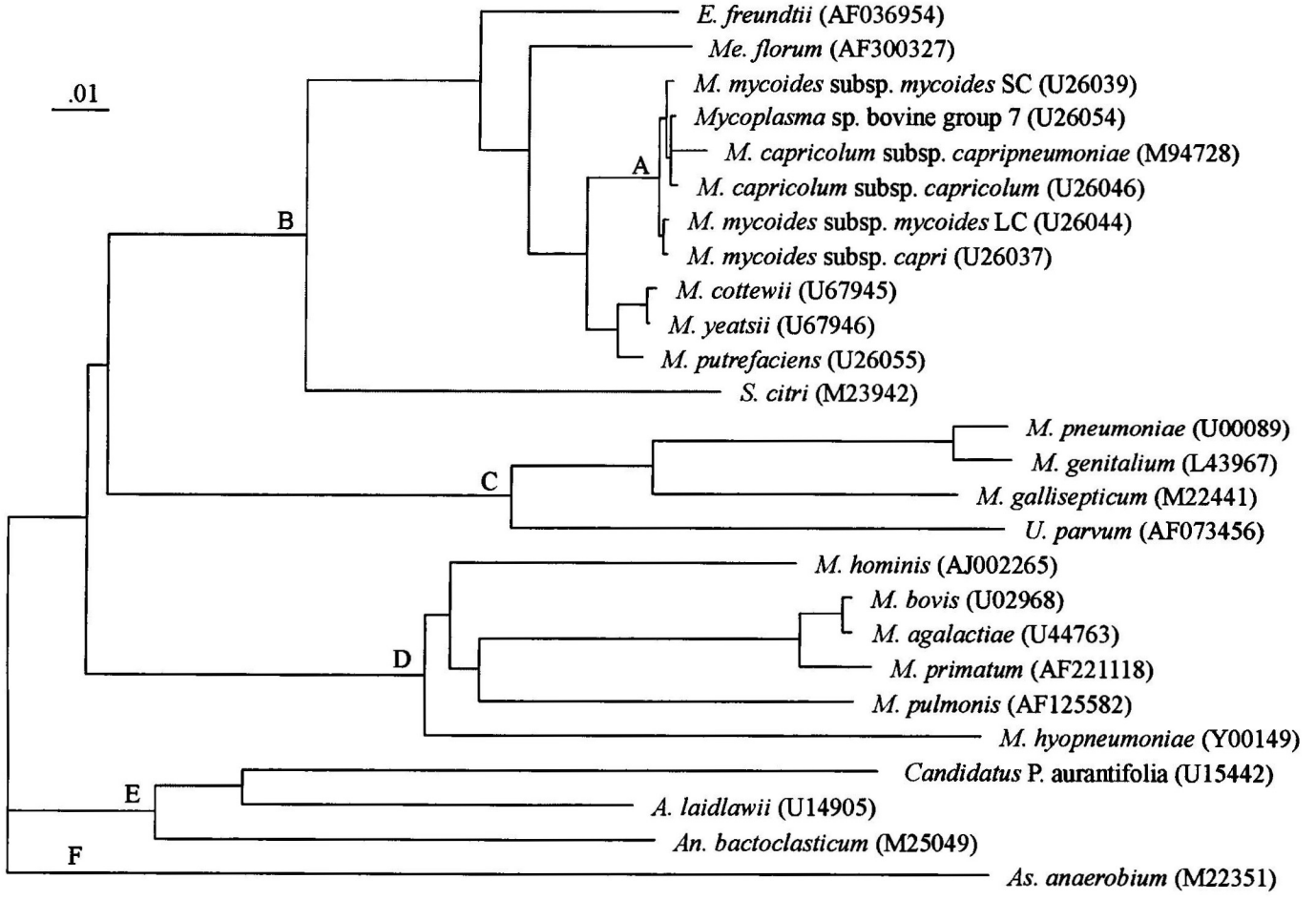
Species	**Reference strain**	**Main hosts (other hosts)**	**Disease**
M. capricolum subsp. *capricolum*	California kid^T	Goats, sheep, (cattle)	Arthritis, mastitis, pneumonia
M. capricolum subsp. *capripneumoniae*	F38^T	Goats, (sheep)	Pleuropneumonia (CCPP)
M. mycoides subsp. *capri*	PG3^T	Goats	Arthritis, Pleuropneumonia
M. mycoides subsp. *mycoides* LC	Y-goat^R	Goats, sheep, (cattle)	arthritis, peritonitis, pleuro-pneumonia, septicaemia
M. mycoides subsp. *mycoides* SC	PG1^T	Cattle, (buffalo, goats, sheep)	Pleuropneumonia (CBPP)
M. sp. bovine group 7	PG50^R	Cattle, (Goats, sheep)	Arthritis, calf pneumonia, mastitis
M. cottewii	VIS^T	Goats	-
M. putrefaciens	KS-1^T	Goats	Arthritis, mastitis
M. yeatsii	GIH^T	Goats	-

^{T} Type strain
^{R} Reference strain

Biochemical properties

All *M. mycoides* SC strains are able to metabolise glucose, N-acetylglucosamine (GlcNAc), oxobutyrate, fructose, pyruvate, and lactate (Abu-Groun et al., 1994). Glucosamine and mannose were only metabolised at high substrate concentrations. The rate of lactate metabolism was also clearly depending on the substrate concentration, which may be explained by the specificity and availability of transport proteins in the cell membrane. Glycerol was efficiently metabolised by all strains apart from the European ones that were deficient in glycerol oxidation (Houshaymi et al., 1997). *M. mycoides* SC cannot metabolise the disaccharides maltose and trehalose.

Figure 3 (right). Phylogenetic tree based on distance matrix analysis of 1434 positions in the 16S rRNA genes. The letters A-F indicate; A, the classical *Mycoplasma mycoides* cluster; B, the spiroplasma group; C, the pneumoniae group; D, the hominis group; E, the anaeroleplasma group; and F, the asteroleplasma group. The genus abbreviations are *E.-Entomoplasma, Me.-Mesoplasma, M.-Mycoplasma, U.-Ureaplasma, P.-Phytoplasma, A.-Acholeplasma, An.-Anaeroleplasma* and *As. Asteroleplasma* (outgroup). The scale bar indicates 1 nucleotide substitution per 100 positions.


.01
E. freundtii (AF036954)
Me. florum (AF300327)
M. mycoides subsp. mycoides SC (U26039)
Mycoplasma sp. bovine group 7 (U26054)
M. capricolum subsp. capripneumoniae (M94728)
M. capricolum subsp. capricolum (U26046)
M. mycoides subsp. mycoides LC (U26044)
M. mycoides subsp. capri (U26037)
M. cottewii (U67945)
M. yeatsii (U67946)
M. putrefaciens (U26055)
S. citri (M23942)
M. pneumoniae (U00089)
M. genitalium (L43967)
M. gallisepticum (M22441)
U. parvum (AF073456)
M. hominis (AJ002265)
M. bovis (U02968)
M. agalactiae (U44763)
M. primatum (AF221118)
M. pulmonis (AF125582)
M. hyopneumoniae (Y00149)
Candidatus P. aurantifolia (U15442)
A. laidlawii (U14905)
An. bactoclasticum (M25049)
As. anaerobium (M22351)
A
B
C
D
E
F

Most biochemical investigations have been performed on "*M. mycoides*", which refers to the subspecies *mycoides* but not to the serotype, or on *M. mycoides* LC. One cannot be sure that the transport systems and metabolic pathways will be identical to those of *M. mycoides* SC, but similarities can be expected and some of the results are therefore reported in this section.

Pathways for de novo synthesis of nucleotides were not found in *M. mycoides*, but interconversion of nuceotides do occur. Uracil and thymine are required for pyrimidine synthesis, suggesting that that methylation of uracil is missing while the nucleotide cytidine triphosphate is formed without supply of cytosine (Mitchell and Finch, 1977). Guanine is the only source needed for purine nucleotide synthesis (Mitchell et al., 1978).

The amino acid requirements of *M. mycoides* are complex but generally all amino acids except cysteine, aspartic acid and glutamic acid, need to be provided by the host or in the growth medium (Rodwell, 2000). Also the vitamins and co-factors thiamine, riboflavin, nicotinic acid, and coenzyme A are required. α-lipoic acid is needed for pyruvate oxidation.

In addition, *M. mycoides* was found to require sterols and fatty acids (Rodwell, 2000). The fatty acids cannot be altered by the mycoplasma and the membrane lipid composition was therefore reflecting the composition of fatty acids in the medium. Sterol is a major constituent of mycoplasma membranes and is required by most mollicutes.

Molecular epidemiology

The discovery of the insertion sequences IS*1296* (Frey et al., 1995) and IS*1634* (Vilei et al., 1999) was a breakthrough for the possibilities to distinguish genomic differences between *M. mycoides* SC strains and to perform molecular epidemiology. Insertion sequences are small mobile DNA elements that are genetically compact (Mahillon and Chandler, 1998). They generally do not code for any other factors than those required for their own transposition. These factors are one or two open reading frames that encode a transposase, and terminal inverted repeats that serve as recognition and cleavage site for the transposase. The target sequence of the integration site is normally duplicated on insertion, leaving direct repeats in the immediate flanking regions of the insertion sequence. As the insertion sequences are often found in several copies, they are known to cause genomic rearrangements in bacteria, such as insertions, deletions and inversions. For this reason, the IS-elements are suitable markers for diagnostics and epidemiological investigations. Fingerprints with IS*1296* and IS*1634* were obtained by Southern blotting of *Hin*dIII digested chromosomal DNA and hybridisation with an IS-specific probe.

IS*1296* was found in three species of the *M. mycoides* cluster namely, *M. mycoides* SC (18-19 copies), *M. mycoides* LC (5 copies), and *Mycoplasma* bg7 (2 copies). Analysis of 63 strains of *M. mycoides* SC resulted in 10 different IS*1296* patters which were divided into two main clusters, one comprising European strains only and the other cluster comprised the type strain PG1, all African strains, and the Australian strains (Cheng et al., 1995). The result supported the conclusion of Poumarat and colleagues (Poumarat and Solsona, 1995), who observed striking differences in restriction patterns from European and African strains and suggested that the reappearance of CBPP in Europe during 1980 originated from an existing, latent, source of infection rather than being imported from Africa. Fingerprinting with IS*1296* also indicated an epidemiological correlation between African and Australian strains as opposed to the suggestion that Australia received the disease from England in 1858 (Provost et al., 1987, ter Laak, 1992). All European isolates except one from Italy, were identical in the IS*1296* pattern regardless of host species and geographical origin. Strains of the African cluster were more heterogeneous and belonged to 8 different groups, where the vaccine strains and the type strain formed individual patterns.

IS*1634* is unique for *M. mycoides* SC and it was reported to be present in approximately 30 copies (Vilei et al., 1999). Fingerprinting with this IS element gave apparently fewer patterns than IS*1296*. Nonetheless, when digested chromosomal fragments were separated with high resolution, there was a region between 2.0 and 3.5 kb where strain variation occurred. The distribution of IS*1634* in the DNA fragments of this region, led to the conclusion that recent outbreaks in Botswana and Tanzania had different origins. The Botswanan isolates resembled north western strains from Senegal and the Ivory Coast, while the Tanzanian isolates grouped with strains from central and eastern Africa (Chad, Sudan, Kenya, Rwanda, and Tanzania) (March et al., 2000). It was furthermore suggested that strains with the west coast pattern may have derived from the ancestral European epizootics while the central and east coast strains were either always present in Africa, or were imported from Asia in the 19th century (March, 2000). The author also suggests that Australia may have been infected from England after all, but the recent epizootic in Europe originates from an infectious strain that has undergone a major chromosomal deletion since 1858. This chromosomal deletion of 8.8 kb was discovered by examining the DNA fragment that distinguished the IS*1296* patterns of European *M. mycoides* SC strains from African strains (Vilei et al., 2000).

Promising results have also been reported for molecular epidemiology based on the techniques pulse field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) (Kusiluka et al., 2001b, Kusiluka et al., 2001a). Analysis of a large number of *M. mycoides* SC isolates from Tanzania and a few reference strains from other regions showed that all Tanzanian isolates had identical PFGE patterns whereas the references generated other patterns. Analysis by AFLP gave similar results. Only one isolate from goat and one from cattle

deviated from the main Tanzanian AFLP pattern although they were indistinguishable from each other. The results led to the conclusion that nearly all CBPP outbreaks in Tanzania since 1990 are due to the spread of one epizootic clone, which is different from the single Kenyan isolate that was tested. Interestingly, there were no differences between isolates from cattle and goats, which indicated cross-transmission between cattle and goats. Surprisingly, AFLP could not separate the vaccine strain T1Sr from the type strain PG1 as found with other methods for molecular epidemiology.

Studies of protein patterns by immunoblotting have also provided useful epidemiological information (Poumarat and Solsona, 1995, Goncalves et al., 1998). Immunoprofiles of most Italian strains significantly differed from those of France, Portugal and Spain in both investigations, which would suggest that the Italian outbreaks had another origin than the others. Again, there was a clear distinction between the African/Australian strains and the European ones. The investigation by Gonçalves et al. also identified five immunodominant membrane proteins that were present in all *M. mycoides* SC strains, regardless of the sera used.

Molecular diagnostics

Serological cross-reactions is a well known problem for CBPP diagnostics. Availability of monoclonal antibodies (Rurangirwa et al., 2000, Le Goff and Thiaucourt, 1998, Brocchi et al., 1993) have improved the serogical methods but not yet fulfilled the requirements of a satisfactory diagnostic system. Complement fixation (CFT), which currently is the recommended diagnostic test by OIE, has proved to be very specific but it clearly lacks sensitivity (Nicholas et al., 2000). A recently developed competitive enzyme-linked immunosorbent assay (cELISA) (Le Goff and Thiaucourt, 1998) offers practical advantages and enables standardisation, but the sensitivity is lower than that of CFT in detecting acute infections,but has a better potential to detect chronic infections than CFT (Amanfu et al., 2000). Thus, there has been and still is an urge to develop alternative methods. As a result, numerous tests such as latex agglutination (Ayling et al., 1999), biochemical tests (Rice et al., 2000) and PCR assays (se below) were published in the last ten years.

The early molecular methods for diagnostics were based on DNA hybridisations. Taylor and his collegues (1992b, 1992a) produced the CAP-21 probe from an uncharacterised chromosomal fragment of *M. capri,* which hybridised to all members of the classical *M. mycoides* cluster. Southern blotting of *Taq*1 digested genomic DNA, distinguished *M. mycoides* SC from the other species. The CAP-21 gene region was sequenced and shortly a PCR method was designed to facilitate the diagnosis (Bashiruddin et al., 1994). It consisted of two sets of primers, one that amplified all members of the classical *M. mycoides* cluster, and

the other that was specific for the three *M. mycoides* subspecies, from which *M. mycoides* SC could be uniquely separated after digestion of the amplicon with *Asn*I. To reduce the manual work of analysing the PCR products, the method was further refined to allow colorimetric detection of the amplicons (Bashiruddin et al., 1999) that allowed automatic screening in an ELISA reader. However, this method can give false positive results for primer dimer and unspecific products, and it still required separate analysis of the amplicon after *Asn*I or *Vsp*I digestion to distinguish *M. mycoides* SC from *M. mycoides* LC.

Another scheme for PCR in the CAP-21 region was introduced by Hotzel et al. (1996). Group specific primers were used in a first reaction to amplify all species of the classical *M. mycoides* cluster, and species specific amplification was performed in a second, nested reaction. Alternatively, *M. mycoides* SC could be identified after digestion of the first amplicon with *Apo*I. Nested PCR gives high sensitivity and specificity and it is particularly useful for samples containing PCR inhibitors, but it also involves a larger risk for contamination.

A direct PCR that was specific for *M. mycoides* SC was developed by Dedieu et al. (1994). Again, the target region was a genomic fragment of unknown function but in this system it was cloned from the type strain PG1 of *M. mycoides* SC. The specificity of the system could be verified by restriction of the PCR product, or by dot blot hybridisation of the amplicon to a digoxygenin labelled probe. Dot blotting significantly increased the sensitivity of the method, although this method is somewhat more labour intensive.

In contrast to the above mentioned methods, Miserez and collegues (1997) presented a nested PCR system that targetted the gene of a well characterised immunodominant lipoprotein P72. Both primer pairs were specific for *M. mycoides* SC and showed high sensitivity on purified DNA samples. However, nested PCR increased the sensitivity more than 10 000-fold on clinical specimens of bronchial lavage.

An arbiltrarily primed (AP) PCR developed by Rawadi et al. (1995) was used to identify each species of the classical *M. mycoides* cluster. The fingerprints of *M. mycoides* SC strains, *M. capripneumoniae* strains, and *Mycoplasma* bg7 strains were well conserved in all tested strains, while strain variation was observed for *M. capricolum*, *M. mycoides* LC, and *M. capri*. The diagnostic potential is not yet clear as there were high requirements of DNA quality and quantity, but the method can be an interesting alternative for epidemiology among the species that generated various AP-patterns.

A direct PCR that discriminated T1, T1/44, and T1Sr vaccine strains from field strains and other vaccine strains was presented by Lorenzon et al (2000). The test was developed to help investigations on CBPP outbreaks among previously

vaccinated cattle. Targets for the PCR primers were the flanking regions of an IS*1296* element that was uniquely present in the T1 strains.

Pathogenicity

Very little is known about the factors that affect the pathogenicity of *M. mycoides* SC. No secreted toxins have been identified, neither receptor molecules on the bacterial surface that mediate binding to host epithelium or induce other cellular responses in the host tissues. The data listed below are factors that are associated with the pathogenesis, but the precise modes of action are still unclear.

A thick galactan layer often referred to as the capsule, surrounds the cell membrane of *M. mycoides* SC. The capsule is mainly composed of the carbohydrate galactose (90%) and to a lesser extent glucose (2-4 %) and lipid (Plackett et al., 1963, Rodwell, 2000). Its role in pathogenesis has been well reviewed in (Nicholas et al., 2000, Nicholas and Bashiruddin, 1995, Egwu et al., 1996) and can be summarised as four functions. Galactan seems to promote binding to the host tissue surfaces. High capsular content is associated with resistance to phagocytosis. Structural similariteis of the capsular galactan to the bovine pneumogalactan produced in the epithelial cells, leads to the formation of autoreactive antibodies and consequently autoimmune responses. Finally, toxic effects from *M. mycoides* SC and its capsule have been observed, and these toxic substances may be accumulated in the capsule or be one of its components.

Oxidative damage of host cells due to secretion of hydrogen peroxide is known for several mycoplasmas (Tryon and Baseman, 1992). Hydrogen peroxide may be produced by metabolising carbohydrates or organic acids. However, *M. mycoides* SC was shown to accumulate hydrogen peroxide after oxidation of glycerol rather than glucose, that only produced trace amounts (Miles et al., 1991). Glycerol oxidation is a two step reaction that involves two enzymes, L-alpha-glycerophosphate (GP) kinase and GP oxidase. European strains of *M. mycoides* SC lack GP oxidase activity and can therefore not oxidise glycerol, while other strains can (Houshaymi et al., 1997). Thus, the production and secretion of hydrogen peroxide may be one factor that explains the differences in pathogenicity between African and European strains.

Lipoproteins and other surface proteins of unknown function, can be considered as putative virulence factors as they may be involved in adhesion and other host cell interactions. One of these is the lipoprotein LppB (Vilei et al., 2000). The *lppB* gene is located in a genomic fragment that was missing in European strains of *M. mycoides* SC and is present in African and Australian strains. The gene region also contained four other open reading frames and one insertion sequence. It was suggested that LppB could be involved in virulence or pathogenesis and account for the differences observed in natural outbreaks of CBPP in Africa

compared to Europe and in experimental infections (Abdo et al., 1998). Several members of the classical *M. mycoides* cluster were found to express LppB.

Two other lipoproteins of unknown function have also been characterised. LppQ is a predominant antigen of 48 kDa whose C-terminal is composed of nine repetitive hydrophobic domains that were predicted to be integral membrane regions. Epitopes of the C-terminal were also present in proteins from other species of the *M. mycoides* cluster. The N-terminal was unique for *M. mycoides* SC. P72 is an immunogenic lipoprotein present in all tested *M. mycoides* SC strains. A related protein in *Mycoplasma* bg7 was also detected with anti-P72 polyclonal serum and the DNA sequence of the P72 gene was similar to a gene in *M capricolum*, which led to the conclusion that P72 is a member of a protein family of the *M. mycoides* cluster where it later was termed LppA (Monnerat et al., 1999).

Variable surface proteins

In 1990 it was reported for the first time that mycoplasmas express surface proteins that can undergo reversible changes to alter the antigenic repertoire in a cell population (Rosengarten and Wise, 1990). These proteins were termed variable surface proteins and it was assumed that a diverse surface architechture is a means to enhance colonisation and to adapt to the host tissue environment at various stages of infection. Variable surface proteins may also assist the mycoplasma population to escape the immune defence of the host, which leads to persistence of infection. Today it is generally believed that antigenic variation is crucial for survival of these wall-less microorganisms in the hostile environment of their hosts. It has been proven that variable proteins are involved in adhesion, hemadsorption, membrane transport, and immunomodulation. (Washburn et al., 1993, Sachse et al., 2000, Le Grand et al., 1996, Mühlradt et al., 1997, Mühlradt et al., 1998, Theiss and Wise, 1997, Markham et al., 1992, Olson et al., 1991, Citti et al., 1997, Neyrolles et al., 1999, Baseman et al., 1982, Noormohammadi et al., 1997).

Phase varaition

The simplest form of surface variation is a reversible on/off switch in protein expression, called phase variation. A wide range of mutations have been described to cause phase variation, for example promoter mutations, changes in putative transcription activators, mutations that lead to gene truncation, frameshift mutations, and DNA inversions or gene conversion that fuse the coding gene to an active promoter (Noormohammadi et al., 2000, Washburn et al., 1998, Lysnyansky et al., 2001b, Theiss and Wise, 1997, Boguslavsky et al., 2000, Glew et al., 1998, Zhang and Wise, 1997, Yogev et al., 1991, Bhugra et al., 1995).

Antigenic variation

Some surface proteins undergo antigenic variation by altering the size and/or epitope composition. These proteins normally contain repetitive units of different structure. At the genetic level, the repetitive sequences function like interchangeable cassettes that can be deleted, inserted or recombined to form a rich variety of protein variants. The perhaps most striking examples are the *vsp* genes of *M. bovis* but there are several others (Zheng et al., 1995, Zhang and Wise, 1996, Yogev et al., 1995, Yogev et al., 1991, Washburn et al., 1998, Lysnyansky et al., 1996, Lysnyansky et al., 1999, Lysnyansky et al., 2001a, Boguslavsky et al., 2000, Boesen et al., 1998, Citti et al., 2000). Variable proteins are often subjected to both antigenic variation and phase variation.

Epitope masking

Eptiope masking is a phenomenon where a surface protein that is constitutively expressed have epitopes that are subject to variable surface exposure, either due to a secondary protein that sterically blocks the accessability of its surface epitopes, or as a consequence of size variation. Several mycoplasma proteins that at first were believed to be phase variable have later been shown to be subjected to epitope masking. In one case, the masking protein per se underwent phase variation (Theiss et al., 1996, Rosengarten and Yogev, 1996, Zhang and Wise, 2001).

Other mechanisms

Differential expression of protein variants from the same gene has also been observed. The molecular mechanism for differential display were not found despite thorough analyses of the gene region and post-transcriptional or post-translational mechanisms were suggested (Calcutt et al., 1999).

Multigene families

Many variable proteins are members of multigene families (Lysnyansky et al., 1999, Citti et al., 2000, Markham et al., 1994, Markham et al., 1999, Flitman-Tene et al., 2000, Glew et al., 2000, Röske et al., 2001, Shen et al., 2000, Noormohammadi et al., 1998). The proteins of a multigene family are often differentially expressed and chromosomal rearrangements occur to allow transcription of one gene instead of the other. Some families are species specific and others are shared with other mycoplasma species. Multigene families can involve as many as 70 genes (Baseggio et al., 1996).

Variation of the glucose specific phosphotransferase system

We have recently discovered an immunodominant, species specific, variable surface protein in *M. mycoides* SC, earlier called protein 3F3 (Persson et al., 2001). The variability was first detected by colony immunostainings with the monoclonal antibody (mAb) 3F3. Positive, negative and sectored colonies of different staining intensity were found in cultures of unicellular origin. The presence of positive and negative clones is a sign of phase variation whereas lighter and darker positive sectors normally indicate size variation or changes in protein conformation. Phase variation protein 3F3 was proved in three strains (Afadé, B17 and T1/44) by Western blotting of clonal lineages.

By performing affinity pannings of a phage display library to mAb 3F3, a region of the gene that encoded the target epitope for mAb 3F3 was identified (as described in III). The remainder of the gene sequence could be extracted from the genome sequence database (IV), although this sequence contained a few polymorphisms. It was later apparent that the gene occurred in two copies in the genome of the type strain PG1 and that the genes differed by two amino acids only. Obviously, a large chromosomal fragment has been duplicated at some stage of the evolution (Persson et al., unpublished).

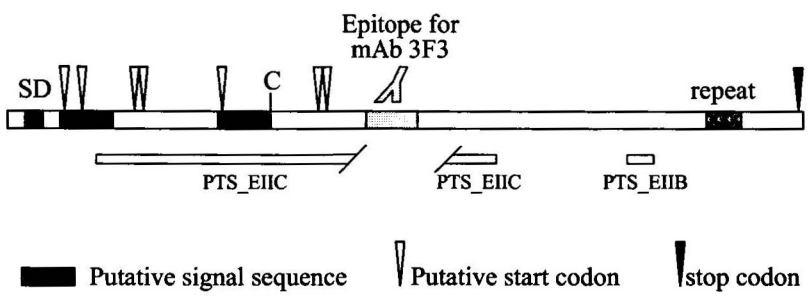


Figure 4. Schematic illustration of the *ptsA* gene showing the location of the shine dalgarno sequence (SD), putative signal sequences, cystein residue for hypothetical lipid attachment (C), and the alternative initiation codons. The binding epitope for mAb 3F3 is located between two parts of the conserved EIIC domain, and the EIIB domain is located 3' of the of the EIIC domain. Two short repetitive motifs are found in the 3'-end of the gene.

The putative genes contained 7 alternative initiation codons, the first preceeded by a putative promoter and ribosome binding site (Fig. 4). Two of the open reading frames were predicted to contain a signal sequence, a finding that suggested expression of two size variants. The longer ORF would encode a native

protein of 676 aa with 10 predicted transmembrane regions including the signal sequence. The shorter ORF encodes a putative prolipoprotein of 532 aa with seven membrane spanning regions.

Similarity searches by BLASTp (Altschul et al., 1997) has shown that the longer ORF contains the EIIB and EIIC domains of the glucose/maltose family of phosphotransferase systems (PTS) (Persson et al., unpublished). In this thesis I will refer to the genes as *ptsA* and the corresponding proteins as PTS-A. The names are preliminary because the components of all PTS systems in *M. mycoides* SC are not fully analysed and annotated yet. The substrate specificity of the PTS-A needs to be experimentally established before it can be concluded that it is glucose specific, and if it has potential to transport any other sugar molecules as well. By structural predictions, PTS-A forms the transport channel across the cell membrane. The EIIC domain catalyses the transfer of a phosphoryl group from EIIB to the sugar substrate.

Southern blotting of digested genomic DNA from 48 *M. mycoides* SC strains and hybridisations with a *ptsA* specific probe were performed to investigate the number of gene copies (Persson et al., unpublished). Most African isolates have two copies of the *ptsA* genes or at least the probing region, while all European and two African isolates had only one copy of *ptsA*. At a closer examination by colony immunostaining with mAb 3F3, strains possessing one *ptsA* gene do not seem to undergo phase variation of the PTS-A protein as weakly and strongly positive colonies are found but no negatives. On the contrary, at least three of the strains with two *ptsA* copies are truly subjected to phase variation as shown earlier.

Most likely the correlation is not due to the number of *ptsA* genes but rather a connection to the capacity of metabolising glycerol. Again, the highly pathogenic African strains can metabolise glycerol while less virulent Europeans strains lack this ability. Glycerol uptake and metabolism may be induced as a result of phase variation to an OFF-state of PTS-A expression. As a consequence, there is an increased H_2O_2 production, which induces tissue damage in the host. Thereby it is possible that phase variation of PTS-A is a mechanism that indirectly affects the pathogenicity of *M. mycoides* SC by oxidative damage, and directly contributes to immune evasion. The glucose specific PTS's of *E. coli* are involved in feedback regulation of substrate import and export, via the PTS itself but also by regulating non-PTS uptake systems, for example the glycerol transporters (Postma et al., 1996).

There seem to be complex mechanisms for variation of the glucose/maltose specific phosphotransferase system. The mechanisms differ between strains, and appear to involve phase variation and likely also antigenic variation. Whether the shorter ORF is expressed or not remains to be determined and also what function it may have. It is also important to find out if variation of PTS-A occurs *in vivo*. It

has been discussed that phase variation of one subunit in a putative ABC transporter in *M. fermentans* may be a way to preserve essential metabolic functions while altering the substrate specificity and contributing to immune evasion (Theiss and Wise, 1997). The multi-protein PTSs are involved in regulation of a variety of metabolic and transcriptional processes and variability of two PTS components may therefore have a wide range of effects.

Genomics

A new era of science was initiated with the large scale DNA sequencing of whole genomes, the era of genomics. *Haemophilus influenze* was the first genome to be completely sequenced, tightly followed by *Mycoplasma genitalium* refs (Fleischmann et al., 1995, Fraser et al., 1995). Both sequences were published in 1995. To date, there are 63 published microbial genomes and another 169 microbial genomes are in progress (http://www.tigr.org/tdb: Accessed 04-03-2002). Out of these, there are 18 species of *Archae*, 32 from *Eucarya* and 182 species of *Bacteria*. Only the projects that have been reported to the Institute for Genomic Research, TIGR, are included in these 232 genome sequencing projects and the real figure may therefore be significantly higher. The impressive collection of sequence data has enabled comparative genomics between various species and strains. Comparative genomics can efficiently reveal information of anything from metabolic pathways to SOS response and pathogenicity factors. It will also increase our knowledge on the evolution of microorganisms.

Among the mycoplasma genomes, full genome sequences are available for *M. genitalium, M. pneumoniae, M. pulmonis,* and *Ureaplasma parvum*. The genomes of *Mycoplasma gallisepticum, Mycoplasma hyopneumoniae, M. mycoides* SC (see IV), *Mycoplasma penetrans, Spiroplasma citri* and *Spiroplasma kunkelii* are also being sequenced but the data has not been released yet. The genome of *M. capricolum* was partly sequenced earlier (Bork et al., 1995). A new project to complete the sequence of *M. capricolum* has been initiated by another research team (K. S. Wise and M. J. Calcutt, personal communication).

M. genitalium is a primate parasite found in ciliated epithelial cells of the genital and respiratory tracts. Its genome of 580,070 bp is the smallest known genome of any free living organism and it codes for 470 proteins or RNA species (Fleischmann et al., 1995). The G+C content was 32% and the coding density 88%. A shotgun sequencing strategy was used and 9,846 sequences were assembled to cover the entire genome.

Mycoplasma pneumoniae is a human pathogen that causes tracheobronchitis and primary atypical pneumonia. The genome, which is 816,394 bp, has a G+C content of 40% and it was sequenced by primer walking (Himmelreich et al., 1996). The procedure required 6385 sequences. Totally 716 genes were identified

constituting 89% of the genome. A large portion of the genome (8%) comprised repetitive DNA elements called RepMP, some of which are associated with the adhesion protein P1.

Mycoplasma pulmonis is the etiological agent of respiratory mycoplasmosis in rats and mice. Hitherto, it is the largest mycoplasma genome that has been sequenced. 13 496 reactions were performed, mainly by shotgun sequencing, to complete the sequence of the 963 879 bp chromosome (Chambaud et al., 2001). The G+C content was 27%. There were 782 coding sequences in the genome and the coding density was 91%. Interestingly, some of the genes that were supposedly essential for the minimal self-replicating cell were missing in *M. pulmonis*.

Ureaplasma parvum was recently renamed and it was earlier known as a biovar parvum of *Ureaplasma urealyticum* (Robertson et al., 2002). This common commensal in humans can cause urogenital infections and it is associated with premature spontaneous delivery and septicaemia, meningitis and pneumonia in newborn infants. The chromosome of *U. parvum* is 751 719 bp and it was sequenced with a complete random and ordered shotgun strategy referred to as CROSS (Glass et al., 2000). 11 291 sequences were assembled in the full genome sequence. The coding density was 93% and the G+C content 26%. Ureaplasmas are unusual in that they generate most of their ATP by hydrolysis of urea. Among the 652 predicted genes in *U. parvum*, several components that are involved in urea metabolism and substrate transport, were identified. The order of genes in *M. genitalium and M. pneumoniae* was highly conserved, but this is not the case in *U. parvum* nor in *M. pulmonis*.

Mycoplasma hyopneumoniae is a porcine pathogen known to cause respiratory disease. To date, there are two small gaps to fill between the contigs in the genome sequence of *M. hyorhinis* (F. C. Minion, personal communications). The size of the chromosome is approximately 900 kb. Annotation of the putative coding sequences has begun and 766 ORFs have been identified. A shotgun sequencing strategy was used.

Mycoplasma gallisepticum is of major concern in poultry industry in that it causes severe respiratory disease in chicken and turkeys. It has come to my attention that the DNA sequencing of its genome is nearly finished and that annotation of the coding sequences is currently going on, but the information has not been confirmed.

I do not know the current status of the genome sequencing projects of *M. penetrans, S. citri*, or *S. kunkelii* (Chambaud et al., 2001). The genome of *S. citri* was earlier reported to be nearly finished (Laigret et al., 2000). A web site of *S. kunkelii* stated that 280 kb of the genome was completed in June 2000 (http: //www.oardc.ohio-state.edu/spiroplasma/genome.htm: Accessed 08-03-2002).

The genome sequencing projects generate a lot of raw data. Although there are many technichal problems associated with the production of genomic libraries, the sequencing and assembly, the main work is to put the raw data into its context, i.e. to define the function of all gene products and their roles in cellular processes. New technologies for large scale analyses such as proteomic facilities and microarray scans will be of great use but functional genomics by mutational analysis and knock-out experiments will still be necessary. Mycoplasmas can be transformed after treatment with polyethylene glycol or by electroporation, however, there have been difficulties to obtain efficient cloning vectors for these organisms and for most mycoplasmas conventional genetic tools are missing (Razin et al., 1998). Mutational analysis is by no means a straight forward and simple procedure to perform and there is a long way before we have a comprehensive understanding of mycoplasmas and mycoplasma pathogenicity.

Aims

The aim of this work was to characterise genetic and structural features of *M. mycoides* SC and to create molecular tools that would facilitate in depth studies this organism. Thereby, I hope to contribute to improved methods for diagnostics and epidemiological investigations, to increased knowledge of the virulence and pathogenicity of this infectious agent, and to aid the development of new and improved vaccines. It was also my purpose to analyse putative virulence factors of *M. mycoides* SC and although the use of these findings is not yet clear, it is an example of fundamental research in the struggle towards the understanding and defeat of this highly pathogenic bacterium. Moreover, by providing the full genome sequence to the public, we wish to stimulate all conceivable research on *M. mycoides* SC, such as studies of the metabolic processes in bacteria with minimal genomes and studies on the bacterial evolution.

Comments on materials and methods

Diagnostic methods (I)

Two different diagnostic methods were designed and evaluated in the study. The first system was based on PCR and laser induced fluorescence (LIF) and a DNA sequencer was used to analyse the PCR products. As no processing of the PCR products was needed, the method was intended for large scale screening of clinical specimens. Other advantages were the accuracy of the analyses and the high sensitivity. Today, the method can be further automated with the use of capillary electrophoresis units, where no manual work is needed for casting and

loading of gels. Internal standards can be added before PCR is performed in 96 well plates (or a larger scale microtiter format) and the plate can be directly transferred to the sequencing unit where the samples are analysed without additional manual work. Although the PCR-LIF system is a convenient method, CBPP is primarily a problem of the developing countries and we also wanted to provide an alternative system that would be robust and easy to perform without expensive equipment. The resulting system was based on PCR and restriction endonuclease analysis (REA) and the products were analysed by conventional agarose gel electrophoresis. Nested PCR could be performed to increase the sensitivity when needed.

Both PCR systems amplified a fragment in the 16S rRNA genes. There were mainly two reasons for choosing these target genes, stability and primer design. The 16S rRNA genes are present in all prokaryotes and the function has remained throughout the evolution. Due to the complex tertiary structure of the 16S rRNA molecules, with stem and loop structures that are essential for the function, the genes have evolved to contain conserved, semi conserved and variable regions. The different regions provide suitable primer targets for group and species specific amplification.

Genome sequencing and insertion sequences (II, IV)

The genome of *M. mycoides* SC was sequenced with a shotgun strategy. All sequencing methods and the softwares used for basecalling, editing, assembly and annotation are described in (IV). Sequence gaps between contigs were filled by primer walking on a chromosomal DNA template.

During the project we have had major problems in the assembly, caused by insertion sequences. Most insertion sequences have now been individually cloned and sequenced. One type of insertion sequence, ISMmy*1*, had not been described before and it was therefore analysed further (II). Occurence of ISMmy*1* in different *M. mycoides* SC strains and representative strains of some other mycoplasma species, was investigated by Southern blotting.

Surface variation (III)

In this investigation, a variable surface protein, Vmm, was characterised. Right before this investigation was initiated, several monoclonals were produced in an Italian-French collaboration (Brocchi et al., 1993) which enabled studies of unique proteins of *M. mycoides* SC. We used one of the monoclonals in this work, and started by performing a thorough investigation of the specificity of mAb 5G1. Western blotting and colony immunostaining to the reference strains of the

classical *M. mycoides* cluster were performed. Altogether, 173 reference strains and isolates of mycoplasmas from ruminants were used in dot immunobinding tests. The tests showed clearly that the target epitope for mAb 5G1 was only present in the *M. mycoides* SC strains and that further characterisation of this protein could reveal information of this highly pathogenic bacterial species.

A phage display library was used to identify the gene that encodes Vmm. Phage display has since the introduction in 1985 (Smith, 1985) proven to be a useful tool in characterisation of proteins and protein interactions. It can briefly be described as a technique where a DNA fragment is fused to the gene for a coat protein of a filamentous phage. The phage particles that are produced at assembly will display the fusion peptide on the surface, and their genomes will contain the partial or complete gene of interest (Fig. 5). By this procedure it is possible to get a direct connection between the selection for a phenotype and isolation of the genotype. The number of publications that concern phage display is today enormous and there is a wide range of applications and vectors in for example antibody libraries, cDNA libraries, oligopeptide libraries, and shotgun libraries of whole genomes (e.g. Crameri and Suter, 1993, Cwirla et al., 1990, Hoogenboom et al., 1998, Burton, 1995, Wilson and Finlay, 1998, Smith and Petrenko, 1997). The method used in this investigation was developed by Jacobsson et al. (1995, 1997, 1998). It involves cloning of randomly fragmented genomic DNA into a gene VIII based phagemid vector (Fig. 6). The protein and gene of interest can be extracted by affinity pannings of the phagemid library to an immobilised ligand. In this study mAb 5G1 was immobilised in microtiter wells and three subsequent pannings were perfomed to enrich the phage that expressed the 5G1 epitope on the surface.

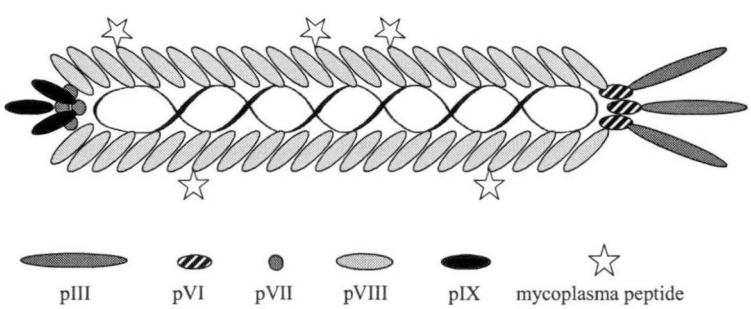


Figure 5. Schematic drawing of a filamentous phage displaying a mycoplasma peptide in fusion to its major coat protein pVIII. The fusion protein is vector encoded while all other proteins derive from the helper phage. A mixture of phage will be produced at assembly, some containing the phagemid vector and others having the helper phage chromosome.

Before constructing a shotgun phage display library of mycoplasma DNA, the unusual codon usage of genus *Mycoplasma* prompted us to select an *E. coli* strain

for propagating the phage, which would suppress the Opal stop codon UGA to tryptophan. Other requirements of the host strain was a pili for the attachment of the virions and it should render a high electrocompetence for successful transformation. Once this was accomplished, a *M. mycoides* SC strain was selected, chromosomal DNA was extracted and used to produce the library. Strain M223/90 (Bölske et al., 1995) was chosen because it is an African low passage strain that was isolated from a naturally infected cow in a region that suffered from a rapidly expanding epidemic of CBPP. The aim was to obtain a library that could reveal the aggressive pathogenicity factors of *M. mycoides* SC.

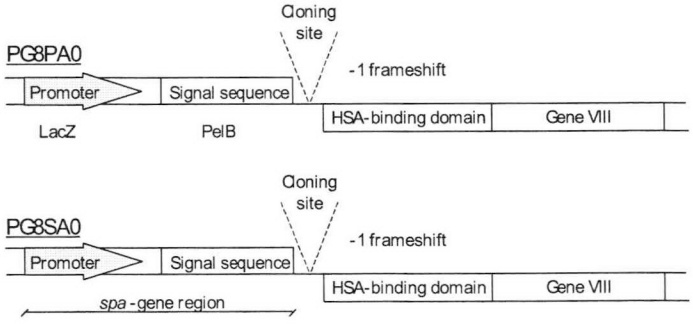


Figure 6. The cloning site of the phagemid vectors PG8PA0 and PG8SA0. The vectors also contain the *Escherichia coli* origin of replication, the phage intergenic region and an ampicillin resistance gene.

The phagemid library contained 10^7 independent clones and 95% of these contained DNA inserts of mycoplasma origin. As sonicated DNA was ligated into the vector, there is statistically only 1 of 18 phage particles that will express mycoplasma peptides since the DNA fragment must be inserted in the correct orientation and restore an open reading frame. This leaves approximately half a million variants of mycoplasma peptides. Most certainly, many of the inserts do not contain DNA of coding regions or do contain stop codons and thus they will not express any fusion protein at all, or not display the true mycoplasma peptides. If we assume that half of the inserts contain DNA of open reading frames, there will be about 250 000 different functional mycoplasma peptides expressed on the phage surface. The genome of *M. mycoides* SC contains 1,067 coding sequences with a coding density of 87%, (IV). In conclusion, I think it is reasonable to believe that the phagemid library will display epitopes from most proteins of *M. mycoides* SC.

Results and discussion

PCR based diagnosis of contagious bovine pleuropneumonia (I)

In the beginning of this study, the entire 16S rRNA genes from 25 *M. mycoides* SC strains that had been isolated in different decades and geographical regions, were sequenced. This served two purposes. With the intention of developing diagnostic PCR systems, it was necessary to find target regions for the primers that were stable, i.e. regions that were identical in all the strains. We also hoped to be able to reflect the evolution of *M. mycoides* SC by analysing polymorphisms between the *rrnA* and *rrnB* operons, as had been done for *M. capripneumoniae* (Pettersson et al., 1998, Heldtander et al., 2001). All strains were identical in the entire 16S rRNA genes with two exceptions, the type strain PG1 and a field sample from Tanzania, both of which had one unique mutation in one of the operons. Obviously, the 16S rRNA genes did not provide enough resolution from a phylogenetic perspective, however the genes indeed provided reliable target regions for diagnostic primers since they were extremely conserved between the strains.

Both PCR systems in (I) were designed to give group specific amplification of the members of the classical *M. mycoides* cluster. In the PCR-LIF system, the species identification was based on a sequence length difference of two basepairs between the 16S rRNA gene of the *rrnA* operon and that of the *rrnB* operon. This sequence length difference is unique for *M. mycoides* SC. Separation of the amplicons in the DNA sequencer resulted in two distinct peaks and the sizes of the fragments were accurately determined. Amplicons of all other species of the *M. mycoides* cluster were equal sized and appeared as a single peak in the electropherogram of the sequencer. The method was highly sensitive and relatively quick to perform.

M. mycoides SC was was also found to have another unique polymorphism in the 16S rRNA genes that coincided with an *Alu*I restriction site. This was used in the PCR-REA system to distinguish the CBPP agent from the other members of the *M. mycoides* cluster. Briefly, when amplicons are digested with *Alu*I and separated by agarose gel electrophoresis, an additional band is observed for the *M. mycoides* SC strains, due to the absence of one of the cleavage site in the 16S *rrnA* gene. The sensitivity of the PCR-REA system was enhanced by performing nested PCR with primers that were complementary to the conserved regions U1 and U8 in the first reaction. PCR-REA was easy to perform and the analyses did not require any sophisticated equipment.

The two PCR systems proved useful for analysing all samples in the current investigation. The PCR-REA has also been successfully used by March et al. (March et al., 2000). However, both methods need to be thoroughly tested on a

wide range of sample materials and the results should be statistically evaluated before any of the methods can be fully established as a diagnostic system. Therefore it might have been more correct to refer to the methods as simply PCR systems and not as diagnostic systems in the article (I). It should also be noted that PCR is presently not accepted as the sole method for diagnosing CBPP.

Many PCR systems that are used for diagnostic purposes contain an engineered control molecule, so called mimic. A mimic molecule is normally a plasmid that contains a region which is amplified with the diagnostic primers although the amplicon is bigger than the expected amplicon of an authentic sample. A carefully titrated amount of mimic is co-amplified in the same tube as the clinical specimen and its only purpose is to verify that no false negative results occur due to inhibitory substances in the reaction. In analysing samples for CBPP, the test material is often nasal swabs, pleural fluid, lung tissue, lymph or lymph nodes, perputial washings or possibly blood samples. None of these materials contain an excessive amount of inhibitory substances for PCR when properly handled (compared to e.g. faeces), and the extra work of including a mimic was therefore not prioritised.

Insertion sequence ISMmy*1* (II)

A novel insertion sequence, ISMmy*1*, was discovered in the process of sequencing the genome of *M. mycoides* SC. All copies of ISMmy*1* in the type strain PG1 of *M. mycoides* SC were sequenced and analysed. ISMmy*1* was found to have all characteristic features of an IS element. Inverted repeats that serve as recognition and clevage sites for IS-encoded transposase were identified at the termini of the element. Most of the sequence of ISMmy*1* was part of an open reading frame that encodes a putative transposase. This transposase was found to contain a DDE motif that is commonly found in transposases, integrases and resolvases (Mahillon and Chandler, 1998). ISMmy*1* was grouped with the IS*4* family of insertion sequences based on protein similarity searches on the transposase and the fact that the DDE motif was similar to the consensus DDE of the IS*4*-type elements. Analysis of the integration sites showed that duplication of the target sequence had occurred where ISMmy*1* was inserted, forming direct repeats that flanked the inverted repeats of ISMmy. There was a predominance of AT dinucleotide iterations in the direct repeats.

The number of ISMmy*1* in various *M. mycoides* SC strains and its occurrence in different mycoplasma species was investigated by Southern blotting. Most *M. mycoides* SC strains had identical ISMmy*1* patterns and possesed seven copies of ISMmy*1*, regardless of the geographical origin or which host species it was isolated from. Unfortunately, the ISMmy*1* could therefore not be used as an epidemiological tool. However, some important variations from the standard pattern were observed. The type strain PG1 deviated in the position of one band,

probably due to an extra restriction site. This could easily be confirmed by performing the analysis with another restriction enzyme but this has not yet been done. The vaccine strain T1Sr lacked one band and the position of another band was changed. This finding is interesting for two reasons, firstly the vaccine strain has low pathogenicity, and secondly, it provides a possibility to distinguish vaccinated animals from naturally infected ones. Also strain Afadé had a unique ISMmy*1* pattern in that it lacked one band on the blot, although it was not the same band that was lacking for T1Sr. Interestingly, Afadé was proved highly pathogenic by experimental infections (Belli et al., 1989, Abdo et al., 1998). Whether significant genomic differences that can explain the difference in pathogenicity can be found in the vicinity of these IS-elements, needs further investigation. The vaccine strain KH3J, that was shown to have unique band patterns in REA (Poumarat and Solsona, 1995) and by IS*1296*-typing (Cheng et al., 1995), did not deviate from the main cluster by ISMmy*1*-typing.

ISMmy*1* or ISMmy*1*-like insertions sequences could not be found in the genomes of any of the mycoplasma species that are closely related to *M. mycoides* SC. Most surprisingly, the more distantly related *M. bovis* contained numerous copies of ISMmy*1*. As both mycoplasmas can form persistent infections in bovines, the findings suggest that horizontal gene transfer has occurred between *M. bovis* and *M. mycoides* SC as a result of coexistence in the same host.

Only five of the 16 nucleotide differences that were found between the consensus transposase genes of ISMmy*1* in *M. bovis* and *M. mycoides* SC, lead to amino acid substitutions. None of the substitutions were found in the DDE motif. For unknown reasons, we failed to sequence the 5' end of the ISMmy*1* transposase genes of *M. bovis* and the last 76 bp of the 3' end, so we cannot fully compare the genes of the two species. The transposase gene in *M. mycoides* SC had two alternative start codons, the longer ORF encodes 470 aa and the shorter ORF 361 aa. The sequence from *M. bovis* covers 280 aa. Five amino acid substitutions of 280 aa corresponds to 98.2% identity. Moreover, the amino acid substitutions did only lead to a changed charge in one position where an acidic aa (E) was substituted for a basic aa (K) in *M. bovis*. In short, there is no doubt that there is significant similarity between the ISMmy*1* elements in these species.

Differentiation between the *M. agalactiae* strains and *M. bovis* strains is a well known problem to anyone who works with these mycoplasmas. In fact, some strains seem to be an intermediate between these two species (Heldtander Königsson et al., 2002). Neither *M. agalactiae* nor *M. primatum* that are phyogenetically close to *M. bovis* (Fig. 3), were found to have ISMmy*1* elements in their genomes. Naturally, the study needs to be extended to include more than the type strains to make such a conclusion, but I find the result very interesting. A PCR directed to ISMmy*1* may offer a quick and simple solution to the strain classification problem.

Variable protein Vmm of *Mycoplasma mycoides* (III)

In this study, we report the discovery of surface antigen variation in *M. mycoides* SC. The variable epitope was proven to be specific for *M. mycoides* SC and as a putative virulence or pathogenicity factor, we found it interesting to perform a detailed analysis of the protein, the encoding gene and the regulation of expression.

Intraclonal variability of Vmm was observed in colony immunostainings with mAb 5G1, as positive, negative and sectored colonies appeared in the same blot. When a single colony was cultured in broth and replated on agar, there was again a mixture of positive and negative colonies. The existence of sectored colonies was an evidence that mutations had occurred during colony growth. Epitope masking effects and size variation could be ruled out in subsequent experiments and it was shown that Vmm underwent reversible phase variation at high frequency.

Vmm is a small protein with an apparent size of 16 kDa in Western blots. Metabolic labelling of lipoproteins with ^{14}C palmitic acid and Triton X-114 fractionation showed that Vmm is a membrane bound lipoprotein. It is present on the bacterial cell surface as confirmed by electron microscopy. Only a few gold labelled Vmm molecules were detected on each cell surface in the electron micrographs, most likely because Vmm is expressed at low levels. We had earlier observed in Western blotting experiments that Vmm was hardly an abundant antigen, nor immunodominant so it was not a surprising finding. However, there is also a possibility that the capsular polysaccharide layer limited the accessability of the surface protein thereby hindering the antibodies from binding. Thus, it could be a methodological problem of culturing the mycoplasmas and preparing sample grids for electron microscopy, that resulted in poor labelling rather than a low number of molecules being present on the surface.

Affinity pannings of the phagemid library to mAb 5G1 efficiently selected phage that displayed the binding epitope for mAb 5G1. Chromosomal DNA of captured phage was isolated, the mycoplasma inserts were sequenced and the sequences were aligned. The consensus sequence was used to search the genome database, and the entire *vmm* gene and its flanking regions was identified. One of the phagemid recombinants happened to contain the whole *vmm* gene, except for the stop codon. The results were verified by immunoblotting of total phage proteins, in which the fusion proteins of the different recombinants were recognised by mAb 5G1.

Analysis of the *vmm* gene revealed an ORF of 177 bp preceeded by a σ^{70}-type promoter and a ribosome binding site (Shine Dalgarno sequence), but a transcription terminator could not be identified. We successfully performed amplification of cDNA in the 5'-end of the *vmm* transcript to define the

transcription start, however the 3'-end of the transcript has not yet been analysed. By comparing the *vmm* gene regions in ON and OFF clones from several *M. mycoides* SC strains, it was found that phase variation was caused by dinucleotide insertions or deletions in a repetitive region of the promoter spacer. Active promoters have a 17 bp spacer and most likely, the insertions or deletions cause conformational changes that destabilises the open RNA polymerase-promoter complex, thereby preventing transcription initiation.

Prediction analyses of the Vmm structure as deduced from the amino acid sequence, were performed. A signal sequence and a signal peptidase II site were located and cleavage was predicted between amino acid residue 23 and 24. The mature lipoprotein was predicted to be attached to the membrane by the lipid chain(s) only. Vmm is very small, containing only 36 aa after processing. It has 10 charged amino acid residues and it was therefore predicted to be hydrophilic. Unlike many other variable surface proteins, there were no dominating repetitive elements in the structure.

Although it was shown that the 5G1 epitope of the Vmm protein is exclusively expressed by *M. mycoides* SC strains, Southern blotting experiments with a *vmm* specific probe showed that the closely related mycoplasmas of the *M. capricolum* species group in the *M. mycoides* cluster have one or more *vmm*-like genes in their genomes. More information on the occurence of Vmm-like proteins in various species is needed and thorough analysis of the *vmm*-like genes should be performed. It is interesting to note that all of the concerned mycoplasma species are known to cause severe animal diseases of ruminants such as arthritis, pleuropneumonia and mastitis. Vmm is presumably one factor that contributes to immunological cross-reactions within the *M. capricolum* species group and it would be very valuable to find out what function Vmm has and if there is a correlation between Vmm and pathogenicity.

The genome of *Mycoplasma mycoides* subsp. *mycoides* SC (IV)

Annotation of the *M. mycoides* SC genome has only just begun and the presented data of the genome sequencing project is by no means complete. Generally, in judging the significance of a similarity search by BLAST, there is a risk to give excessive annotation or under-annotation. Excessive annotation would for example be to assign a substrate specificity of an ABC transporter because there are significant hits to other proteins annotated in this fashion, while the data are only enough to conclude it is an ABC transporter by its typical motifs. Under-annotiation would be the opposite, to give the annotation transporter, when it is clear it must be a sodium/proton antiporter. At this stage, we have deliberately chosen to under-annotate the *M. mycoides* SC genome. All ORFs with indecisive annotations will be reanalysed with appropriate search engines or softwares.

M. mycoides SC is a facultative anaerobe and it has the ability to ferment glucose and other sugars to lactate and acetate via the glycolysis. Coding sequences for all enzymes that are required for glycolysis and gluconeogenesis were identified in the genome. Cytochrome pigments and the components of the tricarboxylic acid cycle are missing like for other mollicutes (Pollack et al., 1997), which means that the respiratory chain is not available for generation of ATP. Therefore the main ATP production is likely to occur by substrate phosphorylation from phosopho-glycerate and pyruvate kinase activities. Most other housekeeping genes have also been identified. Some of these are the DNA and RNA polymerases, 51 ribosomal proteins, the rRNA genes of both operons, 30 tRNA genes, and all tRNA ligases except glutamine tRNA ligase.

Virulence genes of pathogenic bacteria are often located in particular regions of the chromosome termed pathogenicity islands (Hacker et al., 1997). The pathogenicity islands comprise large DNA regions between 30 and 200 kb, they normally carry more than one virulence factor and the G+C content is typically different from the rest of the genome. Pathogenicity islands are often unstable and associated with tRNA genes or insertions sequences at the boundaries and direct repeats in the flanking regions. We have not identifed any pathogenicity islands in *M. mycoides* SC that met all these criteria, however, the genes annotated "hypothetical protein", "conserved hypothetical protein", and "putative lipoprotein" have a tendency to cluster together. The *vmm* gene for example, is surrounded by 10 ORFs of hypothetical proteins or lipoproproteins. Only four of 26 genes in the same region, which is located between two IS*1634* elements, have known function.

The most striking feature of the *M. mycoides* SC genome is the abundance of insertion sequences. Knowing that there are 86 insertions sequences of only three types, it feels obvious that transposition and chromosomal rearrangements occur frequently. However, the IS-typing with IS*1296*, IS*1634*, and ISMmy*1* specific probes in a wide range of strains have indicated the chromosomal structure is relatively stable. It was also discovered that *M. mycoi*des SC seems to possess another two types of IS elements or prophage which need to be further analysed and categorised. Generally, the mysteries of the IS elements remains to be solved; how can a bacterium with such a minimal genome afford to carry all the IS elements? How do they affect the evolution of this pathogen?

Future perspectives

The findings in studies II-IV and the current project on PTS-A open up for many interesting investigations.

Vmm is a very small protein and it would be important for the understanding of its function to determine if it is associated to, or interacts with, another protein of *M. mycoides* SC and if the phase variation thereby regulates a number of processes. We hope to express and purify the fusion protein that was found in one of the phagemid clones and use it as a ligand in new affinity pannings with the phagemid library. Any associated proteins that may be found will be analysed. It is also our intention to investigate whether Vmm is a member of a gene family as commonly found for variable proteins in many other mycoplasmas, and if the protein is co-expressed with other proteins in a poly cistronic operon. Comparative studies of Vmm and Vmm-like proteins of other mycoplasmas may also give interesting results.

Further studies of the variable phosphotransferase system PTS-A will be performed. Attempts to characterise the variability of PTS-A in different strains and the possible connection to glycerol metabolism, will continue. There are also other interesting aspects of PTS-A. It is for example an immunodominant protein with epitopes that are unique for *M. mycoides* SC and it is the target molecule for a widely used diagnostic ELISA.

Molecules that mediate binding to the host epithelium are key factors for virulence. By affinity pannings to lung tissues or bovine epithelial cell lines, it may be possible to reveal proteins that are involved in adhesion.

Insertion sequence typing with the ISMmy*1* specific probe identified several differences between the pathogenic strain Afadé and the vaccine strain T1Sr. In the hunt for pathogenicity factors of *M. mycoides* SC, it may be worth looking into the genes that are located on the restriction fragments that contributed to unique ISMmy*1* patterns and to perform comparative analyses of these strains.

ISMmy*1* may be used as the target molecule in diagnostic PCR to distinguish *M. agalactiae* from *M. bovis*. PCR generally has higher specificity than probing so initially it may be better to screen a large number of strains from both species for the presence of ISMmy*1* by Southern blotting, and then perform a PCR survey. It would also be interesting to fully compare the sequences and integration sites of ISMmy*1* in *M. bovis* to those of *M. mycoides* SC, to see how closely related the elemtents are.

The genome sequence of PG1 can of course lead to an unlimited number of new research projects. Aided by proteomic tools and microarrays, the molecular

characterisation has potential to advance quickly and hopefully the development of new vaccines and eradication of the disease is within reach. Comparative genomics with strains of different pathogenicity may be one approach to reveal virulence factors, however, most of the genes in the genome are housekeeping genes and IS-elements. Thus, it is probably a more cost effective solution to focus on putative pathogenicity islands and surface components in general.

Conclusions

The genome of *M. mycoides* SC has been sequenced. Most housekeeping genes have been identified while the analysis of putative coding sequences is in progress.

A shotgun phage display library expressing peptides of most *M. mycoides* SC proteins, was produced to facilitate studies of proteins and protein expression. The combination of the phage display library and the genome sequence proved to be an efficient tool for molecular studies of *M. mycoides* SC.

Vmm, the Variable protein of *Mycoplasma mycoides*, is a small, surface located lipoprotein that undergoes phase variation. The phase variaiton was caused by dinucleotide insertions or deletions in the promoter spacer. Closely related mycoplasmas possess *vmm*-like genes.

PTS-A, the glucose/maltose specific phosphotransferase system of *M. mycoides* SC is variable. Phase variation of the EIIB/EIIC domains of PTS-A in African strains may affect the pathogenicity by regulating the glycerol metabolism and H_2O_2 production.

Two PCR systems for identification of *M. mycoides* SC were developed. Both methods involve group specific amplification of the members of the classical *M. mycoides* cluster. Differentiation of *M. mycoides* SC is possible after *Alu*I restriction and agarose gel eletrophoresis in one of the systems, and by direct analysis of the amplicons in a DNA sequencer when the other method is used.

A great number of insertions sequences are interspersed throughout the genome of *M. mycoides* SC, comprising 13% of the genome size. One of these, ISMmy*1* was characterised and the occurrence in different strains of *M. myciodes* SC and in some other mycoplasma species was investigated.

References

Abdo, E.M., Nicolet, J., Miserez, R., Goncalves, R., Regalla, J., Griot, C., Bensaide, A., Krampe, M. & Frey, J. 1998. Humoral and bronchial immune responses in cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* small colony type. *Veterinary Microbiology* 59, 109-22.

Abu-Groun, E.A., Taylor, R.R., Varsani, H., Wadher, B.J., Leach, R.H. & Miles, R.J. 1994. Biochemical diversity within the "*Mycoplasma mycoides*" cluster. *Microbiology* 140, 2033-42.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389-402.

Amanfu, W., Sediadie, S., Masupu, K.V., Raborokgwe, M.V., Benkirane, A., Geiger, R. & Thiaucourt, F. 2000. Comparison between c-ELISA and CFT in detecting antibodies to *Mycoplasma mycoides mycoides* biotype SC in cattle affected by CBPP in Botswana. *Annals of the New York Academy of Sciences* 916, 364-9.

Anonymous 1997. Recognising CBPP. A Field Manual for Recognition. EMPRES, FAO, Rome, pp. 1-27.

Anonymous 2000a. *Manual of standards for diagnostic tests and vaccines,* 4th. Office International des Epizooties. Paris.

Anonymous 2000b. Reviving progressive control of CBPP in Africa. In *Report of the second meeting of the FAO/OIE/OAU/IAEA consultative group on contagious bovine pleuropneumonia (CBPP)* Rome, pp. 1-9.

Ayling, R., Regalla, J. & Nicholas, R. 1999. A field test to *Mycoplasma mycoides* subsp. *mycoides* small colony type using the latex slide agglutination test. In *Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics*, Vol. 3 (Eds, Stipkovits, L., Rosengarten, R. and Frey, J.) Office for Official Publications of the European Communities, Luxembourg, pp. 155-158.

Baseggio, N., Glew, M.D., Markham, P.F., Whithear, K.G. & Browning, G.F. 1996. Size and genomic location of the pMGA multigene family of *Mycoplasma gallisepticum*. *Microbiology* 142, 1429-35.

Baseman, J.B., Cole, R.M., Krause, D.C. & Leith, D.K. 1982. Molecular basis for cytadsorption of *Mycoplasma pneumoniae*. *Journal of Bacteriology* 151, 1514-22.

Bashiruddin, J.B., De, S.P., Vacciana, A. & Santini, F.G. 1999. Detection of *Mycoplasma mycoides* subspecies *mycoides* SC in clinical material by a rapid colorimetric PCR. *Molecular and Cellular Probes* 13, 23-28.

Bashiruddin, J.B., Taylor, T.K. & Gould, A.R. 1994. A PCR-based test for the specific identification of *Mycoplasma mycoides* subspecies *mycoides* SC. *Journal of Veterinary Diagnostic Investigation* 6, 428-34.

Belli, P., Poumarat, F., Perrin, M., Longchambon, D. & Martel, J.L. 1989. Experimental reproduction and the course of contagious bovine pleuropneumonia in a group of cattle and goats: anatomoclinical aspects. *Revue d Elevage et de Medecine Veterinaire des Pays Tropicaux* 42, 349-56.

Bellini, S., Giovannini, A., di Francesco, C., Tittarelli, M. & Caporale, V. 1998. Sensitivity and specificity of serological and bacteriological tests for contagious bovine pleuropneumonia. *Revue Scientifique et Technique* 17, 654-659.

Bhugra, B., Voelker, L.L., Zou, N., Yu, H. & Dybvig, K. 1995. Mechanism of antigenic variation in *Mycoplasma pulmonis*: interwoven, site-specific DNA inversions. *Molecular Microbiology* 18, 703-14.

Boesen, T., Emmersen, J., Jensen, L.T., Ladefoged, S.A., Thorsen, P., Birkelund, S. & Christiansen, G. 1998. The *Mycoplasma hominis vaa* gene displays a mosaic gene structure. *Molecular Microbiology* 29, 97-110.

Boguslavsky, S., Menaker, D., Lysnyansky, I., Liu, T., Levisohn, S., Rosengarten, R., Garcia, M. & Yogev, D. 2000. Molecular characterization of the *Mycoplasma gallisepticum pvpA* gene which encodes a putative variable cytadhesin protein. *Infection and Immunity* 68, 3956-64.

Boonstra, E., Lindbaek, M., Fidzani, B. & Bruusgaard, D. 2001. Cattle eradication and malnutrition in under five's: a natural experiment in Botswana. *Public Health Nutrition* 4, 877-82.

Bork, P., Ouzounis, C., Casari, G., Schneider, R., Sander, C., Dolan, M., Gilbert, W. & Gillevet, P.M. 1995. Exploring the *Mycoplasma capricolum* genome: a minimal cell reveals its physiology. *Molecular Microbiology* 16, 955-67.

Bové, J.M., Tully, J.G., Madoff, S., Meymerit, J.-C., Blanchard, A., Chastel, C. & Kobisch, M. 1994. International Organization for Mycoplasmology (IOM). Exhibit on History of Mycoplasmology and IOM. Bordeaux.

Brandao, E. 1995. Isolation and identification of *Mycoplasma mycoides* subspecies *mycoides* SC strains in sheep and goats. *Veterinary Record* 136, 98-9.

Brocchi, E., Gamba, D., Poumarat, F., Martel, J.L. & De Simone, F. 1993. Improvements in the diagnosis of contagious bovine pleuropneumonia through the use of monoclonal antibodies. *Revue Scientifique et Technique* 12, 559-70.

Burton, D.R. 1995. Phage display. *Immunotechnology* 1, 87-94.

Bölske, G. 1995. Respiratory Mycoplasmoses in Goats; Especially with Regard to Diagnosis of Contagious Caprine Pleuropneumonia. *Doctoral thesis*, Veterinary Microbiology, Swedish University of Agricultural Sciences, Uppsala, 64

Bölske, G., Msami, H.M., Gunnarsson, A., Kapaga, A.M. & Loomu, P.M. 1995. Contagious bovine pleuropneumonia in northern Tanzania, culture confirmation and serological studies. *Tropical Animal Health and Production* 27, 193-201.

Calcutt, M.J., Kim, M.F., Karpas, A.B., Muhlradt, P.F. & Wise, K.S. 1999. Differential posttranslational processing confers intraspecies variation of a major surface lipoprotein and a macrophage-activating lipopeptide of *Mycoplasma fermentans*. *Infection and Immunity* 67, 760-71.

Chambaud, I., Heilig, R., Ferris, S., Barbe, V., Samson, D., Galisson, F., Moszer, I., Dybvig, K., Wroblewski, H., Viari, A., Rocha, E.P. & Blanchard, A. 2001. The complete genome sequence of the murine respiratory pathogen *Mycoplasma pulmonis*. *Nucleic Acids Research* 29, 2145-53.

Cheng, X., Nicolet, J., Poumarat, F., Regalla, J., Thiaucourt, F. & Frey, J. 1995. Insertion element IS*1296* in *Mycoplasma mycoides* subsp. *mycoides* small colony identifies a European clonal line distinct from African and Australian strains. *Microbiology* 141, 3221-8.

Citti, C., Kim, M.F. & Wise, K.S. 1997. Elongated versions of Vlp surface lipoproteins protect *Mycoplasma hyorhinis* escape variants from growth-inhibiting host antibodies. *Infection and Immunity* 65, 1773-85.

Citti, C., Watson-McKown, R., Droesse, M. & Wise, K.S. 2000. Gene families encoding phase- and size-variable surface lipoproteins of *Mycoplasma hyorhinis*. *Journal of Bacteriology* 182, 1356-63.

Cottew, G.S., Breard, A., DaMassa, A.J., Erno, H., Leach, R.H., Lefevre, P.C., Rodwell, A.W. & Smith, G.R. 1987. Taxonomy of the *Mycoplasma mycoides* cluster. *Israel Journal of Medical Sciences* 23, 632-5.

Crameri, R. & Suter, M. 1993. Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production. *Gene* 137, 69-75.

Cwirla, S.E., Peters, E.A., Barrett, R.W. & Dower, W.J. 1990. Peptides on phage: a vast library of peptides for identifying ligands. *Proceedings of the National Academy of Sciences of the United States of America* 87, 6378-82.

Dedieu, L., Mady, V. & Lefevre, P.C. 1994. Development of a selective polymerase chain reaction assay for the detection of *Mycoplasma mycoides* subsp. *mycoides* S.C. (contagious bovine pleuropneumonia agent). *Veterinary Microbiology* 42, 327-39.

Edward, D.G. & Freundt, E.A. 1956. The classification and nomenclature of organisms of the pleuropneumonia group. *Journal of General Microbiology* 14, 197-207.

Edward, D.G., Freundt, E.A., Chanock, R.M., Fabricant, J., Hayflick, L., Lemcke, R.M., Rezin, S., Somerson, N.L. & Wittler, R.G. 1967. Recommendations on nomenclature of the order *Mycoplasmatales. Science* 155, 1694-6.

Egwu, G.O., Nicholas, R.A.J., Ameh, J.A. & Bashiruddin, J.B. 1996. Contagious bovine pleuropneumonia: an update. *Veterinary Bulletin* 66, 875-888.

Fleischmann, R.D., Adams, M.D., White, O., Clayton, R.A., Kirkness, E.F., Kerlavage, A.R., Bult, C.J., Tomb, J.F., Dougherty, B.A., Merrick, J.M., McKenney, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J.D., Scott, J., Shirley, R., Liu, L.-I., Glodek, A., Kelley, J.M., Weidman, J.F., Phillips, C.A., Spriggs, T., Hedblom, E., Cotton, M.D., Utterback, T.R., Hanna, M.C., Nguyen, D., Saudek, D.M., Brandon, R.C., Fine, L.D., Fritchman, J.L., Fuhrmann, J.L., Geoghagen, N.S.M., Gnehm, C.L., McDonald, L.A., Small, K.V., Fraser, C.M., Smith, H.O. & Venter, C. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 269, 496-512.

Flitman-Tene, R., Levisohn, S., Lysnyansky, I., Rapoport, E. & Yogev, D. 2000. A chromosomal region of *Mycoplasma agalactiae* containing *vsp*-related genes undergoes in vivo rearrangement in naturally infected animals. *FEMS Microbiology Letters* 191, 205-12.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kerlavage, A.R., Sutton, G., Kelley, J.M., Fritchman, J.L., Weidman, J.F., Small, K.V., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T.R., Saudek, D.M., Phillips, C.A., Merrick, J.M., Tomb, J.-F., Dougerthy, B.A., Bott, K.F., Hu, P.-C., Lucier, T.S., Peterson,

S.N., Smith, H.O., Hutchinson III, C.A. & Venter, C. 1995. The minimal gene complement of *Mycoplasma genitalium*. *Science* 270, 397-403.

Frey, J., Cheng, X., Kuhnert, P. & Nicolet, J. 1995. Identification and characterization of IS*1296* in *Mycoplasma mycoides* subsp. *mycoides* SC and presence in related mycoplasmas. *Gene* 160, 95-100.

Garba, S.A. 1980. Shelf life of wet T1 broth vaccine for contagious bovine pleuropneumonia. *Tropical Animal Health and Production* 12, 189-91.

Glass, J.I., Lefkowitz, E.J., Glass, J.S., Heiner, C.R., Chen, E.Y. & Cassell, G.H. 2000. The complete sequence of the mucosal pathogen *Ureaplasma urealyticum*. *Nature* 407, 757-62.

Glew, M.D., Baseggio, N., Markham, P.F., Browning, G.F. & Walker, I.D. 1998. Expression of the pMGA genes of *Mycoplasma gallisepticum* is controlled by variation in the GAA trinucleotide repeat lengths within the 5' noncoding regions. *Infection and Immunity* 66, 5833-41.

Glew, M.D., 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*. *Infection and Immunity* 68, 4539-48.

Goncalves, R., Regalla, J., Nicolet, J., Frey, J., Nicholas, R., Bashiruddin, J., de Santis, P. & Goncalves, A.P. 1998. Antigen heterogeneity among *Mycoplasma mycoides* subsp. *mycoides* SC isolates: discrimination of major surface proteins. *Veterinary Microbiology* 63, 13-28.

Gourlay, R.N. 1964. Antigenicity of *Mycoplasma mycoides*. *Research in Veterinary Science* 5, 473-482.

Grieco, V., Boldini, M., Luini, M., Finazzi, M., Mandelli, G. & Scanziani, E. 2001. Pathological, immunohistochemical and bacteriological findings in kidneys of cattle with contagious bovine pleuropneumonia (CBPP). *Journal of Comparative Pathology* 124, 95-101.

Hacker, J., Blum-Oehler, G., Muhldorfer, I. & Tschape, H. 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Molecular Microbiology* 23, 1089-97.

Heldtander Königsson, M., Bölske, G. & Johansson, K.-E. 2002. Intraspecific variation in the 16S rRNA gene sequences of *Mycoplasma agalactiae* and *Mycoplasma bovis* strains. *Veterinary Microbiology* 85, 209-220.

Heldtander, M., Pettersson, B., Tully, J.G. & Johansson, K.-E. 1998. Sequences of the 16S rRNA genes and phylogeny of the goat mycoplasmas *Mycoplasma adleri*, *Mycoplasma auris*, *Mycoplasma cottewii* and *Mycoplasma yeatsii*. *International Journal of Systematic Bacteriology* 48, 263-8.

Heldtander, M., Wesonga, H., Bölske, G., Pettersson, B. & Johansson, K.-E. 2001. Genetic diversity and evolution of *Mycoplasma capricolum* subsp. *capripneumoniae* strains from eastern Africa assessed by 16S rDNA sequence analysis. *Veterinary Microbiology* 78, 13-28.

Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B.C. & Herrmann, R. 1996. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic Acids Research* 24, 4420-49.

Hoogenboom, H.R., de Bruine, A.P., Hufton, S.E., Hoet, R.M., Arends, J.W. & Roovers, R.C. 1998. Antibody phage display technology and its applications. *Immunotechnology* 4, 1-20.

Hotzel, H., Sachse, K. & Pfutzner, H. 1996. A PCR scheme for differentiation of organisms belonging to the *Mycoplasma mycoides* cluster. *Veterinary Microbiology* 49, 31-43.

Houshaymi, B.M., Miles, R.J. & Nicholas, R.A. 1997. Oxidation of glycerol differentiates African from European isolates of *Mycoplasma mycoides* subspecies *mycoides* SC (small colony). *Veterinary Record* 140, 182-3.

Hutyra, F., Marek, J. & Manninger, R. 1938. Contagious Bovine Pleuro-pneumonia of Cattle. In *Special pathology and therapeutics of the diseases of domestic animals* (Eds, Greig, J. R., Mohler, J. R. and Eichhorn, A.) Balliere, Tindal and Cox, London, pp. 455-472.

Jacobsson, K. & Frykberg, L. 1995. Cloning of ligand-binding domains of bacterial receptors by phage display. *Biotechniques* 18, 878-85.

Jacobsson, K. & Frykberg, L. 1998. Gene VIII-based, phage-display vectors for selection against complex mixtures of ligands. *Biotechniques* 24, 294-301.

Jacobsson, K., Jonsson, H., Lindmark, H., Guss, B., Lindberg, M. & Frykberg, L. 1997. Shot-gun phage display mapping of two streptococcal cell-surface proteins. *Microbiological Research* 152, 121-8.

Johansson, K.-E. & Pettersson, B. 2002. Taxonomy of *Mollicutes*. In *Molecular Biology and Pathogenicity of Mycoplasmas* (Eds, Razin, S. and Herrmann, R.) Kluwer Academic/ Plenum Publishers, London, UK, pp. In press.

Kusiluka, L.J.M., Ojeniyi, B., Friis, N.F., Kokotovic, B. & Ahrens, P. 2001a. Molecular analysis of field strains of *Mycoplasma capricolum* subspecies *capripneumoniae* and *Mycoplasma mycoides* subspecies *mycoides*, small colony type isolated from goats in Tanzania. *Veterinary Microbiology* 82, 27-37.

Kusiluka, L.J.M., Ojeniyi, B., Friis, N.F., Kokotovic, B. & Ahrens, P. 2001b. Molecular epidemiology of contagious bovine pleuropneumonia in Tanzania based on amplified fragment length polymorphism and pulsed-field gel electrophoresis analysis. *Journal of Veterinary Medicine. Series B* 48, 303-312.

Laigret, F., Carle, P., Carrère, N., Garnier, M. & Bové, J.M. 2000. Toward the complete genome sequence of *Spiroplasma citri*. In *13th international congress of the International Organization for Mycoplasmology,* Fukuoka, pp. 48.

Le Goff, C. & Thiaucourt, F. 1998. A competitive ELISA for the specific diagnosis of contagious bovine pleuropneumonia (CBPP). *Veterinary Microbiology* 60, 179-91.

Le Grand, D., Solsona, M., Rosengarten, R. & Poumarat, F. 1996. Adaptive surface antigen variation in *Mycoplasma bovis* to the host immune response. *FEMS Microbiology Letters* 144, 267-75.

Lorenzon, S., David, A., Nadew, M., Wesonga, H. & Thiaucourt, F. 2000. Specific PCR identification of the T1 vaccine strains for contagious bovine pleuropneumonia. *Molecular and Cellular Probes* 14, 205-10.

Lysnyansky, I., Ron, Y., Sachse, K. & Yogev, D. 2001a. Intrachromosomal recombination within the *vsp* locus of *Mycoplasma bovis* generates a chimeric variable surface lipoprotein antigen. *Infection and Immunity* 69, 3703-12.

Lysnyansky, I., Ron, Y. & Yogev, D. 2001b. Juxtaposition of an active promoter to *vsp* genes via site-specific DNA inversions generates antigenic variation in *Mycoplasma bovis*. *Journal of Bacteriology* 183, 5698-708.

Lysnyansky, I., Rosengarten, R. & Yogev, D. 1996. Phenotypic switching of variable surface lipoproteins in *Mycoplasma bovis* involves high-frequency chromosomal rearrangements. *Journal of Bacteriology* 178, 5395-401.

Lysnyansky, I., Sachse, K., Rosenbusch, R., Levisohn, S. & Yogev, D. 1999. The *vsp* locus of *Mycoplasma bovis*: gene organization and structural features. *Journal of Bacteriology* 181, 5734-41.

Mahillon, J. & Chandler, M. 1998. Insertion sequences. *Microbiology and Molecular Biology Reviews* 62, 725-74.

Maniloff, J. 1996. The minimal cell genome: "on being the right size". *Proceedings of the National Academy of Sciences of the United States of America* 93, 10004-6.

March, J.B. 2000. Differentiation of *Mycoplasma mycoides mycoides* SC strains/isolates - prospects for molecular epidemiology. FAO/OIE/OAU/IAEA, Rome, Italy, pp. 53-58.

March, J.B., Clark, J. & Brodlie, M. 2000. Characterization of strains of *Mycoplasma mycoides* subsp. *mycoides* small colony type isolated from recent outbreaks of contagious bovine pleuropneumonia in Botswana and Tanzania: evidence for a new biotype. *Journal of Clinical Microbiology* 38, 1419-25.

Markham, P.F., Duffy, M.F., Glew, M.D. & Browning, G.F. 1999. A gene family in *Mycoplasma imitans* closely related to the pMGA family of *Mycoplasma gallisepticum*. *Microbiology* 145, 2095-103.

Markham, P.F., Glew, M.D., Brandon, M.R., Walker, I.D. & Whithear, K.G. 1992. Characterization of a major hemagglutinin protein from *Mycoplasma gallisepticum*. *Infection and Immunity* 60, 3885-91.

Markham, P.F., Glew, M.D., Sykes, J.E., Bowden, T.R., Pollocks, T.D., Browning, G.F., Whithear, K.G. & Walker, I.D. 1994. The organisation of the multigene family which encodes the major cell surface protein, pMGA, of *Mycoplasma gallisepticum*. *FEBS Letters* 352, 347-52.

Masiga, W.N., Domenech, J. & Windsor, R.S. 1996. Manifestation and epidemiology of contagious bovine pleuropneumonia in Africa. *Revue Scientifique et Technique* 15, 1283-308.

Masiga, W.N., Windsor, R.S. & Read, W.C. 1972. A new mode of spread of contagious bovine pleuropneumonia? *Veterinary Record* 90, 247-8.

Miles, R.J. 1983. Effect of some cultural factors on T1 broth vaccine for contagious bovine pleuropneumonia. *Tropical Animal Health and Production* 15, 144-8.

Miles, R.J., Taylor, R.R. & Varsani, H. 1991. Oxygen uptake and H_2O_2 production by fermentative *Mycoplasma* spp. *Journal of Medical Microbiology* 34, 219-23.

Miserez, R., Pilloud, T., Cheng, X., Nicolet, J., Griot, C. & Frey, J. 1997. Development of a sensitive nested PCR method for the specific detection of *Mycoplasma mycoides* subsp. *mycoides* SC. *Molecular and Cellular Probes* 11, 103-11.

Mitchell, A. & Finch, L.R. 1977. Pathways of nucleotide biosynthesis in *Mycoplasma mycoides* subsp. *mycoides*. *Journal of Bacteriology* 130, 1047-54.

Mitchell, A., Sin, I.L. & Finch, L.R. 1978. Enzymes of purine metabolism in *Mycoplasma mycoides* subsp. *mycoides*. *Journal of Bacteriology* 134, 706-12.

Monnerat, M.P., Thiaucourt, F., Poveda, J.B., Nicolet, J. & Frey, J. 1999. Genetic and serological analysis of lipoprotein LppA in *Mycoplasma mycoides* subsp. *mycoides* LC and *Mycoplasma mycoides* subsp. *capri*. *Clinical and Diagnostic Laboratory Immunology* 6, 224-30.

Mühlradt, P.F., Kiess, M., Meyer, H., Sussmuth, R. & Jung, G. 1997. Isolation, structure elucidation, and synthesis of a macrophage stimulatory lipopeptide from *Mycoplasma fermentans* acting at picomolar concentration. *Journal of Experimental Medicine* 185, 1951-8.

Mühlradt, P.F., Kiess, M., Meyer, H., Sussmuth, R. & Jung, G. 1998. Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis*. *Infection and Immunity* 66, 4804-10.

Neyrolles, O., Chambaud, I., Ferris, S., Prevost, M.C., Sasaki, T., Montagnier, L. & Blanchard, A. 1999. Phase variations of the *Mycoplasma penetrans* main surface lipoprotein increase antigenic diversity. *Infection and Immunity* 67, 1569-78.

Nicholas, R., Bashiruddin, J., Ayling, R. & Miles, R. 2000. Contagious bovine pleuropneumonia: a review of recent developments. *Veterinary Bulletin* 70, 827-838.

Nicholas, R. & Palmer, N. 1994. Contagious bovine pleuropneumonia in Europe. *State Veterinary Journal* 4, 14-16.

Nicholas, R.A. & Bashiruddin, J.B. 1995. *Mycoplasma mycoides* subspecies *mycoides* (small colony variant): the agent of contagious bovine pleuropneumonia and member of the "*Mycoplasma mycoides* cluster". *Journal of Comparative Pathology* 113, 1-27.

Nocard & Roux 1898. Le microbe de la péripneumonie. *Annals de l'institut Pasteur* 12, 240-262.

Noormohammadi, A.H., Markham, P.F., Duffy, M.F., Whithear, K.G. & Browning, G.F. 1998. Multigene families encoding the major hemagglutinins in phylogenetically distinct mycoplasmas. *Infection and Immunity* 66, 3470-5.

Noormohammadi, A.H., Markham, P.F., Kanci, A., Whithear, K.G. & Browning, G.F. 2000. A novel mechanism for control of antigenic variation in the haemagglutinin gene family of *Mycoplasma synoviae*. *Molecular Microbiology* 35, 911-23.

Noormohammadi, A.H., Markham, P.F., Whithear, K.G., Walker, I.D., Gurevich, V.A., Ley, D.H. & Browning, G.F. 1997. *Mycoplasma synoviae* has two distinct phase-variable major membrane antigens, one of which is a putative hemagglutinin. *Infection and Immunity* 65, 2542-7.

OIE, 2002a. Handistatus II, Multiannual animal disease status. Office International des Epizooties, Paris. (*http://www.oie.int/hs2/sit_mald_freq_pl.asp?c_cont=6&c_mald=7*: Accessed 2002-02-13).

Olson, L.D., Shane, S.W., Karpas, A.A., Cunningham, T.M., Probst, P.S. & Barile, M.F. 1991. Monoclonal antibodies to surface antigens of a pathogenic *Mycoplasma hominis* strain. *Infection and Immunity* 59, 1683-9.

Persson, A., Solsona, M., Le Grand, D., Citti, C., Johansson, K.-E. & Poumarat, F. 2001. Genetic and antigenic aspects on intraclonal variability of *Mycoplasma mycoides* subsp. *mycoides* SC. In *Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics*, Vol. 5 (Eds, Poveda, J. B., Fernández, A., Frey, J. and Johansson, K.-E.) Office for Official Publications of the European Communities, Luxembourg, pp. 14-17.

Pettersson, B., Bölske, G., Thiaucourt, F., Uhlén, M. & Johansson, K.-E. 1998. Molecular evolution of *Mycoplasma capricolum* subsp. *capripneumoniae* strains, based on polymorphisms in the 16S rRNA genes. *Journal of Bacteriology* 180, 2350-8.

Pettersson, B., Leitner, T., Ronaghi, M., Bölske, G., Uhlén, M. & Johansson, K.-E. 1996. Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. *Journal of Bacteriology* 178, 4131-42.

Plackett, P., Buttery, S.H. & Cottew, G.S. 1963. Carbohydrates of some *Mycoplasma* strains. In *Recent Progress in Microbiology. 8th International Congress for Micobiology*, Vol. 8 Montreal, pp. 535-547.

Pollack, J.D., Williams, M.V. & McElhaney, R.N. 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. *Critical Reviews in Microbiology* 23, 269-354.

Postma, P.W., Lengeler, J.W. & Jacobson, G.R. 1996. Phosphoenolpyruvate: Carbohydrate Phosphotransferase Systems. In *Escherichia coli and Salmonella*, Vol. 2 (Eds, Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. and Umbarger, H. E.) ASM Press, Washington, pp. 1149-1174.

Poumarat, F. & Solsona, M. 1995. Molecular epidemiology of *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony, the agent of contagious bovine pleuropneumonia. *Veterinary Microbiology* 47, 305-15.

Provost, A., Perreau, P., Breard, A., Le Goff, C., Martel, J.L. & Cottew, G.S. 1987. Contagious bovine pleuropneumonia. In *Revue Scientifique et Technique*, Vol. 6 Office International des Epizooties, Paris, pp. 625-679.

Rawadi, G., Lemercier, B. & Roulland-Dussoix, D. 1995. Application of an arbitrarily-primed polymerase chain reaction to mycoplasma identification and typing within the *Mycoplasma mycoides* cluster. *Journal of Applied Bacteriology* 78, 586-92.

Razin, S. 1992. Mycoplasma Taxonomy and Ecology. In *Mycoplasmas: Molecular Biology and Pathogenesis* (Eds, Maniloff, J., McElhaney, R. N., Finch, L. R. and Baseman, J. B.) American Society for Microbiology, Washington, D.C.

Razin, S., Yogev, D. & Naot, Y. 1998. Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular Biology Reviews* 62, 1094-156.

Rice, P., Houshaymi, B.M., Nicholas, R.A. & Miles, R.J. 2000. A rapid biochemical test to aid identification of *Mycoplasma mycoides* subsp. *mycoides* small colony (SC) strains. *Letters in Applied Microbiology* 30, 70-4.

Robertson, J.A., Stemke, G.W., Davis Jr., J.W., Harasawa, R., Thirkell, D., Kong, F., Shepard, M.C. & Ford, D. 2002. Proposal of *Ureaplasma parvum* sp. nov. and Emended Description of *Ureaplasma urealyticum* (Shepard et al. 1974) Robertson et al. 2001. *International Journal of Systematic and Evolutionary Microbiology* 52, 587-597.

Rodwell, A.W. 2000. Biochemical studies on *Mycoplasma mycoides* and other mycoplasmas. In *Of vets, viruses and Vaccines. The story of the animal health research laboratory, Parkville* (Ed, Bucher, B. W.) Csiro Publishing, pp. chapter 9.

Rosengarten, R. & Wise, K.S. 1990. Phenotypic switching in mycoplasmas: phase variation of diverse surface lipoproteins. *Science* 247, 315-8.

Rosengarten, R. & Yogev, D. 1996. Variant colony surface antigenic phenotypes within mycoplasma strain populations: implications for species identification and strain standardization. *Journal of Clinical Microbiology* 34, 149-58.

Rurangirwa, F.R., Shompole, P.S., Wambugu, A.N. & McGuire, T.C. 2000. Monoclonal antibody differentiation of *Mycoplasma mycoides* subsp. *mycoides* small-colony strains causing contagious bovine pleuropneumonia from less important large-colony strains. *Clinical and Diagnostic Laboratory Immunology* 7, 519-21.

Rweyemamu, M., Paskin, R., Benkirane, A., Martin, V., Roeder, P. & Wojciechowski, K. 2000. Emerging diseases of Africa and the Middle East. *Annals of the New York Academy of Sciences* 916, 61-70.

Röske, K., Blanchard, A., Chambaud, I., Citti, C., Helbig, J.H., Prevost, M.C., Rosengarten, R. & Jacobs, E. 2001. Phase variation among major surface antigens of *Mycoplasma penetrans*. *Infection and Immunity* 69, 7642-51.

Sachse, K., Helbig, J.H., Lysnyansky, I., Grajetzki, C., Muller, W., Jacobs, E. & Yogev, D. 2000. Epitope mapping of immunogenic and adhesive structures in repetitive domains of *Mycoplasma bovis* variable surface lipoproteins. *Infection and Immunity* 68, 680-7.

Scanziani, E., Paltrinieri, S., Boldini, M., Grieco, V., Monaci, C., Giusti, A. & Mandelli, G. 1997. Histological and immunohistochemical findings in thoracic lymph nodes of cattle with contagious bovine pleuropneumonia. *Journal of Comparative Pathology* 117, 127-136.

Scudamore, J. 1995. Contagious bovine pleuropneumonia. *State Veterinary Journal* 5, 13-16.

Shen, X., Gumulak, J., Yu, H., French, C.T., Zou, N. & Dybvig, K. 2000. Gene rearrangements in the *vsa* locus of *Mycoplasma pulmonis*. *Journal of Bacteriology* 182, 2900-8.

Smith, G.P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228, 1315-7.

Smith, G.P. & Petrenko, V.A. 1997. Phage display. *Chemical Reviews* 97, 391-410.

The *Spiroplasma* Web Page, 2000. The *Spiroplasma kunkelii* genome sequencing project. Ohio State University, Columbus, OH. (*http://www.oardc.ohio-state.edu/spiroplasma/genome.htm*: Accessed 08-03-2002).

Stradaioli, G., Sylla, L., Mazzarelli, F., Zelli, R., Rawadi, G. & Monaci, M. 1999. *Mycoplasma mycoides* subsp. *mycoides* SC identification by PCR in sperm of seminal vesiculitis-affected bulls. *Veterinary Research* 30, 457-66.

Taylor, T.K., Bashiruddin, J.B. & Gould, A.R. 1992a. Application of a diagnostic DNA probe for the differentiation of the two types of *Mycoplasma mycoides* subspecies *mycoides*. *Research in Veterinary Science* 53, 154-9.

Taylor, T.K., Bashiruddin, J.B. & Gould, A.R. 1992b. Relationships between members of the *Mycoplasma mycoides* cluster as shown by DNA probes and sequence analysis. *International Journal of Systematic Bacteriology* 42, 593-601.

ter Laak, E.A. 1992. Contagious bovine pleuropneumonia. A review. *Veterinary Quarterly* 14, 104-110.

Theiss, P., Karpas, A. & Wise, K.S. 1996. Antigenic topology of the P29 surface lipoprotein of *Mycoplasma fermentans*: differential display of epitopes results in high-frequency phase variation. *Infection and Immunity* 64, 1800-9.

Theiss, P. & Wise, K.S. 1997. Localized frameshift mutation generates selective, high-frequency phase variation of a surface lipoprotein encoded by a mycoplasma ABC transporter operon. *Journal of Bacteriology* 179, 4013-22.

Thiaucourt, F., Lorenzon, S., David, A., Tulasne, J.J. & Domenech, J. 1998. Vaccination against contagious bovine pleuropneumonia and the use of molecular tools in epidemiology. *Annals of the New York Academy of Sciences* 849, 146-51.

Thiaucourt, F., Yaya, A., Wesonga, H., Huebschle, O.J.B., Tulasne, J.J., Provost, A., House, J.A., Kocan, K.M. & Gibbs, E.P.J. 2000. Contagious bovine pleuropneumonia. A reassessment of the efficacy of vaccines used in Africa. In *Tropical veterinary diseases: Control and prevention in the context of the New World Order. Proceedings of the Fifth Biennial Conference of the Society for Tropical Veterinary Medicine, held on June 12 16, 1999, in Key West, Florida, USA. Annals of the New York Academy of Sciences. 2000, 916: 71 80; 22 ref.*

TIGR Microbial Database, 2002b. Published complete microbial genomes. Microbial genomes and chromosomes in progress. The Institute for Genomic Research, Rockville, MD. (*http://www.tigr.org/tdb*: Accessed 04-03-2002).

Tryon, V.V. & Baseman, J.B. 1992. Pathogenic determinants and mechanisms. In *Mycoplasmas: Molecular Biology and Pathogenesis* (Eds, Maniloff, J., McElhaney, R. N., Finch, L. R. and Baseman, J. B.) American Society for Microbiology, Washington, DC.

Waite, E.R. & March, J.B. 2001. Effect of HEPES buffer systems upon the pH, growth and survival of *Mycoplasma mycoides* subsp. *mycoides* small colony (MmmSC) vaccine cultures. *FEMS Microbiology Letters* 201, 291-4.

Washburn, L.R., Hirsch, S. & Voelker, L.L. 1993. Mechanisms of attachment of *Mycoplasma arthritidis* to host cells in vitro. *Infection and Immunity* 61, 2670-80.

Washburn, L.R., Weaver, K.E., Weaver, E.J., Donelan, W. & Al-Sheboul, S. 1998. Molecular characterization of *Mycoplasma arthritidis* variable surface protein MAA2. *Infection and Immunity* 66, 2576-86.

Weisburg, W.G., Tully, J.G., Rose, D.L., Petzel, J.P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T.G., Van Etten, J., Maniloff, J. & Woese, C.R. 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *Journal of Bacteriology* 171, 6455-67.

Wesonga, H.O. & Thiaucourt, F. 2000. Experimental studies on the efficacy of T1sr and T1/44 vaccine strains of *Mycoplasma mycoides* subspecies *mycoides* (small colony) against a field isolate causing contagious bovine pleuropneumonia in Kenya - effect of a revaccination. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux* 53, 313-318.

Vilei, E.M., Abdo, E.M., Nicolet, J., Botelho, A., Goncalves, R. & Frey, J. 2000. Genomic and antigenic differences between the European and African/Australian clusters of *Mycoplasma mycoides* subsp. *mycoides* SC. *Microbiology* 146, 477-86.

Vilei, E.M., Nicolet, J. & Frey, J. 1999. IS*1634*, a novel insertion element creating long, variable-length direct repeats which is specific for *Mycoplasma mycoides* subsp. *mycoides* small- colony type. *Journal of Bacteriology* 181, 1319-23.

Wilson, D.R. & Finlay, B.B. 1998. Phage display: applications, innovations, and issues in phage and host biology. *Canadian Journal of Microbiology* 44, 313-29.

Windsor, R.S. 1978. An investigation into the viability of broth cultures of the T1 strain of *Mycoplasma mycoides* sub-species *mycoides*. *Research in Veterinary Science* 24, 109-12.

Windsor, R.S. 2000. The eradication of contagious bovine pleuropneumonia from south western Africa. A plan for action. *Annals of the New York Academy of Sciences* 916, 326-32.

Windsor, R.S. & Wood, A. 1998. Contagious bovine pleuropneumonia. The costs of control in Central/southern Africa. *Annals of the New York Academy of Sciences* 849, 299-306.

Yogev, D., Rosengarten, R., Watson-McKown, R. & Wise, K.S. 1991. Molecular basis of *Mycoplasma* surface antigenic variation: a novel set of divergent genes undergo spontaneous mutation of periodic coding regions and 5' regulatory sequences. *EMBO Journal* 10, 4069-79.

Yogev, D., Watson-McKown, R., Rosengarten, R., Im, J. & Wise, K.S. 1995. Increased structural and combinatorial diversity in an extended family of genes encoding Vlp surface proteins of *Mycoplasma hyorhinis*. *Journal of Bacteriology* 177, 5636-43.

Zhang, Q. & Wise, K.S. 1996. Molecular basis of size and antigenic variation of a *Mycoplasma hominis* adhesin encoded by divergent *vaa* genes. *Infection and Immunity* 64, 2737-44.

Zhang, Q. & Wise, K.S. 1997. Localized reversible frameshift mutation in an adhesin gene confers a phase-variable adherence phenotype in mycoplasma. *Molecular Microbiology* 25, 859-69.

Zhang, Q. & Wise, K.S. 2001. Coupled phase-variable expression and epitope masking of selective surface lipoproteins increase surface phenotypic diversity in *Mycoplasma hominis*. *Infection and Immunity* 69, 5177-81.

Zheng, X., Teng, L.J., Watson, H.L., Glass, J.I., Blanchard, A. & Cassell, G.H. 1995. Small repeating units within the *Ureaplasma urealyticum* MB antigen gene encode serovar specificity and are associated with antigen size variation. *Infection and Immunity* 63, 891-8.

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