

The ostrich mycoplasma *Ms01*:
The identification, isolation, and modification of the *P100*
vaccine candidate gene and immunity elicited by poultry
mycoplasma vaccines

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Declaration

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Summary

The South African ostrich industry is currently being threatened by respiratory disease in feedlot ostriches with dramatic production losses. Three ostrich-specific mycoplasmas, *Ms01*, *Ms02* and *Ms03* were identified to be associated with respiratory disease in ostriches in South Africa. There is currently no registered mycoplasma vaccine available for use in ostriches. In order to prevent mycoplasma infections in South African ostriches, the ostrich industry has launched an investigation into possible strategies for vaccine development. This thesis describes different strategies for the establishment of immunity in ostriches against the ostrich-specific mycoplasmas. Firstly, the effectiveness of existing poultry mycoplasma vaccines to provide protection in ostriches against ostrich mycoplasma infections was tested. To this end, ostriches received primary and secondary vaccinations with poultry mycoplasma vaccines against *Mycoplasma synoviae* or *Mycoplasma gallicepticum*, respectively, after which protection against ostrich-specific mycoplasma was evaluated. Even though the specific identity of the ostrich-specific mycoplasmas (*Ms01*, *Ms02*, and/or *Ms03*) responsible for subsequent infection of immunized ostriches was not determined, it was concluded that poultry mycoplasma vaccines do not provide protection against these mycoplasma infections in ostriches. This appears to be the result of low levels of antibody cross-reactivity between mycoplasmas, highlighting the necessity for the development of specific vaccines against each of the individual ostrich-specific mycoplasmas.

Secondly, the development of a DNA vaccine against *Ms01* was investigated. With the aim of developing an *Ms01*-specific DNA vaccine, the entire *Ms01* genome was sequenced using GS20 sequencing technology. Bioinformatic searches were launched for the identification of an appropriate vaccine candidate gene in the *Ms01* genome. The *P100* gene, showing a high degree of homology with the *P100* gene of the human pathogen *M. hominis*, was subsequently identified. After successful cloning, and modification of ten specific codons within the gene to correct for alternative codon usage, the modified *P100* gene of *Ms01* is now ready for insertion into a suitable DNA vaccine vector, for subsequent use as a DNA vaccine in ostriches.

Opsomming

Die Suid-Afrikaanse volstruisbedryf word huidiglik bedreig deur respiratoriese siektes in voerkraal volstruise wat aansienlike produksieverliese tot gevolg het. Drie volstruis-spesifieke mikoplasmas, *Ms01*, *Ms02* en *Ms03* is geïdentifiseer wat 'n rol te speel in respiratoriese siektes in volstruise in Suid-Afrika. Daar is huidiglik geen geregistreerde mikoplasma entstof beskikbaar vir gebruik in volstruise nie. Ten einde mikoplasma infeksies in volstruise te voorkom, het die Suid-Afrikaanse volstruisbedryf 'n ondersoek geloods na moontlike strategieë vir entstof ontwikkeling. Hierdie tesis handel oor benaderinge om immuniteit in volstruise teen die volstruis-spesifieke mikoplasmas te induseer. Eerstens is die effektiwiteit van bestaande pluimvee mikoplasma entstowwe getoets vir beskerming in volstruise teen volstruis-spesifieke mikoplasmas. Met dit ten doel, is volstruise twee maal met pluimvee entstowwe teen *Mycoplasma synoviae* of *Mycoplasma gallisepticum* onderskeidelik geënt, waarna die beskerming teen *Ms01* geëvalueer is. Alhoewel die presiese identiteit van die volstruis-spesifieke mikoplasmas (*Ms01*, *Ms02* en/of *Ms03*) verantwoordelik vir die daaropvolgende infeksies in geïmmuniseerde volstruise nie bepaal is nie, is dit gevind dat die toediening van pluimvee entstowwe nie beskerming gebied het teen hierdie mikoplasma infeksies in volstruise nie. Dit blyk die gevolg te wees van die lae vlakke van antiliggaam kruis-reaktiwiteit tussen mikoplasmas, en beklemtoon dat die ontwikkeling van spesifieke entstowwe vir elk van die volstruis-spesifieke mikoplasmas individueel uitgevoer sal moet word.

Tweedens is die ontwikkeling van 'n DNA entstof teen *Ms01* ondersoek. Met die doel om 'n *Ms01*-spesifieke DNA entstof te ontwikkel, is die volledige *Ms01* genoomvolgorde bepaal deur gebruik te maak van "GS20" volgordebepalingtegnologie. Daarna is bioinformatika soektogte geloods vir die identifisering van 'n geskikte entstof kandidaat geen in die *Ms01* genoom. Die *P100* geen, wat hoë homologie toon met die menslike patogeen *M. hominis* se *P100* geen, is geïdentifiseer in *Ms01*. Na suksesvolle klonering, en die modifisering van tien spesifieke kodons in die geen, is die gemodifiseerde *P100* geen van *Ms01* nou geskik vir invoeging in 'n geskikte DNA entstof vektor, vir daaropvolgende gebruik as DNA entstof in volstruise.

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Abbreviations

A	adenine
ABC	ATP-binding cassette
ABTS	2,2'-Azino-di(3-ethylbenzthiazoline-6-sulphonic acid)
APC	antigen presenting cell
AVPO	streptavidin horse radish peroxidase
BGH	bovine growth hormone
BLAST	basic local alignment search tool
bp	base pairs
C	cytosine
CI	consistency index
CMV	cytomegalovirus
CpG	cytidine-phosphate-guanosine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DMF	N,N-dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetra-acetic acid di-sodium salt
ELISA	enzyme-linked immunosorbent assay
emPCR	emulsion polymerase chain reaction
G	guanine
G+C	guanine and cytosine
gDNA	genomic deoxyribonucleic acid
GLM	General Linear Models
GS	genome sequencing
HRP	horse radish peroxidase
ID	intra-dermal
IDT	Integrated DNA Technologies
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	intramuscular
kDa	kilodalton
LB	Luria-Bertani

LSD	least significant difference
MG	<i>Mycoplasma gallisepticum</i>
MG-Bac	Mycoplasma Gallisepticum Bacterin
MHC	major histocompatibility complex
mol%	molecular percentage
mRNA	messenger ribonucleic acid
<i>Ms</i>	<i>Mycoplasma struthionis</i>
MS	<i>Mycoplasma synoviae</i>
MS-Bac	Mycoplasma Synoviae Bacterin
NCBI	National Center for Biotechnology Information
NDV	Newcastle disease virus
Opp	oligopeptide permease
ORF	open reading frame
oriC	origin of replication
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RBS	ribosomal-binding site
RI	retention index
rRNA	ribosomal ribonucleic acid
SAS	Statistical Analysis System
SD	Shine-Dalgarno
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulfate
SV40	simian virus 40
T	thymine
TAE	Tris-acetate EDTA
TE	Tris-EDTA
T _m	melting temperature
TNF	tumor necrosis factor
UV	ultraviolet

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Chapter 1 – Introduction

South Africa is the undisputed world leader in the ostrich trade. Large scale commercial ostrich farming originated in South Africa in the mid-eighteen hundreds (1864), reaching a peak in the early nineteen hundreds (1913) when ostrich feathers became South Africa's fourth largest export product, closely behind gold, diamonds and wool (History Of: Ostriches and Oudtshoorn, 2004). In 1986, South Africa exported a record high of 90 000 ostrich hides to the United States alone, and by 1992, 95% of the ostriches slaughtered worldwide were processed in South Africa. Today, ostrich farming is still regarded as one of the top trades in South Africa, ranking in the top twenty agro-based industries, with the total investment in ostrich production and processing activities exceeding R2.1 billion. The industry is mainly export driven, with 90% of all leather and meat products being exported, amounting to an annual export income of R1.2 billion. Currently, South Africa has 558 registered export farms producing 300 000 slaughter birds annually, and creating employment for more than 20 000 workers, lending to the significant economic and socio-economic value of the industry (The South African Ostrich Industry, 2004).

A major attribute of the ostrich industry, is its high profit potential brought about by the variety of products obtained from a bird. Initially the focus of the ostrich trade was on the production of feathers only, much later the skin was included, and only relatively recently meat (Huchzermeyer, 2002). The value of a slaughter bird in South Africa can generally be broken down as 10% feathers, 20% meat, and 70% skin. Ostrich feathers are commonly used for cleaning purposes, and also serve as decorations and are quite popular in the fashion industry. Ostrich meat is regarded the healthiest of all red meats with low fat (<2%), cholesterol and calorie content, while still retaining a high protein content. Therefore, ostrich meat has gained considerable popularity in recent years with increased consumer awareness concerning a healthy lifestyle. Furthermore, ostrich leather is considered to be one of the most luxurious leathers, on a par with other exotic leathers such as crocodile and snake leather (Ostrich products, 2004).

Owing to South Africa's historic advantage, as well as the favorable natural conditions, South Africa should be able to maintain its world leadership in the ostrich trade provided that certain conditions, such as disease control and export regulations, are met. The South African ostrich industry is currently being threatened by respiratory disease in feedlot ostriches resulting in up to 30% production losses (personal communication, Dr. A. Olivier). Other than the dramatic production losses, a further concern involves the transmission of mycoplasmas to other countries via contaminated products. Therefore mycoplasma infections may place constraints on the export of ostrich products, thereby potentially having a considerable economic impact. Recently, three ostrich-specific mycoplasmas, *Ms01*, *Ms02* and *Ms03* (*Ms*, *Mycoplasma struthionis* after their host, *Struthio camelus*) were identified to be associated with respiratory disease in ostriches in South Africa (Botes *et al.*, 2005). Strategies for the control of mycoplasma infections in ostriches include prevention by strict biosecurity practices, and treatment with a

limited range of antibiotics. However, there is currently no registered mycoplasma vaccine available for use in ostriches.

In order to prevent mycoplasma infections in South African ostriches, the ostrich industry has launched an investigation into possible strategies for vaccine development. Their investigation includes conventional approaches to vaccine development (whole-organism vaccines), undertaken at Onderstepoort Veterinary Institute, Pretoria (not part of this study), as well as a more novel approach to vaccine development, namely DNA vaccine development (described in this study). As alternative to vaccine development, the use of existing poultry mycoplasma vaccines to provide protection against mycoplasma infections in ostriches has been suggested.

The objectives of this study were:

- Testing the effectiveness of poultry mycoplasma vaccines against *Mycoplasma synoviae* and *Mycoplasma gallisepticum* in providing protection in ostriches against the ostrich-specific mycoplasmas *Ms01*, *Ms02* and *Ms03*.
- The identification, isolation and modification of a DNA vaccine candidate gene in the ostrich mycoplasma *Ms01* for subsequent DNA vaccine development against this mycoplasma.

In this thesis, Chapter 2 contains a literature review of the classification, evolution, phylogeny, genome characteristics, morphology, biochemistry, distribution, and pathogenesis of mycoplasmas. An overview of poultry and ostrich-specific mycoplasmas is given, as well as strategies for the development of new vaccines. Vaccine trials with existing poultry mycoplasma vaccines in ostriches are described in Chapter 3. In Chapter 4, the identification, isolation and modification of a possible DNA vaccine candidate gene of the ostrich-specific mycoplasma, *Ms01*, is described. A conclusion and future perspectives are given in Chapter 5, followed by a reference list and appropriate addenda including the statistical analysis of the ELISA results using the Statistical Analysis System (SAS), the nucleotide/amino acid sequence of the *P100* gene of *Ms01*, and the alignment of the *P100* gene sequence after site-directed mutagenesis (SDM).

Chapter 2 – Characteristics, pathogenicity and host specificity of mycoplasmas, and general approaches to vaccine development

2.1 Introduction

Mycoplasmas are cell wall-less bacteria known to be the smallest cellular organisms capable of self-reproduction. They are commensals as well as parasites of a wide range of hosts, in many cases causing disease (Razin, 1985). In order to develop new strategies for the prevention and control of infection with pathogenic mycoplasma species, it is necessary to have a clear understanding of their cellular mechanisms, and in particular, their mode of pathogenesis. In this literature review, the characteristics of mycoplasmas in general, including their classification, evolution, phylogenetic relationships, genome characteristics, morphology, biochemistry, distribution, as well as their pathogenicity, will be discussed. The focus will then be shifted to avian mycoplasmas, more specifically the two major pathogens of commercial poultry *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS), as well as the recently identified pathogenic ostrich-specific mycoplasmas *Ms01*, *Ms02* and *Ms03*. The epidemiology of these pathogens, as well as currently available treatments will be outlined, followed by a summary of strategies for the development of vaccines against mycoplasmas.

2.2 Taxonomy

Phenotypically, mycoplasmas are mainly distinguished from other bacteria by their complete lack of a cell wall (Razin, 1985). Furthermore, mycoplasmas are known for their minute size and uniquely small genome with their low guanine-and-cytosine (G+C) content, as well as a strict requirement for exogenous sterol (Weisburg *et al.*, 1989; Razin *et al.*, 1998; Bradbury, 2005). It is these most distinctive features that form the basis for the classification of mycoplasmas. Taxonomically, the lack of a cell wall is used to separate them from other bacteria, into a distinct class of prokaryotes named *Mollicutes* (derived from the Latin words ‘*mollis*’, meaning soft, and ‘*cutes*’, meaning skin) (Weisburg *et al.*, 1989; Razin *et al.*, 1998). Based on differences in morphology, genome size, and nutritional requirements, members of the class *Mollicutes* comprise five orders with the best studied genera being found in *Acholeplasmatales* (*Acholeplasma*), *Anaeroplasmatales* (*Anaeroplasma*, *Asteroleplasma*), *Entomoplasmatales* (*Entomoplasma*, *Mesoplasma*, *Spiroplasma*), and *Mycoplasmatales* (*Mycoplasma*, *Ureaplasma*) (Weisburg *et al.*, 1989; Razin *et al.*, 1998; Bradbury, 2005). A summary of the classification of the genus *Mycoplasma* within the class *Mollicutes* is given in Table 2.1. As a general rule, members of the orders *Acholeplasmatales*, *Anaeroplasmatales* and *Entomoplasmatales* are considered phylogenetically early mollicutes and accordingly have larger genome sizes than the phylogenetically more recently evolved *Mycoplasmatales* which often possess smaller genomes (Razin *et al.*, 1998). Furthermore, the requirement for exogenous sterol served as an important taxonomic criterion to distinguish the sterol-nonrequiring mollicutes, *Acholeplasma* and *Asteroleplasma*, from the sterol-requiring ones (Razin *et al.*, 1998; Weisburg *et al.*, 1989).

The majority of mollicutes that are of veterinary importance belong to the genus *Mycoplasma* (derived from the Greek words ‘*mykes*’ for fungus, which is ironic since mycoplasmas are not fungi, and ‘*plasma*’ for something formed or molded) (Bradbury, 2005). To date, more than 100 mycoplasma species have been identified, making this the largest genus within the class *Mollicutes*. It is therefore not surprising that the terms ‘mycoplasma’ and ‘mollicute’ are often used interchangeably to refer to any member within the class *Mollicutes* (Razin *et al.*, 1998). To avoid confusion, and since the genus *Mycoplasma* is the focus of this study, the term ‘mycoplasma’, and not ‘mollicute’, will be used for the remainder of this thesis.

2.3 Evolution

The origin of mycoplasmas was, for many years, quite a controversial topic. Given their unusually small size, both physically and genomically, along with the general simplicity they exhibit, it is understandable that some scientists proposed them to be a primitive life form, possibly preceding present-day bacteria in evolution. Others however, suggest that mycoplasmas were simply wall-less variants of typical bacteria (Woese *et al.*, 1980; Weisburg *et al.*, 1989). However, from nucleic acid hybridization and sequencing studies, it is known today that mycoplasmas originated by degenerate evolution from a low G+C content Gram-positive branch of walled eubacteria. This mode of mycoplasma evolution was accompanied by the loss of a substantial amount genomic sequence, ultimately resulting in the dramatic reduction in the genome size of mycoplasmas, and their consequent obligate parasitic lifestyle (Dubvig and Voelker, 1996; Razin *et al.*, 1998; Rocha and Blanchard, 2000).

Comparative genomics confirmed that the reduction in genome size associated with the degenerate evolution of mycoplasmas did not result from increased gene density or reduction in gene size, but did indeed result from the loss of ‘non-essential’ genes, an event often referred to as ‘gene-saving’. Genes involved in the gene-savings event included those encoding proteins involved in bacterial cell wall synthesis, as well as genes encoding enzymes involved in many anabolic pathways (Razin *et al.*, 1998). This resulted in the two main events of mycoplasma evolution; (i) the loss of a cell wall, (ii) and the loss of various metabolic capabilities (Woese *et al.*, 1980). The number of genes encoding enzymes involved in DNA replication and repair, transcription and translation and cellular processes such as cell division, cell killing, and protein secretion were also reduced. However, the amount of gene-saving in these categories was more restricted in order for mycoplasmas to preserve their own ‘housekeeping’ capabilities (Razin *et al.*, 1998). Accordingly it has been suggested that degenerate evolution of mycoplasmas, has resulted in a model for the minimum number of genes required for sustaining self-replicating life (Razin, 1985; Maniloff, 1992; Dubvig and Voelker, 1996; Maniloff, 1996). Examining the genomic data of mycoplasmas may therefore help to define the genes which are essential for life (Razin *et al.*, 1998).

TABLE 2.1 Summary of the major characteristics of members of the class *Mollicutes*, illustrating the classification of the genus *Mycoplasmas* within the class *Mollicutes*

Classification Class: <i>Mollicutes</i>	No. of species	Genome size (kb)	Mol% G+C	Unique nutritional requirements / special features	Sterol requirement	Habitat
Order: <i>Acholeplasmatales</i> Family: <i>Acholeplasmataceae</i> Genus: <i>Acholeplasma</i>	13	1500-1650	26-36	Optimum growth at 30°C-37°C	No	Animals, insects and plant surfaces
Order: <i>Anaeroplasmatales</i> Family: <i>Anaeroplasmataceae</i> Genus: <i>Anaeroplasma</i> <i>Asteroleplasma</i>	4 1	1500-1600 1500	29-34 40	Oxygen sensitive anaerobes	Sometimes Yes No	Rumens of cattle and sheep
Order: <i>Entomoplasmatales</i> Family: <i>Spiroplasmataceae</i> Genus: <i>Spiroplasma</i> <i>Entomoplasma</i> <i>Mesoplasma</i>	22 5 12	780-2220 790-1140 870-1100	24-31 27-29 27-30	Optimum growth at 30°C	Yes	Plants and insects
Order: <i>Mycoplasmatales</i> Family: <i>Mycoplasmataceae</i> Genus: <i>Mycoplasma</i> * <i>Ureaplasma</i>	120< 6	580-1350 760-1170	23-41 27-30	Optimum growth at 37°C Uses urea as energy source	Yes	Humans and animals
Undefined <i>Phytoplasma</i>	Not defined	530-1350	23-29	Optimum growth at 30°C	No	Plants and insects

*Class: *Mollicutes*, on basis of lack of a cell wall; Oder: *Mycoplasmatales*, based on exogenous sterol requirement; Family: *Mycoplasmataceae*, based on genome size; Genus: *Mycoplasma*

(Table adapted from: Robinson and Freundt, 1987; Razin *et al.*, 1998; Prescott *et al.*, 2002; Kleven, 2008)

2.4 Phylogeny

Based on sequence analysis of the conserved 16S ribosomal RNA (rRNA) genes, the phylogenetic relationship between mycoplasmas and bacteria has been established (Woese *et al.*, 1980). These analyses revealed mycoplasmas to be related to a branch of Gram-positive eubacteria with low G+C composition, and a clostridial phenotype (*Clostridium innocuum*, and *C. ramosum*) (Razin, 1985; Weisburg *et al.*, 1989). The genus *Mycoplasma* is further subdivided into four phylogenetic groups based on 16S rRNA gene sequence analysis; (i) the anaeroplasma group, (ii) the spiroplasma group, (iii) the pneumoniae group, and (iv) the hominis group (Dubvig and Voelker, 1996), which was also retrieved in our phylogenetic analysis as is shown in Figure 2.1.

2.5 Characteristics of the mycoplasmal genome

2.5.1 Genome size

The circular double-stranded genome of mycoplasmas is the smallest reported of all self-replicating cellular organisms, ranging in size from 580 kilobases (kb) in *M. genitalium* to 1380 kb in *M. mycoides* subsp. *mycoides* (Dubvig and Voelker, 1996; Razin *et al.*, 1998). The considerable amount of variability that exists in the genome sizes of different mycoplasma species, is possibly a result of high number of repetitive DNA elements found in mycoplasma genomes (Razin *et al.*, 1998).

2.5.2 Repetitive elements

Although repetitive DNA elements is not a feature expected to be found in a minimal genome, many mycoplasma species have been shown to harbour a high frequency of such elements. Repeated DNA sequences in the mycoplasmal genome include both multiple copies of protein-coding regions, as well as insertion sequence elements. Interestingly many of these repetitive elements are homologous to genes encoding major surface antigens, and may therefore promote DNA rearrangements associated with antigenic variation (see *Antigenic variation*, section 2.8.2.1) (Dubvig and Voelker, 1996; Razin *et al.*, 1998).

2.5.3 Base composition and codon usage

The mycoplasma genome is further known for its extremely low G+C content typically ranging from 23 to 41 mol%. The distribution of G+C along the mycoplasma genome is uneven, with coding regions generally being more G-C rich than the non-coding regions (Weisburg *et al.*, 1989; Razin *et al.*, 1998).

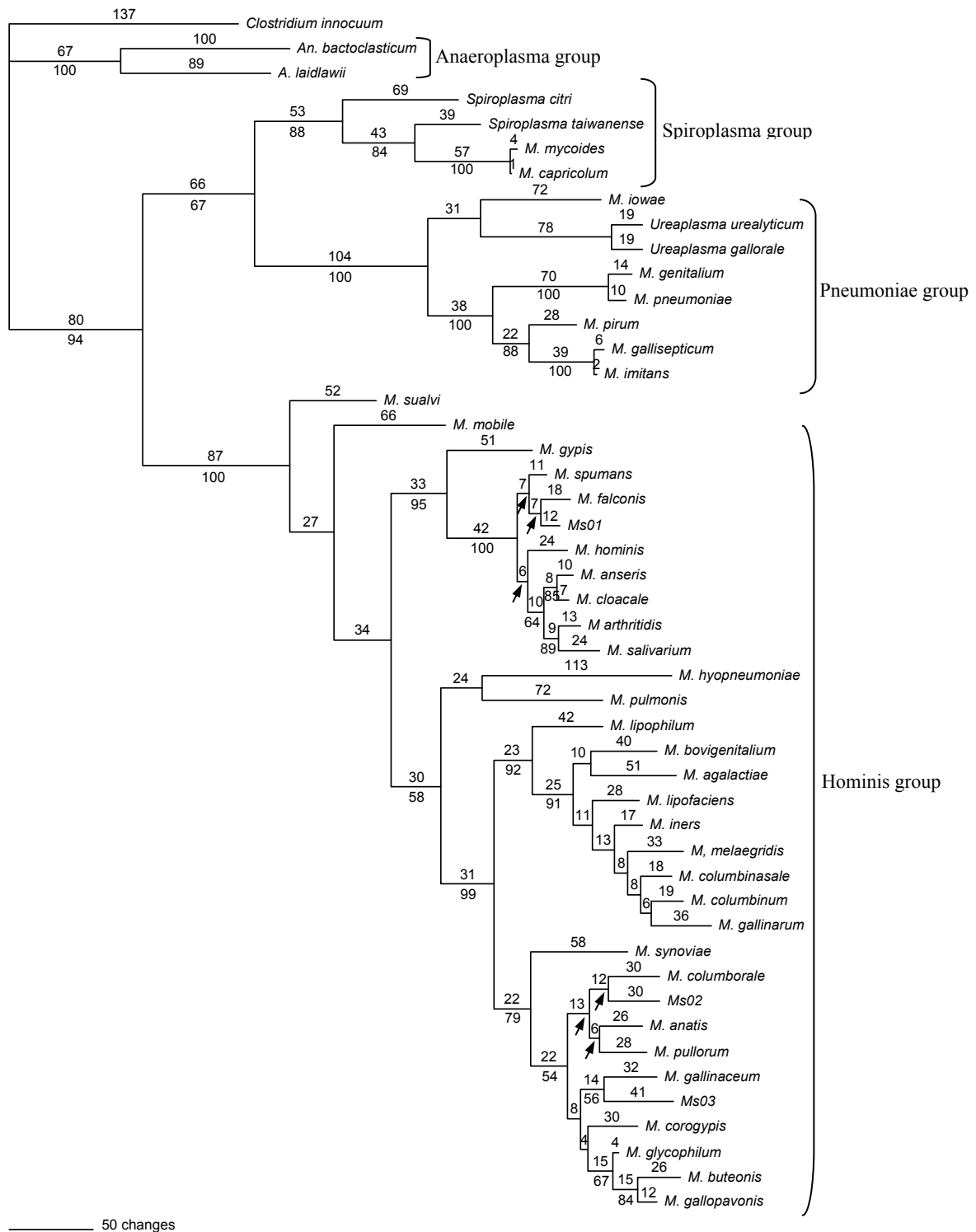


Figure 2.1 Phylogenetic tree of mycoplasmas based on analysis of 16S rRNA gene sequences. This tree represents one of twelve of the shortest trees retrieved in a heuristic search (CI = 0.401, RI = 0.703). Those branches that collapse in the strict consensus tree are indicated with arrows. Branch lengths and bootstrap values are indicated above and below the line respectively.

This characteristic base composition of the mycoplasma genome is manifested in their unique codon usage. Accordingly, mycoplasmas have evolved to preferentially use adenine (A)- and thymine (T)-rich codons (Razin, 1985). Indeed, codon usage data indicate that approximately 90% of codons in the

majority of mycoplasma genomes have an A or T in the third nucleotide position. This has the result that during translation, most mycoplasmas employ the alternative genetic code, known as the mold mitochondrial genetic code. In this code, the universally assigned termination codon TGA, encodes tryptophan instead, encoded by TGG in the universal genetic code (Dubvig and Voelker, 1996; Razin *et al.*, 1998; Söll and RajBhandary, 2006). Such an adaptation in codon usage has obvious practical implications when cloned mycoplasma genes are expressed in heterologous systems, as premature truncation of gene products will occur where the mycoplasma tryptophan codon will be read as a termination codon (Dubvig and Voelker, 1996; Razin *et al.*, 1998). Codon bias is not limited to the third nucleotide position, and is also evident in the first and second codon position, where it has a considerable effect on amino acid composition. For instance, relative to an organism such as *Escherichia coli* with a G+C content approximately 50 mol%, mycoplasmas have fewer GGN, CCN, GCN, and CGN codons. Therefore, mycoplasma proteins generally contain fewer glycine, proline, alanine and arginine residues. In contrast, mycoplasmas tend to have a high percentage AAN, TTY, TAY and ATN codons, resulting in an abundance of asparagine, lysine, phenylalanine, tyrosine, and isoleucine residues in mycoplasma proteins. In highly conserved proteins, mycoplasmas often have lysine residues (codons AAA and AAG) at amino acid positions that have arginine (codons AGA and AGG and CGN) in other organisms (Dubvig and Voelker, 1996).

2.5.4 DNA methylation

As is the case in other prokaryotic genomes, some of the adenine and cytosine residues in the mycoplasma genome may be methylated, resulting in 6-methyladenine and 5-methylcytosine (Razin *et al.*, 1998). In mycoplasmas, the adenine residue (A) at the GATC site is often methylated, while in others the cytosine residue (C) is methylated. Even though the exact biological function of DNA methylation is not clear, this phenomenon in prokaryotic genomes is suggested to provide protection of their DNA against the endonuclease activity of competing microbes within a given environment (Razin, 1985; Dubvig and Voelker, 1996; Xai, 2003).

2.5.5 Gene arrangement

Comparative analysis of the gene order in the genomes of *M. gallisepticum*, *M. hyopneumoniae* and *M. pulmonis*, revealed that there was no fixed arrangement of genes in these genomes. It was found however, that the order of genes within an operon encoding the cytoadhesin proteins GapA, CrmA, CrmB and CrmC, remained the same between the respective species, with only the genes adjacent to the operon varying (Van der Merwe, 2006).

2.5.6 Regulation of gene expression

2.5.6.1 Regulation of transcription

During the transcription of mycoplasma genes, expression signals largely resemble those of Gram-positive bacteria. Two RNA polymerase promoter areas, known as the -10 (Pribnow box) (TATAAT) and -35 regions (TTGACA/TTGNNN), have been identified in mycoplasma, both of which are similar to bacterial promoter consensus sequences recognized by the vegetative sigma factor σ^A . In addition, mycoplasma RNA polymerases show structural similarity to other prokaryote polymerases, although its activity is relatively insensitive to the antibiotic rifampin (Dubvig and Voelker, 1996).

2.5.6.2 Regulation of translation

With the exception of the stop codon TGA encoding tryptophan in most mycoplasmas, the translation of messenger RNA (mRNA) of mycoplasmas otherwise resembles that of Gram-positive bacteria. Nucleotide sequence data indicate that coding regions of most mycoplasma genes begin with an ATG start codon, with GTG and TTG serving as alternative start codons (Dubvig and Voelker, 1996). This is in agreement with most prokaryotes, as the translation initiation codon ATG interacts more tightly with the initiation transcript RNA (tRNA) than to the other initiation codons, therefore being the preferred initiation codon in frequently expressed genes (Sakai *et al.*, 2001). Furthermore, the mRNA of most mycoplasma genes contains a ribosome-binding site (RBS) similar to the Shine-Dalgarno (SD) sequence of Gram-positive bacteria. The typical mycoplasmal RBS has the sequence 5'-AGAAAGGAGG-3' (SD-like sequence) and is usually located four to ten bases upstream of the start codon, (Chen *et al.*, 1994; Dubvig and Voelker, 1996). The extent to which the SD sequence is conserved correlates with the translation efficiency of a gene. For frequently expressed genes, the ribosome needs to recognise the SD sequence more efficiently than in the case of rarely expressed genes. It should be mentioned that no SD-like sequence has been identified in *M. genitalium* or *M. pneumoniae*, suggesting that the translation process of these species does not depend heavily on these factors (Sakai *et al.*, 2001; Madeira and Gabriel, 2007).

2.5.6.3 Nature and posttranslational modification of expressed proteins

As mycoplasmas lack a cell wall and are bound by a plasma membrane only, there is no periplasmic space and proteins that are not cytoplasmic are either membrane bound or secreted. For protein secretion, mycoplasmas possess a typical eubacterial signal sequence ((-4)-VAASC-(+1)) that directs proteins into a secretory pathway to transport them across the plasma membrane (Henrich *et al.*, 1999). Posttranslational modification of mycoplasma proteins includes phosphorylation and isoprenylation, the function of which is not completely clear. In general, protein phosphorylation, through the action of kinases, phosphotransferases and phosphatases, is a mechanism for regulating intracellular signalling, modulating cellular events by interconverting between active and inactive protein forms. Therefore, in mycoplasmas,

the phosphorylation of cytoskeletal proteins may regulate activities such as cytoadherence, gliding motility, and cell division in the same manner (Razin *et al.*, 1998).

2.6 Morphology and Biochemistry

2.6.1 Cell size, shape and motility and reproduction

One of mycoplasmas' most distinctive features is their unusually small cell size, ranging from 0.3-0.8 μm in diameter (Weisburg *et al.*, 1989; Prescott *et al.*, 2002). Their lack of a cell wall and inability to synthesize peptidoglycan precursors render mycoplasmas completely resistant to penicillin and other antibiotics targeting cell wall synthesis, but susceptible to lysis by osmotic shock and detergent treatment (Prescott *et al.*, 2002). Since mycoplasmas are bound by a plasma membrane only, they are pleomorphic, varying in shape from spherical or pear-shaped organisms, to branched or helical filaments. An important group of pathogenic mycoplasmas have a flask shape with a protruding tip structure that mediates attachment to the host (see *Host cell attachment and ABC transporters as virulence factors*, section 2.8.1). The ability of mycoplasmas to maintain their respective cell shapes in the absence of a rigid cell wall is suggested to be made possible by a network of interconnected cytoskeleton-associated proteins, as well as by the incorporation of exogenous sterols into the plasma membrane as a stabilizing factor. The cytoskeleton is also thought to participate in cell division, motility, as well as the asymmetric distribution of adhesins and other membrane proteins along the cell surface (Razin *et al.*, 1998). Although mycoplasmas are generally considered to be non-motile, some species have been shown to exhibit gliding motility on liquid-covered solid surfaces. The exact mechanism of their motility has not been described, however some kind of chemotactic behaviour with a protruding structure in the direction of movement, has been suggested (Dybvig and Voelker, 1996; Razin *et al.*, 1998). The mode of reproduction of mycoplasmas is essentially not different from that of other prokaryotes dividing by binary fission. For typical binary fission to occur, cytoplasmic division must be fully synchronized with genome replication, and in mycoplasmas the cytoplasmic division may lag behind genome replication, resulting in the formation of multinucleated filaments. The factors coordinating the cell division process in mycoplasmas are to date not clearly understood (Razin *et al.*, 1998).

2.6.2 Metabolism

The loss of many of their biosynthetic pathways during degenerative evolution accounts for mycoplasmas' parasitic lifestyle (Prescott *et al.*, 2002). Analysis of sequenced mycoplasma genomes indicate that mycoplasmal genes encode a large number of proteins with functions related to catabolism and to metabolite transport, with few proteins related to anabolic pathways. Accordingly, mycoplasmas lack the capacity to synthesize molecules such as cholesterol, fatty acids, some amino acids, purines and pyrimidines, and therefore need to acquire these and other nutrients from their host (Dybvig and Voelker, 1996; Henrich *et al.*, 1999; Prescott *et al.*, 2002). As far as catabolic metabolism is concerned, mycoplasmas depend largely on glycolysis and lactic acid fermentation as a means of synthesizing ATP,

while others catabolize arginine or urea. The pentose phosphate pathway seems functional in at least some mycoplasmas, while none appear to have the complete tricarboxylic acid cycle. The electron transport system is flavin terminated, thus ATP is produced by substrate-level phosphorylation, a less efficient mechanism than oxidative phosphorylation (Prescott *et al.*, 2002; Razin *et al.*, 1998).

2.6.3 ABC transporters

Knowledge of the transport proteins of an organism can aid in the understanding of the metabolic capabilities of the organism. For example, the combination of transporters in a given organism can shed light on its lifestyle (Ren and Paulsen, 2005). Not surprisingly then, for a parasitic organism that must acquire most of its cellular building blocks from its host, a substantial number of transport proteins are encoded by the mycoplasma genome. Three types of transport systems have been identified to be involved in transport across the mycoplasma cell membrane, namely the ATP-binding cassette (ABC) transporter system, the phosphotransferase transport system, and facilitated diffusion by transmembrane proteins functioning as specific carriers. Of these, mycoplasmas depend mainly on ABC transporters which are involved in the import and export of a large variety of substrates, including sugars, peptides, proteins and toxins (Razin *et al.*, 1998).

2.6.3.1 Structure and assembly of ABC transporters

ABC transporters are widespread among living organisms, comprising one of the largest protein families. Structurally, ABC transporters are remarkably conserved in terms of the primary sequence and the organization of domains. Characteristic to ABC transporters is a highly conserved ATPase domain which binds and hydrolyzes ATP to provide energy for the import and export of a wide variety of substrates. This ATP-binding domain, also known as an ATP-binding cassette, forms the defining structural feature of ABC transporters, and contains two highly conserved motifs, the Walker A or P-loop (GXXXXGKT/S) and Walker B (RXXXGXXLZZZD) motifs (where X is any amino acid, and Z represents a hydrophobic residue), which together form a structure for ATP binding. The ATP-binding domain further contains a highly conserved signature sequence known as the C motif of linker peptide (LSGGQ/R/KQR) that is specific to ABC transporters and is located at the N-terminal with respect to the Walker B motif. The ATP-binding domain is further associated with a hydrophobic membrane-spanning domain, typically consisting of six putative α -helix membrane-spanning segments that constitute the channel through which substrate may be transported (Henrich *et al.*, 1999). In addition, ABC transporters may also include additional proteins with specific functions. In the case of Gram-positive bacteria and mycoplasmas, such proteins include substrate-binding proteins anchored to the outside of the cell via lipid groups, binding substrate and then delivering it to the membrane-spanning import complex (Garmory and Titball, 2004).

2.6.3.2 *The physiological role of ABC transporters*

This superfamily of ABC transporters has a wide range of functions in bacteria, allowing them to survive in many different environments. Some ABC transporters are importers responsible for the uptake a wide variety of substrates, including sugars and other carbohydrates, amino acids, di-, tri- and oligopeptides, polyamines, and inorganic ions. Others function as exporters and are responsible for the export of proteins, such as proteases and hemolysin, polysaccharides, and toxins, as well as the secretion of antibiotics in antibiotic-producing and drug-resistant bacteria (Razin et al., 1998; Garmory and Titball, 2004; Davidson and Maloney, 2007).

2.6.3.3 *The oligopeptide permease system of *M. hominis**

The oligopeptide permease (Opp) system is an ABC transporter responsible for the import of oligopeptides into bacteria (Henrich *et al.*, 1999). In *M. hominis*, the Opp system consists of four core domains, the OppBCDF domains, and a cytodherence-associated lipoprotein, P100, functioning as the substrate-binding domain OppA. The OppB and OppC subunits are integral membrane-spanning domains and possess conserved hydrophobic motifs characteristic to bacterial permeases (RTAK-KGLXXXI/VZXXHZLR in the OppB domain, and XAAXXZGAXXXRXIFXHILP in the OppC domain). Each domain typically contains six membrane-spanning α -helices forming the permease pathway for the transport of oligopeptides through the membrane. The OppD and OppF subunits are the peripheral ATPase domains that bind and hydrolyze ATP for the active transport of oligopeptides (Henrich *et al.*, 1999; Hopfe and Henrich, 2004). Uncharacteristic of a substrate-binding domain, the P100/OppA domain of *M. hominis* has been shown to contain the highly conserved Walker A and Walker B motifs, characteristic of the ATP-binding (OppD and OppF) domains. Therefore, in addition to the substrate-binding role, as well as its association with cytodherence, the P100/OppA domain is also described as the main ecto-ATPase of *M. hominis*. The role of the ecto-ATPase activity of the P100/OppA domain is unclear, however, several hypotheses for its physiological function exist. These include: (i) protection from the cytolytic effect of extracellular ATP by allowing splitting of the ATP released in the vicinity by the colonized cells, (ii) regulation of ecto-kinase substrate concentration, (iii) involvement in signal transduction, as well as (iv) possible involvement in cytodhesion (Hopfe and Henrich, 2004). Although the physiological role of the P100/OppA protein in *M. hominis* is largely speculative, no P100/OppA-deficient mutants have been identified to date, suggesting that P100/OppA plays an essential role in the vitality of the organism (Hopfe and Henrich, 2004).

2.6.4 *In vitro* cultivation

The difficulty with which mycoplasmas are cultivated *in vitro* is a major impediment in mycoplasma research. The most common explanation for mycoplasmas' weak cultivation properties are their numerous nutritional requirements brought about by the scarcity of genes involved in their biosynthetic pathways (Dubvig and Voelker, 1996; Razin *et al.*, 1998). To overcome these deficiencies, mycoplasmas

generally require a complex protein-rich growth medium containing serum, which provides the fatty acids and cholesterol required for membrane synthesis. In addition, mycoplasma growth medium often contain yeast derived components, as well as various sugars or arginine as primary energy source. Penicillin and thallium acetate are also often included to inhibit contaminant growth (Razin *et al.*, 1998; Kleven, 2008). Mycoplasmas demonstrate optimal growth at 37°C-38°C, and exhibit markedly diverse atmospheric requirements. Most mycoplasma species are facultative anaerobes usually favoring an anaerobic state, while many species also flourish in aerobic environments, with yet another group being obligate anaerobes (Razin *et al.*, 1998; Weisburg *et al.*, 1989; Prescott *et al.*, 2002).

Even in the most complex growth media, mycoplasmas still exhibit poor and slow growth rates (Kleven, 1998), raising the question whether the lack of growth in a rich medium is not rather due to the presence of a component or components that are toxic to mycoplasmas, thereby inhibiting their growth. However, the reason for mycoplasmas problematic *in vitro* cultivation remains unresolved (Razin *et al.*, 1998). When grown on agar, mycoplasmas form colonies with a characteristic “fried egg” appearance; growing into the medium surface at the centre while spreading outward on the surface at the colony edges, possibly reflecting their facultative anaerobic atmospheric requirements (Kleven, 1998).

2.7 Distribution and host specificity

Mycoplasmas are widely distributed in nature as saprophytes, as well as commensals and parasites of a broad range of mammalian, bird, reptile, insect, plant and fish hosts, with the list of hosts known to harbour mycoplasmas continuously increasing. In general, mycoplasmas tend to exhibit rather strict host and tissue specificity, a feature thought to reflect their nutritionally fastidious nature and obligate parasitic lifestyle. However, numerous reports of mycoplasmas crossing species barriers, as well as mycoplasmas being isolated from sites other than their normal specified niches, reflect a greater than expected adaptability of mycoplasmas to different environments (Dybvig and Voelker, 1996; Razin *et al.*, 1998; Pitcher and Nicholas, 2005). The primary habitats of mycoplasmas in animals are the mucous surfaces of the respiratory and urogenital tracts, the eyes, alimentary canal, mammary glands, and joints (Razin *et al.*, 1998; Rocha and Blanchard, 2000).

2.8 Pathogenicity of mycoplasmas

Despite mycoplasmas’ small size and general simplicity, many species have the ability to cause adverse effects in their hosts (Bradbury, 2005). Relatively little is known about the pathogenesis of mycoplasma infections, however, it is thought to be a complex and multifactorial process (Lockaby *et al.*, 1998; Kleven, 2008).

2.8.1 Host cell attachment and ABC transporters as virulence factor

Many mycoplasma species are well-recognized respiratory pathogens. As a first step to pathogenesis, mycoplasmas must adhere to and colonize the epithelial linings of the host they infect (Razin *et al.*,

1998), in many cases resulting in diseases, such as contagious bovine pleuropneumoniae in cattle caused by *M. mycoides*, chronic respiratory disease in chickens caused by *M. gallisepticum*, and pneumoniae in swine caused by *M. hyopneumoniae*. Attachment of mycoplasmas to the epithelial surfaces of their host is regarded to be a critical step during mycoplasma infections. This event, often also referred to as cytoadherence or adhesion, plays a key role as virulence factor during mycoplasma infection, particularly in cases where the pathogens are confined to the mucosal surfaces of their host (Kleven, 2008). Mycoplasma cytoadhesins are generally large integral membrane proteins having regions exposed on the mycoplasma cell surface (Henrich *et al.*, 1993; Dybvig and Voelker, 1996; Razin *et al.*, 1998; Evans *et al.*, 2005). Some mycoplasma species related to the human pathogen *M. pneumoniae*, including *M. genitalium* and *M. gallisepticum*, possess a specialized attachment organelle or tip structure that facilitates attachment to host cells (Henrich *et al.*, 1993; Dybvig and Voelker, 1996; Razin *et al.*, 1998). The best studied cytoadhesin is the P1 protein of *M. pneumoniae* (Dybvig and Voelker, 1996). The P1 protein is surface-localized, 165 kilodalton (kDa), trypsin-sensitive protein that clusters at the terminus of the attachment organelle of *M. pneumoniae* (Su *et al.*, 1987). Other well-known attachment proteins in mycoplasmas include the MgPa adhesin of *M. genitalium*, the GapA adhesin of *M. gallisepticum*, as well as the cytoadherence associated P100 protein of *M. hominis*. Like the majority of mycoplasmas, *M. hominis* lacks a well-defined attachment tip structure. The cytoadherence properties of such species are not well understood (Henrich *et al.*, 1993; Dybvig and Voelker, 1996). In addition, little is known about the ligand-receptor interactions that promote attachment to host cells. Two different types of receptors, sialoglycoproteins and sulfated glycolipids, have however been implicated (Razin *et al.*, 1998).

Since loss of cytoadherence have been shown to prevent infecting mycoplasmas from colonizing their target tissue and causing disease, attachment of mycoplasmas to their respective host cells is considered an initial and crucial step for colonisation and subsequent infection. Therefore, the membrane proteins that mediate this adhesion are regarded to be a crucial part of mycoplasmas' pathogenicity (Henrich *et al.*, 1993; Lockaby *et al.*, 1998).

ABC transporters have also been suggested to play an important role in the virulence of pathogenic organisms. Their association with virulence is most likely a reflection of their involvement in nutrient uptake, but may also indirectly result from associated substrate and/or host cell attachment (Garmory and Titball, 2004).

2.8.2 Evasion of the host's immune system

The immune system functions to protect an organism from foreign invading agents that may cause damage to the host. In order to persist and cause disease, some pathogens have developed means to evade the humoral immune system of their host (Evans *et al.*, 2005). Two well-known routes of evading the host's immune system are (i) antigenic variation, and (ii) internalization of the microbe into non-phagocytic host cells.

2.8.2.1 Antigenic variation

The pathogenesis of mycoplasmas is complicated by their ability to alter their antigenic profile by varying the expression of major immunogenic surface proteins, thereby evading the host's immune system, (Evans *et al.*, 2005; Kleven, 2008). Multiple surface exposed membrane proteins have been implicated in antigenic variation (Dybvig and Voelker, 1996; Evans *et al.*, 2005). Of these, lipoproteins are regarded the primary source of variation. The membranes of mycoplasmas contain an unusually high number of lipoproteins that are attached to the membrane via a lipid moiety or via hydrophobic amino acids, with a portion of the protein on the outer surface of the cell. Although the function of most lipoproteins in mycoplasmas is unknown, some, at least, are thought to undergo antigenic variation, resulting in a changing mosaic of antigenic structures of the cell surface (Dybvig and Voelker, 1996; Kleven, 1998; Rocha and Blanchard, 2002). Antigenic variation may be achieved by the on/off switching of multiple copies within a gene family, thereby resulting in alternate expression of the genes encoding antigens (Dybvig and Voelker, 1996; Kleven, 1998). Furthermore, genes encoding attachment proteins often contain repetitive elements that allow homologous recombination and genomic rearrangements, thereby also contributing to antigenic variation (Dybvig and Voelker, 1996; Razin *et al.*, 1998). This feature of mycoplasmas provides one possible explanation for how mycoplasmas manage to persist in a host and cause disease, often in spite of strong immune responses (Dybvig and Voelker, 1996; Kleven, 1998; Rocha and Blanchard, 2002).

2.8.2.2 Intracellular location

Most animal mycoplasmas are considered to be non-invasive surface parasites. Some species, such as *M. fermentans*, *M. genitalium*, *M. hominis* and *M. penetrans*, however, have the ability to penetrate and survive within the cells of their respective hosts (Razin *et al.*, 1998; Evans *et al.*, 2005). The suggested mechanism by which mycoplasmas enter their host cells involves initial attachment of the pathogen to the surface of the host cell. Host cell attachment is followed by certain cytoskeletal changes including; rearrangement of the microtubule and microfilament proteins, aggregation of tubulin and α -actinin, and condensation of phosphorylated proteins. This demonstrates yet another example of where adherence to their host cells plays a key role in mycoplasma pathogenesis, being the signal that prompts cytoskeletal changes (Razin *et al.*, 1998).

Entry into host cells allows mycoplasmas to persist in their host by evading the humoral immune system of the host, as well as exposure to antibiotics, promoting the establishment of chronic infection states. This may account, to some extent, for the difficulty with which mycoplasmas are eradicated from infected hosts (Razin *et al.*, 1998; Kleven, 2008).

2.8.3 Other possible virulence causal factors

2.8.3.1 Cell damage and disruption

During respiratory disease, mycoplasma colonization of the tracheal epithelial surface results in the loss of cilia movement, erosion of ciliated epithelial cells, and hypertrophy of nonciliated basal epithelial cells. Factors suggested to play a role in the cell damage and disruption include (i) the production of hydrogen peroxide and other toxic metabolic end products of mycoplasmas, and (ii) possible toxic extracellular components of the mycoplasma membrane (Lockaby *et al.*, 1998). In the case of invasive mycoplasmas, entry into the host cells may affect the normal cell function and integrity of the host cell, resulting in potential cell lysis, cell disruption and necrosis. In addition, exposure of the host cells' cytoplasm and nucleus to mycoplasmal endonucleases may cause chromosomal damage (Razin *et al.*, 1998). A less-documented factor also suggested to contribute to the pathogenesis of mycoplasmas is immune-mediated host injury through the stimulation of the hosts' autoimmune responses (Lockaby *et al.*, 1998).

2.8.3.2 Concurrent infections

Mycoplasmas are well-known for their tendency to have single or multiple interactions with other disease causing organisms such as Newcastle disease virus (NDV), Infectious bronchitis virus, and/or bacteria such as *E. coli*. These interactions often have the result that mild or even subclinical mycoplasma infections are aggravated, resulting in severe disease (Kleven, 1998).

2.8.3.3 Environmental factors

Mycoplasma infections, especially respiratory infections, are known to be notably affected by environmental factors, increasing the severity of diseases. Temperature fluctuation, as typically experienced during the change of seasons, humidity, atmospheric ammonia, and dust, have all been found to have important interactions with infecting mycoplasmas in producing respiratory disease (Kleven, 1998).

2.9 Mycoplasmas infecting domestic poultry

More than a dozen mycoplasma species are known to infect commercial poultry, of which the most prominent pathogenic species are MG, MS, *M. meleagridis*, and *M. iowae* (Kleven, 1998). Of these, MG and MS are considered the most important as they are the most widespread in commercial poultry, and as such are being the only ones listed by the World Organisation for Animal Health (OIE) (Kleven, 2008).

2.9.1 Epidemiology

2.9.1.1 Natural host

In general, poultry mycoplasmas tend to be host-specific and are not known to infect mammalian or other avian hosts (Kleven, 1998). However, MG is known to infect a wide range of bird species, of which

gallinaceous birds are most susceptible, while MS are almost exclusively restricted to chickens and turkeys (Kleven, 1998; Evans *et al.*, 2005).

2.9.1.2 Infection and transmission

MG is regarded the most economically important mycoplasma infecting commercial poultry, and is the leading cause of respiratory disease in chickens and infectious sinusitis in turkeys (Kleven, 1998; Evans *et al.*, 2005). MS is known to cause respiratory disease in chickens and turkeys that may result in airsacculitis and synovitis where spreading to the joints is thought to occur through the bloodstream (Kleven, 1998; Lockaby *et al.*, 1998). Both MG and MS infections are highly transmissible, being both spread vertically by egg-transmission, and horizontally through close contact between birds (Kleven, 1998; Evans *et al.*, 2005).

2.9.2 Clinical signs

Poultry mycoplasmas vary widely in virulence, displaying a wide variety of clinical manifestations, making them difficult to diagnose. A possible explanation for this is the high incidence of intraspecies variability that exists among different strains, as well as mycoplasmas' ability to interact with other disease-causing organisms and environmental factors (Kleven, 1998). The clinical signs of MG in infected poultry vary from subclinical to obvious respiratory signs including coryza, conjunctivitis (nasal exudate and swollen eyelids), rales, sinusitis, and severe air sac lesions ultimately resulting in increased mortality, downgrading of meat-type birds, reduced egg production and hatchability, higher feed usage and slow growth rates (Evans *et al.*, 2005). Birds infected with MS display signs of infectious synovitis manifested by pale combs, lameness and slow growth. Swelling may occur around the joints with viscous exudate in the joints and along the tendon sheaths, as well as greenish droppings containing large amounts of urates commonly being observed. In addition, milder clinical signs and lesions of respiratory disease, similar to those observed with MG, are often observed during MS infections (Kleven, 1998).

2.9.3 Diagnosis

MG and MS disease in chickens and turkeys may superficially resemble respiratory disease caused by other pathogens such as NDV and avian infectious bronchitis. For diagnostic purposes, MG and MS can be identified by immunological methods after isolation from mycoplasma media, immunofluorescence of colonies on agar, detection of their DNA in field samples and/or cultures by species-specific PCR, or isolated from other or unknown species by sequencing of the 16S rRNA gene (Kleven, 2008).

2.9.4 Prevention, treatment and control

Control of poultry mycoplasma infections is based on three general aspects: prevention, treatment, and vaccination. The preferred method for the control of mycoplasma infections in poultry is the maintenance of a mycoplasma-free flock as mycoplasmas pathogenic for poultry are transmitted vertically between birds. Although an affective biosecurity program in combination with consistent monitoring for signs of

infection should be adequate, ever increasing population density is however a common cause of lapses in biosecurity (Kleven, 2008).

Even though mycoplasmas are completely resistant to antibiotics that affect cell wall synthesis (Kleven, 1998), a limited range of antibiotics affecting protein production can be used to reduce the effects of MG and MS infections (Evans *et al.*, 2005). The two most commonly used antibiotics in poultry are tylosin and tetracycline. These antibiotics are employed as prophylactic treatment to respiratory disease associated with MG and MS in chickens and turkeys, and to reduce egg transmission of mycoplasma infection. A treatment program in infected birds typically consists of continuous medication in the feed, or treatment for 5-7 days each month. Although antibiotic treatment has proved to be an effective tool in preventing production losses associated with poultry mycoplasma infections, it has been shown to be ineffective at clearing mycoplasma infections, and should not be considered as a long-term solution as resistance may develop (Evans *et al.*, 2005; Kleven, 2008).

In situations where maintaining flocks free of MG and/or MS infection is not feasible, vaccination can be a viable option (Kleven, 2008). There are currently several live attenuated MG vaccines approved and commercially available (including F strain (FVAX-MG, Schering-Plough Animal Health), 6/85 (Mycovac-L, Intervet Inc), and ts-11 (MG vaccine, Merial Select)), to prevent egg-production losses in commercial layers, and to reduce egg transmission in breeding stock (Evans *et al.*, 2005). It is important that vaccination take place before field challenge occurs; one dose often being sufficient for vaccinated birds to remain permanent carriers. Administration of the vaccines may vary from vaccine to vaccine, and different methods including intramuscular or subcutaneous injection, intranasal or eyedrop administration, as well as aerosol and drinking water administration are employed. A number of inactivated, oil-emulsion bacterins against MG and MS respectively, reported to prevent respiratory disease, airsacculitis, egg production losses, and reducing egg transmission in poultry, are also commercially available. In the case of these bacterins, two doses, subcutaneously administered, are necessary to provide longterm protection (Kleven, 2008).

2.10 Mycoplasmas infecting ostriches

Mycoplasmas have been implicated, together with other pathogens such as *E. coli*, *Pseudomonas aeruginosa*, *Pasteurella* species, and *Avibacterium paragallinarum*, in certain clinical syndromes in feedlot ostriches in South Africa (Botes *et al.*, 2005; Verwoerd, 2000). Based on earlier research, poultry mycoplasmas were believed to be responsible for mycoplasma associated diseases in ostriches (Verwoerd, 2000). However, recent analysis of the 16S rRNA gene sequences of mycoplasmas isolated from ostriches in the Oudtshoorn district, revealed that ostriches in this district harbour three unique ostrich-specific mycoplasmas, named *Ms01*, *Ms02* and *Ms03* (until formally described) (Botes *et al.*, 2005). Phylogenetic analysis of the 16S rRNA gene sequences of these ostrich-specific mycoplasmas revealed them to be rather divergent from each other, falling in two different phylogenetic mycoplasma groupings (Figure 2.1, section 2.4). *Ms01* appears to be distinct from *Ms02* and *Ms03*, falling in a

different clade of the phylogenetic tree with *M. falconis* being its closest relative. On the other hand, *Ms02* and *Ms03* were found to be grouped together, and in the same clade as *M. synoviae*.

2.10.1 Ostrich-specific mycoplasmas

2.10.1.1 Infection and contributing factors

Ostrich-specific mycoplasmas are primarily associated with infections of the respiratory tract, causing inflammation of the nose, trachea and air sacs, as well as severe lung lesions. Infection of the respiratory tract of ostriches may have many direct and indirect consequences, including increased treatment costs, erosion disease, downgrading of carcasses, and increased susceptibility to secondary infections with pathogens such as *E. coli*, *Pseudomonas aeruginosa*, *Pasteurella* species, *Bordetella avium* and *Avibacterium paragallinarum*. These secondary infections commonly results in the build-up of pus in the sinuses and air sacs, fever, pneumoniae and septic infection results, which ultimately leads to higher mortality rates and productions losses (Botes *et al.*, 2005).

2.10.1.2 Clinical signs

Clinical signs of ostrich-specific mycoplasma infection in ostriches include nasal exudates, swollen sinuses, foamy eyes, rattle sounds in the throat, shaking of the head as well as excessive swallowing (Respiratory sickness in ostriches: Air sac infection, 2006).

2.10.1.3 Contributing factors

Factors that contribute to the incidence of ostrich-specific mycoplasma infections in ostriches include adverse weather conditions, stress, poor hygiene and lack of biosecurity. A higher incidence of mycoplasma infections in ostriches is recorded annually during the months of autumn and spring when temperature fluctuations occur. Furthermore, windy and wet weather, as typically experienced during the winter months in the Western Cape, causes an increase in the severity of mycoplasma infections by increasing the susceptibility of ostriches to secondary infections. Stress, brought about by transport of the birds, change in feed and high population density, as well as poor hygiene, such as dirty water troughs and moldy feed, are also said to be contributing factors to mycoplasma infections. Finally, poor biosecurity programs, such as mixing birds from different sources, presents the risk of mycoplasma spreading from infected to non-infected birds (Kleven, 1998; Respiratory sickness in ostriches: Air sac infection, 2006).

2.10.1.4 Prevention, treatment and control

Apart from good farming and biosecurity practises, there are currently no means of preventing infections of ostriches with ostrich-specific mycoplasmas. Furthermore, control of mycoplasma infections in ostriches is complicated by the fact that carrier conditions exist, that is, ostriches infected with mycoplasmas often do not show any symptoms. In addition, concealing tactics employed by these

pathogens allow them to evade the host's immune system, thereby rendering them difficult to eradicate. A number of antibiotics, such as tylosin, oxytetracycline, doxycycline, and advocin, are currently being employed to control *Ms01*, *Ms02* and *Ms03* infections in ostriches (Respiratory sickness in ostriches: Air sac infection, 2006). Although the use of these antibiotics has been shown to be effective in managing mycoplasma infections in ostriches, the mycoplasmas cannot be eradicated. For this reason, there is an urgent need for the development of a vaccine(s) against the ostrich-specific mycoplasmas *Ms01*, *Ms02* and *Ms03*.

2.11 Strategies in mycoplasma vaccine development

The concept of vaccination was first demonstrated over 200 years ago when Edward Jenner showed that prior exposure to cowpox could prevent infection by smallpox in humans. Over the last century, vaccines against a wide variety of infectious agents have been developed (Gurunathan *et al.*, 2000). Presently, numerous types of vaccines exist, including conventional whole-organism vaccines, as well as toxoids and protein-subunit vaccines. More innovative vaccines include conjugate and recombinant vector vaccines, as well as the more recently developed DNA vaccines.

2.11.1 Conventional vaccines

Most vaccines today are still whole-organism vaccines, being either (i) killed organism vaccines, typically consisting of a chemically or heat inactivated form of a previously virulent pathogen, or (ii) live, attenuated organism vaccines, consisting of disabled previously virulent organisms, or closely related less virulent strains of an organism. Live attenuated vaccines have the advantage of producing potent and long-lasting cell-mediated and humoral immunity, as these vaccines resemble natural infection closely. However, the risk for attenuated pathogens to mutate back to virulent wild-type strains exists. In contrast, killed organism vaccines are non-infectious, but also less immunogenic than attenuated vaccines, and produce humoral immunity only (Lechmann and Liang, 2000).

Whole-organism vaccine development requires the *in vitro* cultivation of the pathogen in large quantities. This approach has been successful in the development of whole-organism vaccines against the economically important poultry mycoplasmas MS and MG. These vaccines were used in the immunization trials in ostriches in this study to assess their efficacy in providing cross-protection against ostrich-specific mycoplasmas (see Chapter 3). However, the feasibility of whole-organism vaccine development against the ostrich-specific mycoplasmas is hindered due to the weak *in vitro* cultivation properties of mycoplasmas in general. For this reason, the alternative of DNA vaccine development was pursued in this study (see Chapter 4), and will be outlined in the following section.

2.11.2 DNA vaccines

The use of DNA rather than whole organisms to provide immunity against invading pathogens is a relatively new approach to vaccine development (Razin, 1985; Robinson and Torres, 1997; Garmory *et*

al., 2003). Historically, DNA vaccination is based on the influential study by Wolff and colleagues demonstrating that direct immunization with naked DNA resulted in the *in vivo* expression of the foreign protein within the muscle cells of mice (Wolff *et al.*, 1990). Present day DNA vaccines are constructed using recombinant DNA technology where a gene encoding a desired antigen is cloned into a eukaryotic expression vector. The recombinant plasmid is subsequently amplified in bacteria, followed by purification of the plasmid, after which the plasmid DNA is inoculated directly into the animal to be vaccinated. The plasmid DNA is taken up by the cells of the vaccinated animal, expressed, and the resulting foreign protein processed and presented to the immune system, thereby eliciting an immune response (Robinson and Torres, 1997; Garmory *et al.*, 2003).

2.11.2.1 Basic requirements for a DNA vaccine expression vector

The efficacy of a DNA vaccine greatly relies on the components of the expression vector employed. Therefore, an important first consideration when optimising the efficacy of a DNA vaccine is the choice of an appropriate expression vector that would allow optimal expression of the foreign DNA in eukaryotic cells (Gurunathan *et al.*, 2000). An example of a typical DNA vaccine expression vector is shown in Figure 2.2. The basic requirements of a DNA vaccine expression vector include: (i) a strong eukaryotic promoter, such as the most commonly employed virally derived promoters from the immediate-early region of the cytomegalovirus (CMV), (ii) a cloning site downstream of the promoter for insertion of the antigenic gene or genes, (iii) a polyadenylation signal, such as that isolated from the simian virus 40 (SV40), to provide stabilization of the mRNA transcripts, (iv) a selectable marker, such as a bacterial antibiotic resistance gene, which allows for plasmid selection during growth in bacterial cells, and (v) a bacterial origin of replication (*ori*) with a high copy number, enabling high yields of plasmid DNA upon purification from transformed cultures (Robinson and Torres, 1997; Garmory *et al.*, 2003; Gurunathan *et al.*, 2000).

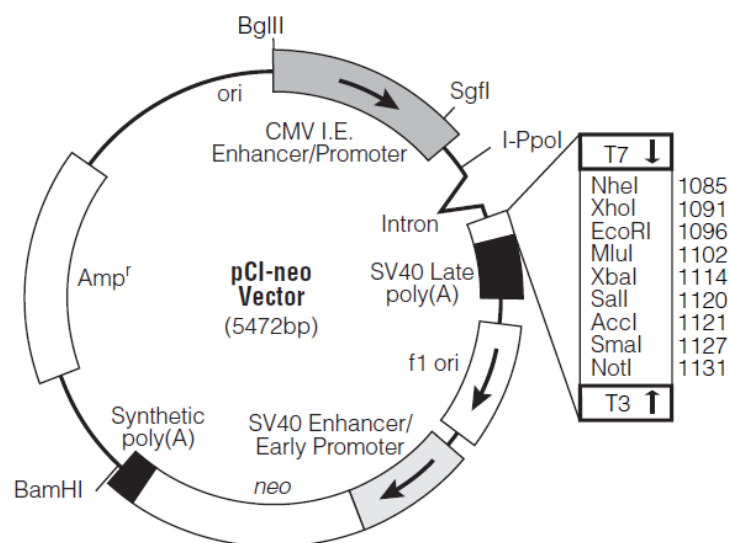


Figure 2.2 Example of a mammalian expression vector (pCI-neo, Promega) used in a typical DNA vaccine strategy.

2.11.2.2 Optimization of immunogenicity of DNA vaccines

A further important consideration when optimising the efficacy of a DNA vaccine is the optimization of the immunogenicity following administration. A number of components have been found to play an important role in the immunogenicity of DNA vaccines, one being the presence of unmethylated cytidine-phosphate-guanosine (CpG) motifs. These motifs are present at 20-fold higher frequencies in bacterial DNA than mammalian DNA, and are known to stimulate monocytes and macrophages to produce a variety of cytokines including interleukin (IL)-12, tumor necrosis factor (TNF)- α , and interferon (IFN)- α/β . These cytokines then act on natural killer cells to induce lytic activity and IFN- γ secretion. The CpG motifs can also stimulate the production of IL-6 that in turn promotes B-cell activation and subsequent antibody secretion. In addition, T-cells are also stimulated directly or indirectly by CpG motifs, depending on their baseline activation state. Since CpG motifs play such a prominent immunostimulatory role, incorporation of these motifs into the backbone of a vaccine vector, could serve to mobilize the immune response against the DNA-expressed antigen (Robinson and Torres, 1997; Gurunathan *et al.*, 2000; Garmory *et al.*, 2003).

The Kozak sequence is a consensus sequence flanking the AUG initiation codon within mRNA shown to play a role in the optimal translation efficiency of expressed mammalian genes, by influencing its recognition by eukaryotic ribosomes. Therefore, since many prokaryotic genes and some eukaryotic genes do not possess such a Kozak sequence, the expression level of these genes might be increased by the insertion of such a sequence (Garmory *et al.*, 2003).

Furthermore, the route of administration of DNA vaccines is also an important consideration as it plays a crucial role in determining the type of immune response elicited. Administration includes intramuscular (IM), intradermal (ID), subcutaneous, intravenous, intraperitoneal, oral, vaginal, intranasal, as well as non-invasive gene-gun delivery to the skin. The most commonly used methods are IM or ID saline injection, as well as gene-gun delivery; where the skin of the host is bombarded with DNA-coated gold beads (Robinson and Torres, 1997; Garmory *et al.*, 2003). Vaccination via gene-gun delivery initiates an immune response by transfecting epidermal Langerhans cells that move in the lymph from the skin to draining lymph nodes. Although this type of delivery is considered to be the best as it results in the transfection of the largest number of cells, it has obvious practical implications concerning the cost effectiveness of DNA vaccination, and does not seem practical for large scale implementation. Following IM injection, most DNA expression occurs in skeletal muscle, whereas following ID inoculations, most expression occurs in keratinocytes. In addition, DNA appears to move as free DNA through the blood to the spleen where professional antigen presenting cells (APCs) initiate an immune response (Robinson and Torres, 1997). Administration of DNA to the mucosal surfaces of their hosts as DNA drops in liposomes or in microspheres has been found to be less consistent and successful than IM, ID or gene-gun delivery. However, mucosal methods of DNA delivery hold promise for raising responses that selectively protect

the respiratory and intestinal surfaces that are major portals for the entry of pathogens (Robinson and Torres, 1997).

2.11.2.3 Dosage

The amount of DNA needed for IM and ID inoculation, DNA being introduced outside the cell, is up to 100 to 1000 times more than needed to raise an immune response during gene-gun bombardments, when DNA is shot directly into the cells (Robinson and Torres, 1997). Interestingly, the amount of DNA required to raise an immune response is suggested to be independent of the size of the vaccinated animal, with fairly similar doses of DNA being used to raise responses in mice, calves, and monkeys. Most immunizations of DNA into mice have used between 1 and 100 µg of DNA, while immunizations into monkeys and calves range from 10 µg to 1 mg of DNA. Gene-gun inoculation requires the least amount of DNA ranging from 10ng to 10 µg in mice. Although much remains to be determined regarding the dosage of DNA vaccines, the relative independence of the dosage and the size of the animal suggest that similar numbers of APCs are able to induce immune responses throughout the animal kingdom (Robinson and Torres, 1997).

2.11.2.4 DNA vaccine raised immune responses

Many factors may affect the efficiency and nature of a DNA-induced immune response, including the type of expression vector employed, the method of DNA delivery, as well as the type of antigen presentation (B lymphocyte, T lymphocyte, or both) to the hosts' immune system (Robinson and Torres, 1997). An illustration of the suggested mechanism by which DNA vaccines elicit immunity upon IM administration, is shown in Figure 2.3.

Once a gene encoding an appropriate antigenic protein has been identified and isolated, it is subsequently inserted into a suitable eukaryotic expression vector. This is followed by mass production in bacteria, plasmid DNA isolation, and subsequent inoculation of the purified recombinant plasmid DNA directly into the animal to be vaccinated. The mechanisms by which the antigen is produced within the cells of the immunized animal, is unclear. However, following the processes of antigen production and processing, the pathogen-derived peptides are suggested to be presented to the immune system by both the major histocompatibility complex (MHC) class I molecules (stimulating CD8⁺ T-cells) as well as MHC class II molecules (stimulating CD4⁺ T-cells) of local APCs, thereby inducing both cellular and humoral immunity (Oshop *et al.*, 2002). It should be noted that even though the principle of DNA vaccination is relatively simple, many details regarding the mechanisms of action are still unknown.

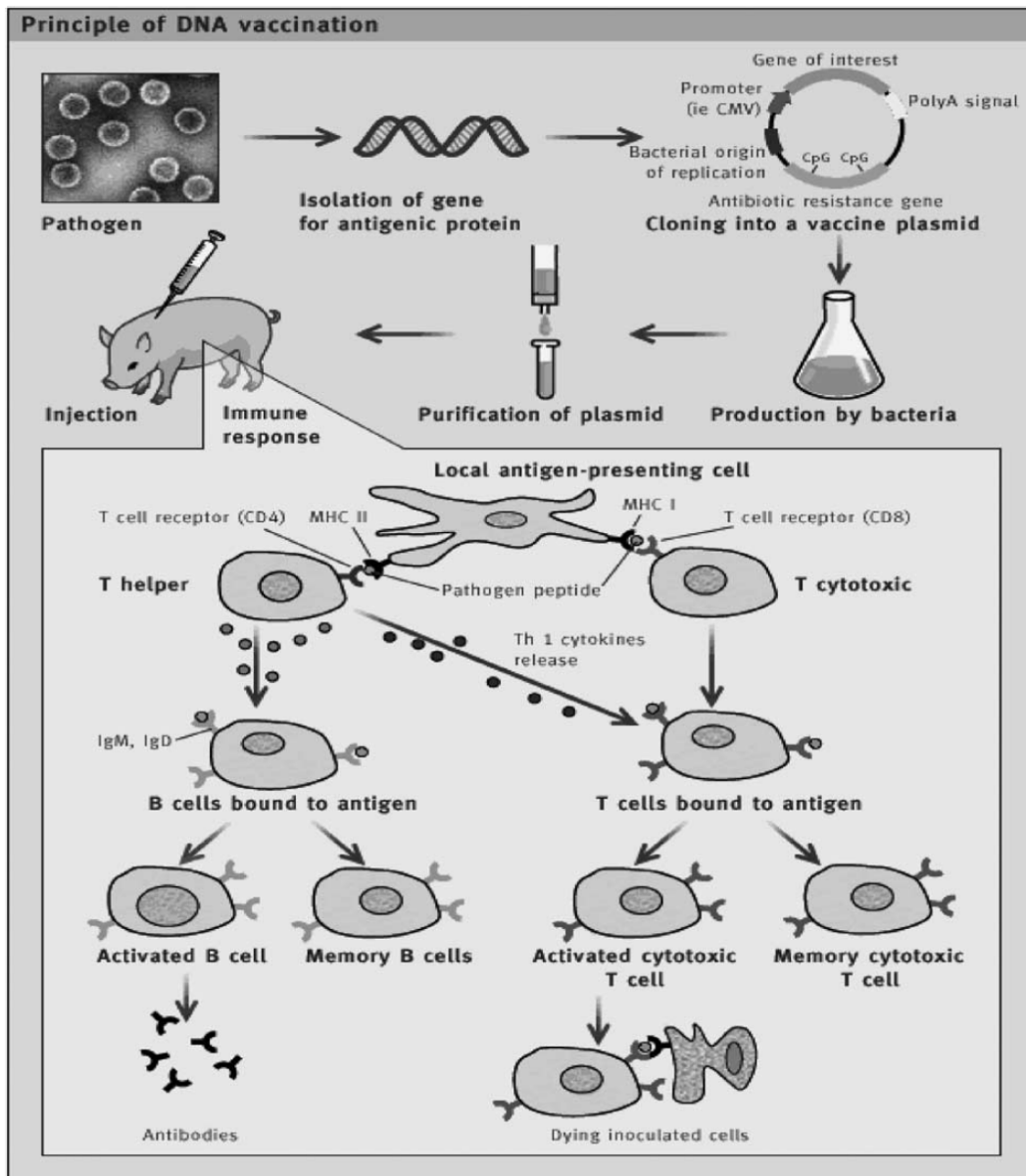


Figure 2.3 Principle mechanism of induced immunity by DNA vaccines (Oshop *et al.*, 2002).

2.11.3 Advantages of DNA vaccinology

A major advantage of immunization with DNA vaccines stems from their ability to activate both humoral and cellular immunity. In the case of extracellular viral and bacterial infections, protection is mediated by the humoral immune response, i.e. through the production of antibodies blocking the activity of extracellular forms of invading pathogens. On the other hand, intracellular pathogens are controlled by cell-mediated immunity, killing off pathogen-infected cells. However, in some cases (such as malaria, and possibly mycoplasmas) both humoral and cellular immune responses may be required to provide protection against the given pathogen. Accordingly, the ability of DNA vaccines to induce both humoral and cellular immunity is the major attribute of this strategy. Although DNA vaccines mimic the effects of live attenuated vaccines in this way, DNA vaccines have the advantage of not posing risk of infection, thereby undermining the safety concerns associated with live vaccines (Robinson and Torres, 1997;

Gurunathan *et al.*, 2000). Another advantage of DNA vaccines is the elimination of the need for large-scale *in vitro* cultivation of pathogens for the development of the vaccine (Rappuoli, 2001). Therefore, the route of DNA vaccine development is especially attractive for use against fastidious or non-cultivable pathogens, a problem often associated with mycoplasmas. Furthermore, DNA vaccines are relatively simple and cost-effective to develop, and require greatly simplified transport and storage needs due to the stability of DNA over a wide temperature range (Gurunathan *et al.*, 2000; Robinson and Torres, 1997).

2.11.4 Candidate genes for DNA vaccine development

The first step in DNA vaccine development is the identification of an appropriate candidate gene, i.e. a gene encoding a protein with good immunogenic properties. Structures of a pathogen interacting with the host, as well as proteins associated with the virulence of pathogens, are thought to be especially immunogenic (Henrich *et al.*, 1993). Accordingly, genes encoding membrane proteins, such as proteins mediating attachment of mycoplasmas to their host cells, and ABC-transporters responsible for the uptake of various nutrients from their host, are generally considered as good candidates for DNA vaccine development (Garmory and Titball, 2004).

In order to identify such DNA vaccine candidate genes, information concerning the genetic makeup of the target pathogen is required. To this end, the genetic information of a wide range of organisms, including many pathogens, for which genomes have been completely or partly sequenced, are available on genetic databases such as GenBank, which contains an annotated collection of all publicly available DNA sequences. A combination of bioinformatic approaches and recombinant DNA technology can then be used to identify and isolate genes to serve as vaccine candidates. Genomic libraries (also known as DNA libraries) are commonly employed in cases where little or no genetic information is available for a given pathogen. In order to construct a genomic library, the genomic DNA is fragmented, each fragment cloned and replicated separately in bacteria, and the clones screened to identify individual genes. Where the construction of a genomic library is not possible, as was the case in this study, alternative techniques for generating genomic data, such as whole genome sequencing, can be considered.

2.11.5 Whole-genome sequencing of mycoplasma genomes

The sequencing of whole bacterial and viral genomes can potentially play an important role in the development of new antibiotics and vaccines. Comparison of the entire genome sequences of pathogens can lead to the identification of conserved antigenic regions, and therefore the identification of possible candidate genes to be used in subunit vaccine development (Leamon *et al.*, 2007). Whole-genome sequencing usually requires the cloning of DNA fragments into bacterial vectors, amplification and purification of individual templates, followed by Sanger sequencing, using fluorescent chain-terminating nucleotide analogues, and either slab gel or capillary electrophoresis (Margulies *et al.*, 2005).

Since the late 1980s, several laboratories initiated independent projects focusing on the sequencing and genetic mapping of different mycoplasma genomes. The first large-scale studies directed at sequencing entire mycoplasma genomes were initiated in the early nineties. One of the first mycoplasma genomes to enter such a study was that of *M. capricolum*. However, by 1995, less than a quarter of the 1000 kb genome of *M. capricolum* had been sequenced successfully. The genome of *M. pneumoniae* was sequenced by first constructing a cosmid library, followed by sequencing both DNA strands in a directed fashion by primer walking, limiting random (shotgun) sequencing to a minimum. The 800 kb genome of *M. pneumoniae* was subsequently sequenced successfully over a period of three years. For the sequencing of the 580 kb genome of *M. genitalium*, an application of whole-genome shotgun sequencing was employed. Random fragmentation of genomic DNA (gDNA), followed by cloning and sequencing of the individual fragments, and the subsequent reassembly of the overlapping sequenced fragments, resulted in the entire *M. genitalium* genome being sequenced in under 6 months (Razin *et al.*, 1998).

It is evident that whole-genome sequencing can become a rather time consuming, strenuous and expensive process, and although alternative sequencing methods have been described, no technology had displaced the use of bacterial vectors and Sanger sequencing as the main generators of sequence information (Margulies *et al.*, 2005). Recently however, Margulies and colleagues (2005) described the novel 454 Sequencing System that circumvents subcloning into bacteria, as well as the handling of individual clones (Leamon *et al.*, 2007).

2.11.5.1 The 454 Sequencing System using GS20 sequencing technology

The 454 Sequencing System is based on GS20 sequencing technology that allows high-throughput, sequencing-by-synthesis to be performed in parallel. The system combines an emulsion-based method to isolate and clonally amplify DNA fragments *in vitro*, with modified pyrosequencing in picoliter-sized wells. Consequently, the 454 Sequencing System provides significantly higher throughput than any of the other existing sequencing technologies (Rogers and Venter, 2005), and allows high accuracy whole-genome sequencing at relatively low cost and with reduced effort and time (Margulies *et al.*, 2005).

An illustration of the GS20 sequencing method is shown in Figure 2.4. In order to sequence the entire genome of an organism, a DNA library is first created by shearing the isolated gDNA of the organism to be sequenced into fragments between 300 and 800 bp in length (Figure 2.4 A). Next, specialized A- and B-adaptors carrying priming sequences are ligated to the ends of the fragments (Figure 2.4 B). The B-adaptor carries a biotin tag that allows binding of the individual fragments to the surface of streptavidin coated capture beads in a water-in-oil emulsion. The simultaneous amplification of fragments is achieved by isolating individual DNA-carrying beads in separate aqueous droplets, each droplet serving as a separate microreactor in which parallel DNA amplification takes place (Figure 2.4 C). Each individual fragment is subsequently clonally amplified by emulsion PCR (emPCR), yielding approximately 10^7 copies of a template per bead (Figure 2.4 D). Following emPCR, the capture beads containing clonally amplified fragments, together with enzyme beads containing immobilized ATP, sulphurylase and

luciferase necessary for pyrosequencing, are loaded into the open wells of a fiber optic picotitre slide. The slide is subsequently mounted in a flow chamber through which sequencing reagent containing the individual nucleotides (A, T, G and C) flow cyclically (Figure 2.4 E). Upon incorporation of a nucleotide into the growing DNA strand, inorganic pyrophosphate is released and converted to ATP by the sulfurylase. ATP in turn serves as substrate for luciferase, which generates photons proportional to the quantity of nucleotides incorporated in the elongating DNA strand (Figure 2.4 F).

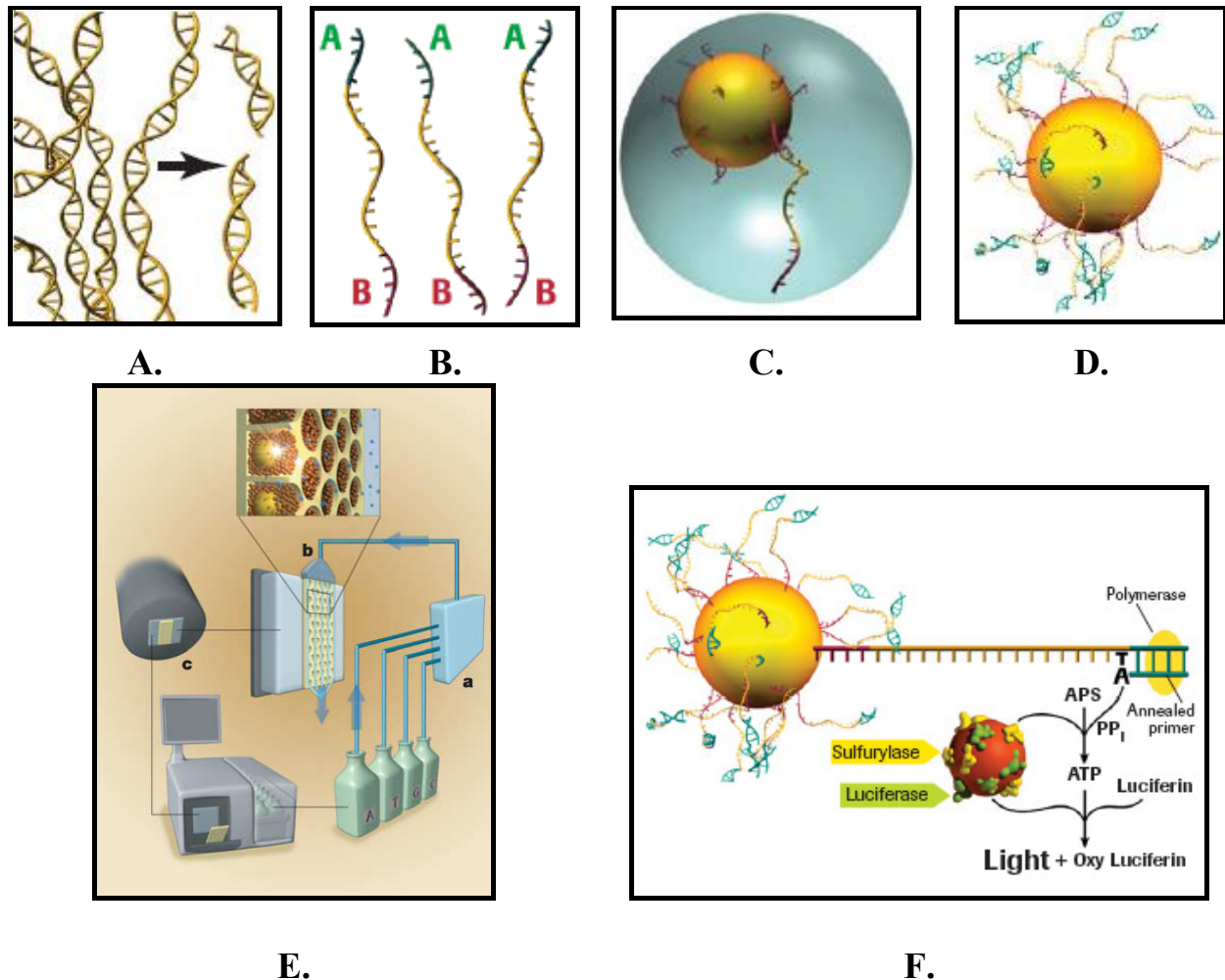


Figure 2.4 Schematic illustration of the GS20 method. (A). Shearing of the isolated gDNA into 300-800 bp fragments. (B). Ligation of specialized A- and B-adaptors to individual fragments. (C). Water-in-oil emulsion forming the microreactor for emPCR. (D). Multiple clonally amplified copies of a single fragment contained on an individual capture bead to be sequenced. (E). Overview of the 454 Sequencing system. (F). The sequencing-by-synthesis process based on pyrosequencing (Margulies *et al.*, 2005; Leamon *et al.*, 2007).

The resulting light signal is captured by a charge-coupled device (CCD) camera, allowing the capture of emitted photons from the bottom of each individual well, and the signal to be converted into nucleotide sequence. The resulting nucleotide sequences are subsequently subjected to assembly using a *de novo* shotgun sequence assembler program that forms part of the GS20 data processing software package. The assembler consists of a series of modules: the 'Overlapper', which finds and creates overlaps between reads; the 'Unitigger', which constructs larger contigs (contiguous sequences) of overlapping sequence

reads; and the ‘Multialigner’, which generates consensus calls and quality scores for the bases within each contig (Margulies *et al.*, 2005).

GS20 sequencing is an effective method through which whole genomes of organisms can be sequenced, and was employed in this study for the sequencing of the genome of the ostrich-specific mycoplasma *Ms01*, and suitable DNA vaccine candidate genes were subsequently identified in the whole genome sequence data (see Chapter 4).

Chapter 3 – Poultry Mycoplasma Vaccine Trials in Ostriches

3.1 Introduction

Of the more than a dozen mycoplasma species known to infect poultry, the most widespread and prominent mycoplasmas infecting commercial poultry are MG and MS. Vaccines available for the control of MG and MS infections in chickens and turkeys include inactivated, oil-emulsified bacterins, live vaccines, as well as recombinant vaccines, all of which have been shown to prevent airsacculitis, respiratory disease, egg production losses, as well as vertical transmission of disease between birds (Kleven, 2008).

There are currently no mycoplasma vaccines available for use in ostriches. For this reason, a new strategy based on the close phylogenetic relationship of the two ostrich-specific mycoplasmas *Ms02* and *Ms03*, to the poultry mycoplasma MS (Botes *et al.*, 2005) was developed. This entails the use of a vaccine against MS to provide possible protection against mycoplasma infections in ostriches. This approach was further supported by a preliminary study using immunofluorescence, in which it was found that the natural anti-mycoplasma antibodies in ostriches showed cross-reactivity with MS (Morley, personal communication, 1999). For these reasons, a preliminary study using commercially available poultry mycoplasma vaccines to prevent mycoplasma infections in ostriches, was launched (Van der Merwe, 2006). The study showed that MS and MG vaccines did elicit immune responses in three different groups of ostrich chicks aged three months, four to five months and six to seven months. However protection could not be assessed as only one vaccination was given, and the vaccinated birds were not challenged.

In this study, trials, using vaccines against MG and MS, were again launched in order to determine whether commercially available poultry mycoplasma vaccines can provide protection against mycoplasma infections in ostriches. To this end, 8-10 week old ostrich chicks received initial and booster vaccinations, followed by assessment of the resulting antibody responses by an enzyme-linked immunosorbent assay (ELISA). Resistance to infection with ostrich mycoplasmas was assessed by field challenge of the immunized ostriches after booster vaccinations, followed by visual inspection of the birds.

3.2 Materials and Methods

3.2.1 Poultry mycoplasma vaccine trials at Oudtshoorn

In these vaccine trials, the two inactivated oil-emulsion vaccines “Mycoplasma Synoviae Bacterin” (MS-Bac), and “Mycoplasma Gallisepticum Bacterin” (MG-Bac) were used. MS-Bac is commonly administered to laying and breeding birds to prevent egg production losses, as well as respiratory disease, and leg abnormalities associated with MS in chickens and turkeys. MG-Bac, on the other hand, prevents egg production losses and respiratory disease associated with MG in many bird species, especially

gallinaceous birds. Prior to use, the oil was removed by centrifugation to prevent the occurrence of granulomas and abscesses following subcutaneous immunization as oil-emulsion vaccines are known to cause these unacceptable side-effects in ostriches.

Ostriches from two farms in the Oudtshoorn district, the Kwessie and the Schoeman farmstead, where a high annual incidence of mycoplasma infections was reported, were included in the trials. A total of 140 and 77 ostrich chicks (8-10 weeks old, 15-20 kg), none of which showed symptoms of mycoplasma infection, were selected from the Kwessie and Schoeman farms, respectively. Chicks from the Kwessie farm were in a better condition than chicks from the Schoeman farm, where in many cases the masses of chicks from the Kwessie farm exceeded 20 kg. On each farm, ostriches were divided into three groups: group A and group B were vaccinated with MS-Bac and MG-Bac respectively, while ostriches in the control group underwent no vaccination.

The general strategy that was followed was to vaccinate the ostrich chicks in the summer months during which there is generally a low risk of natural mycoplasma infections. Immunity was then assessed in autumn when there is a much higher risk of mycoplasma infections.

3.2.2 Immunizing schedule and serum sample collection

On the first day of the vaccine trials, designated day 0, pre-immunization serum samples were collected from all of the ostriches in all the groups, followed by subcutaneous immunization in group A and group B with 1 ml oil-free MS-Bac and MG-Bac respectively, while the control group received no vaccine.

Trials were influenced by adverse weather condition in the Oudtshoorn vicinity during the trial period, which made access to these farms impossible on the dates that were originally planned for serum sample collection. As a result, serum collection dates had to be adjusted, and were not taken at weekly intervals as originally planned. In some instances, the collection dates of serum samples were missed, which subsequently influenced the interpretation of the results.

Serum samples were collected from each ostrich in each group on days 6, 14, and 21 on the Kwessie farm, and on day 7 on the Schoeman farm, after the first immunization. Thirty-eight days after the commencement of the vaccine trial, the ostriches in group A and B on the Kwessie farm received a second subcutaneous vaccination with 1 ml oil-free MS-Bac and MG-Bac respectively, following serum samples being collected. Ostriches in the control group received no vaccine. Serum samples were subsequently collected from each ostrich in each group on day 52, 62, 83 and 111 on the Kwessie farm. Twenty-seven days after the commencement of the vaccine trial, the ostriches in groups A and B on the Schoeman farm received a second subcutaneous vaccination with 1 ml oil-free MS-Bac and MG-Bac respectively, following serum samples being collected. Ostriches in the control group once again received no vaccine. Serum samples were subsequently collected from each ostrich in each group on day

34, 45, 53 and 83 on the Schoeman farm. The collected serum samples were stored at 4°C for immediate use, and at -20°C for long-term storage.

3.2.3 Field challenge with ostrich mycoplasmas *Ms01*, *Ms02* and *Ms03*

After the final serum samples were collected, immunized ostriches and those in the control group were mixed with unimmunized flock on the respective farms during the high risk autumn period. The reason for this approach of challenging immunized birds was that re-infection with cultivated mycoplasmas was not possible due to the poor cultivation properties of mycoplasmas. Thus, the infection of the vaccinated and control birds was based on natural horizontal transfer of mycoplasmas from one bird to another in a natural field situation. Eight weeks after exposure to unimmunized flock, the ostriches were visually inspected for symptoms usually associated with mycoplasma infections such as nasal exudates, foamy eyes and swollen sinuses.

3.2.4 Enzyme-linked immunosorbent assay

In order to assess the serum antibody production in response to vaccination, the ELISA technique was employed. This technique can provide the relative antibody concentration of a sample, and was chosen for its simplicity, specificity, sensitivity, commercial availability and adaptability. Two commercially available ELISA kits for the detection of MS and MG antibodies in chicken and turkey serum were used. The microtitre plates were coated with the respective antigen by the manufacturer. In the ELISA, antibodies present in the serum of the vaccinated ostriches were allowed to bind to the immobilized antigen in the wells of the microtitre plate. For the detection of the bound antibodies, labeled specific secondary antibodies, i.e. biotinylated rabbit anti-ostrich immunoglobulin (Ig) antibodies, were used. These secondary antibodies were used since the use of antibodies raised against poultry immunoglobulins, as those supplied in the kits, do not react well with ostrich antibodies (Blignaut *et al.*, 2000). A horse radish peroxidase (HRP) - Streptavidin conjugate was used for the detection of the bound biotinylated rabbit anti-ostrich Ig antibodies. This system was chosen for its high sensitivity and the low background levels. The colorless substrate 2,2'-Azino-di(3-ethylbenzthiazoline sulphonic acid-6) (ABTS) is converted to a green product in the presence of hydrogen peroxide (H₂O₂), which was then measured spectrophotometrically to determine the antibody concentration of a serum sample.

For use in the ELISA, rabbit anti-ostrich Ig was first isolated and biotinylated, followed by a modified protocol of the ELISA technique with MS and MG Antibody Test kits, FlockChek Ms and Mg respectively (IDEXX Laboratories, Dehteq), after which the results were analyzed statistically.

3.2.4.1 Isolation and biotinylation of rabbit anti-ostrich Ig

To precipitate the Ig fraction, 500 µl of high titre rabbit anti-ostrich Ig serum was added to 1 ml of phosphate buffered saline (PBS, pH 7.2) in a 50 ml JA-20 centrifuge tube. A volume of 1.5 ml saturated

ammonium sulphate was added, and the mixture kept at 4°C for 20 min followed by centrifugation at 27 200 x g for 20 min. After centrifugation, the supernatant was decanted and the pellet redissolved in 1 ml PBS. A volume of 1 ml saturated ammonium sulphate was again added, and the mixture kept at 4°C for 20 min followed by centrifugation at 27 200 x g for 20 min. The supernatant was decanted and the remaining pellet redissolved in 500 µl PBS. The resulting Ig fraction was dialyzed overnight against carbonate buffer (0.1 M, pH 8.3) at 4°C, replacing the buffer with fresh carbonate buffer approximately 8 hours after starting dialysis.

Following isolation, the Ig concentration was determined by measuring the absorbance at 280 nm. To this end, the Ig sample was diluted 1:10 in carbonate buffer. The rabbit anti-ostrich Ig solution was subsequently diluted with carbonate buffer to a final concentration of 5 mg/ml. For biotinylation, 2 mg biotinamidocaproate N-hydroxysuccinimide ester (Sigma) was dissolved in 1 ml N,N-dimethylformamide (DMF). The solution was then slowly added to the Ig fraction (250 µl biotinylation reagent per 1 ml Ig fraction) while stirring gently for 2 hours at room temperature. The prepared conjugate was subsequently dialyzed overnight against PBS at 4°C, replacing the solution with fresh PBS approximately 8 hours after starting dialysis. Finally, glycerol was added in a 1:1 ratio to the biotinylated rabbit anti-ostrich Ig preparation, mixed thoroughly and stored at -20°C.

3.2.4.2 Detection of humoral Ig antibodies to MS and MG in ostrich serum

Only the 96-well microtitre antigen coated plates and sample diluent buffer (Reagent 5) of the MS and MG Antibody Test kits were used (for reasons previously stated reasons in section 3.2.4). All serum samples from ostriches immunized with MS-Bac or MG-Bac, as well as unimmunized control birds, were assayed with both the MS and MG antigen coated plates.

Serum samples collected on days 0, 6, 14, 21, 38, 52, 62, 83 and 111 (Kwessie farm), and days 0, 7, 27, 34, 45, 53, 83 (Schoeman farm) were diluted 1:500 with diluent buffer from the respective kits. The first column on each plate served as a control; containing all reagents except the ostrich serum to be analyzed. Furthermore, to assess the efficacy of the isolated rabbit anti-ostrich Ig (section 3.2.4.1) a positive control of ostrich serum previously shown to possess a strong antibody titre was included (results not shown). Of the diluted sera, 100 µl was pipetted in duplicate into the wells of each MS and MG antigen coated microtitre plates respectively, and the plates incubated at 37°C for 3 hours. Following incubation, the serum was decanted and the wells washed three times with PBS-Tween (200 µl/well, PBS buffer with 0.1% Tween-20).

Biotinylated rabbit anti-ostrich Ig was diluted 1:100 in Casein-Tween buffer (0.01 M Tris-HCl, pH 7.6, containing 0.5% casein, 0.15 M NaCl, 0.02% thiomersal, 0.1% Tween) and added to the microtitre plates, 100 µl/well, followed by incubation of the plates at 37°C for 1 hour. After incubation, the contents of the plates were decanted and the wells washed with PBS-Tween as described above.

Streptavidin horse radish peroxidase (AVPO) was diluted 1:100 in Casein-Tween buffer and added to the microtitre plates, 100 µl/well, followed by incubation of the plates at 37°C for 1 hour. After incubation, the contents of the plates were once again decanted and the wells washed with PBS-Tween as described above.

Finally, substrate solution (0.05% ABTS, 0.015% H₂O₂ in 0.1 M citrate buffer, pH5) was added to all wells of the microtitre plates, 100 µl/well, followed by incubation of the plates at 37°C for 30 min. The absorbance of each well of the microtitre plates was subsequently measured spectrophotometrically at 405 nm on a Labsystems Multiskan MS microtitre plate reader.

3.2.4.3 Statistical analysis

The antibody titres in response to the different vaccines as measured by ELISA were followed over time and these results were analyzed using the General Linear Models procedure in the Statistical Analysis System (SAS, Cary, NC) program (v 6.2) and a least significant differences (LSD) value was calculated.

3.3 Results

3.3.1 Antibody responses to MS and MG vaccines in ostriches

The average titre value of each of the immunized groups of ostriches was plotted against time. In the interpretation of the immune response data, a minimum antibody level sufficient to provide protection against mycoplasma infections in ostriches has not been determined. However, a cut-off value of 0.2 proved to be a good indicator of protection against NDV in the vaccination trials in ostriches done by Blignaut *et al.* (2000). This cut-off value was also arbitrarily used in these vaccine trials, and was used to calculate the fraction and percentages of birds that showed a positive antibody response (titre \geq 0.2) in each group at each time point.

3.3.2.1 Antibody response obtained from the vaccine trials conducted on the Kwessie farm

The average titre values representing the antibody response elicited in the ostriches on the Kwessie farm following immunization with the poultry mycoplasma vaccines against MS and MG as obtained from the MS Antibody Test kit plates are shown in Figure 3.1.

Ostriches in group A, responded to immunization with the MS vaccine with both a primary and secondary antibody response, while no antibodies could be detected in the serum samples from ostriches in group B, immunized with the MG vaccine and analysed with the MS Antibody Test kit plates. Three weeks after the initial immunization, a peak of serum antibody productions was observed in ostriches from Group A, with 16% of birds responding with titres higher than 0.2 (see Table 3.1, day 21). The responses of Group B and the control group did not differ significantly from each other, showing very little response, differing significantly from Group A at 21 days after the first immunization.

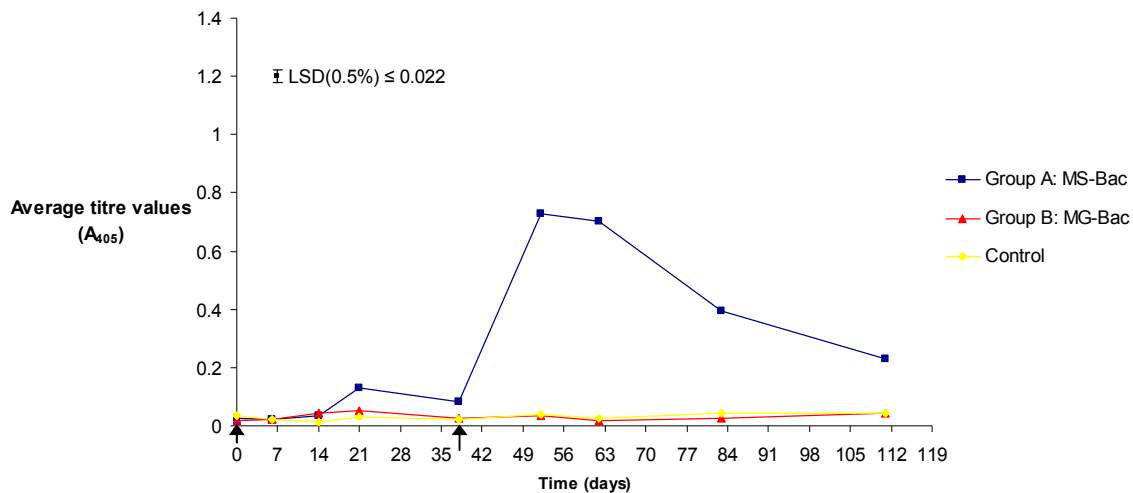


Figure 3.1 Antibody responses, as determined with MS Antibody Test kit plates, elicited in ostriches from the Kwessie farm after immunization with poultry mycoplasma vaccines. Groups A and B were vaccinated with 1 ml oil-free MS-Bac and MG-Bac respectively, and the control group received no vaccine. The days on which the initial (day 0) and second (day 38) immunizations took place, are indicated with arrows. The LSD value for this trial was 0.022.

After the second immunization titre values of birds in group A increased rapidly, reaching a peak after 52 days with 92% of birds responding with titres higher than 0.2 (see Table 3.1, day 52). Although serum antibody levels dropped after 111 days, ostriches in group A still showed high levels of circulating antibody with 39% of birds maintaining a positive, presumably protective, antibody response (see Table 3.1, day 111). No immune response was detected in ostriches in the control group and group B, immunized with MG vaccine.

Table 3.1 Antibody responses detected in serum samples of ostriches from the Kwessie farm as detected with the MS Antibody Test kit plates. Positive responses ($0.2 \leq$ titre) are indicated as fractions and percentages for group A immunized with MS-Bac, group G immunized with MG-Bac, and the control group which received no vaccine.

Day	Group A		Group B		Control group	
	Fraction	%	Fraction	%	Fraction	%
0	0/50	0	0/50	0	0/40	0
6	0/50	0	0/50	0	0/40	0
14	2/50	4	2/50	4	0/40	0
21	6/50	16	2/50	4	0/40	0
38	5/50	10	1/50	2	0/40	0
52	33/37	89	1/39	2.6	0/33	0
62	33/36	89	0/37	0	0/30	0
83	23/36	64	0/33	0	0/27	0
111	14/36	39	0/34	0	0/30	0

The average titre values representing the antibody response elicited in the ostriches on the Kwessie farm following immunization with the poultry mycoplasma vaccines against MS and MG as obtained from the MG Antibody Test kit plates are shown in Figure 3.2.

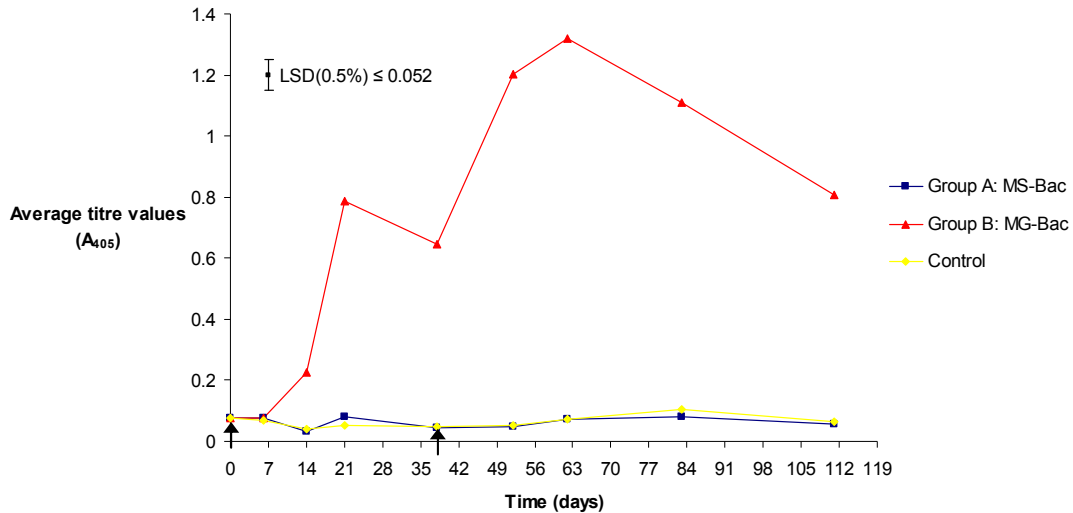


Figure 3.2 Antibody responses, as determined with MG Antibody Test kit plates, elicited in ostriches from the Kwessie farm after immunization with poultry mycoplasma vaccines. Groups A and B were vaccinated with 1 ml oil-free MS-Bac and MG-Bac respectively, and the control group received no vaccine. The days on which the initial (day 0) and second (day 38) immunizations took place, are indicated with arrows. The LSD value for this trial was 0.052.

Ostriches in group B, responded to immunization with the MG vaccine with both a primary and secondary antibody response, while no antibodies could be detected in the serum samples from ostriches in group A, immunized with the MS vaccine and analysed with the MG Antibody Test kit plates. Two weeks after the initial immunization, more than half (56%) of birds in group B showed a positive antibody response, reaching a peak after 21 days when 86% of the birds responded with titres higher than 0.2 (see Table 3.2, day 21). The responses of Group A and the control group did not differ significantly from each other, showing very little response, differing significantly from Group B from 14 days after the first immunization. After the second immunization, titre values of birds in group B increased rapidly reaching a peak after 62 days with 97% of birds responding with titres higher than 0.2 (see Table 3.2, day 62). Although serum antibody levels dropped after 111 days, ostriches in group B still showed high levels of circulating antibody with 91% of birds maintaining a positive antibody response (see Table 3.2, day 111). No immune response was detected in ostriches in the control group and group A, immunized with MS vaccine.

Table 3.2 Antibody responses detected in serum samples of ostriches from the Kwessie farm as detected with the MG Antibody Test kit plates. Positive responses ($0.2 \leq \text{titre}$) are indicated as fractions and percentages for group A immunized with MS-Bac, group G immunized with MG-Bac, and the control group which received no vaccine.

Day	Group A		Group B		Control group	
	Fraction	%	Fraction	%	Fraction	%
0	0/50	0	0/50	0	0/40	0
6	0/50	0	0/50	0	0/40	0
14	0/50	0	28/50	56	0/40	0
21	0/50	0	44/50	88	0/40	0
38	0/50	0	41/50	82	1/40	2.5
52	0/37	0	36/39	92	0/33	0
62	0/36	0	36/37	97	0/30	0
83	0/36	0	32/33	97	0/27	0
111	0/36	0	31/34	91	0/30	0

Thus both vaccines elicited antibody responses in the immunized ostriches. It is tempting to wish to make a comparison between the antibody responses of ostriches immunized with the MS and MG vaccine and measured with ELISAs against MS and MG respectively (comparison of group A in Figure 3.1 with group B in Figure 3.2). However, this is not statistically comparable due to the fact that two different ELISAs were used for the detection of these antibody responses.

3.3.2.2 Antibody response results obtained from the vaccine trials conducted on the Schoeman farm

The average titre values representing the antibody response elicited in the ostriches on the Schoeman farm following immunization with the poultry mycoplasma vaccines against MS and MG as obtained from the MS Antibody Test kit plates are shown in Figure 3.3.

The MS vaccine elicited an immune response in ostriches in group A, while no antibodies could be detected in serum samples from ostriches in group B, immunized with the MG vaccine when testing with the MS Antibody Test kit plates. From the available data points, a clear primary and secondary antibody response cannot be distinguished, in all likelihood because the serum samples were not collected at the peak of the primary immune response.

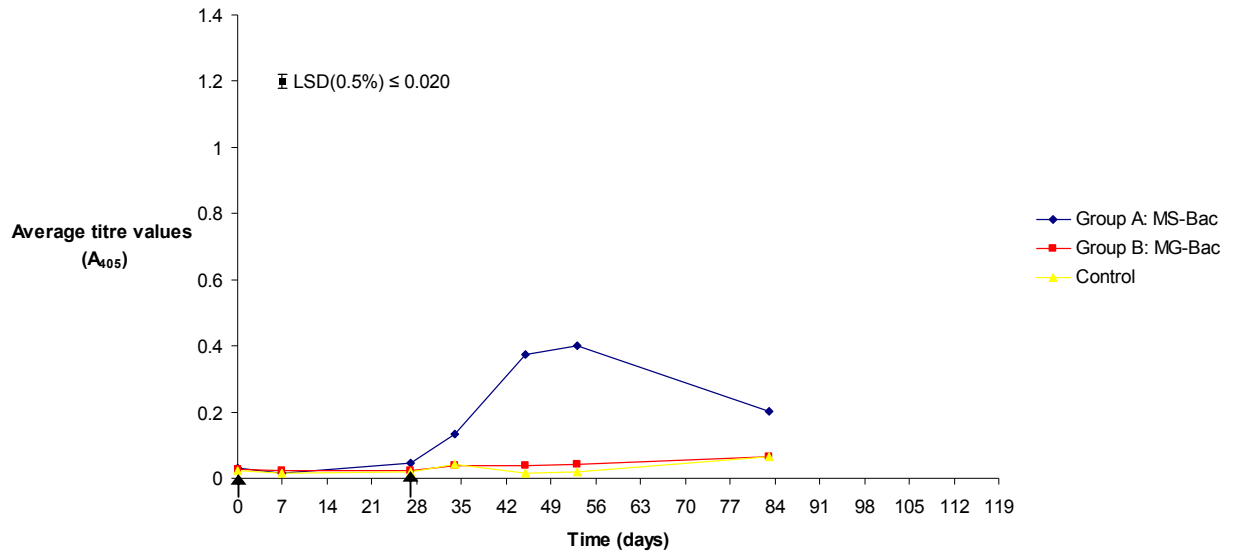


Figure 3.3 Antibody responses, as determined with MS Antibody Test kit plates, elicited in ostriches from the Schoeman farm after immunization with poultry mycoplasma vaccines. Groups A and B were vaccinated with 1 ml oil-free MS-Bac and MG-Bac respectively, and the control group received no vaccine. The days on which the initial (day 0) and second (day 27) immunizations took place, are indicated with arrows. The LSD value for this trial was 0.020

Ostriches in group A reached their peak of serum antibody production after the second immunization when 58% of birds in group A responded with a titre value higher than 0.2 (see Table 3.3, day 53). The responses of group B and the control group did not differ significantly from each other, but did differ significantly from group A. Although serum antibody levels dropped after 84 days, ostriches in group A still showed high levels of circulating antibody with 26% of birds maintaining a positive antibody response (see Table 3.3, day 83). No immune response was detected in ostriches in the control group and group B, immunized with MG vaccine.

Table 3.3 Antibody responses detected in serum samples of ostriches from the Schmoeman farm as detected with the MS Antibody Test kit plates. Positive responses ($0.2 \leq \text{titre}$) are indicated as fractions and percentages for group A immunized with MS-Bac, group G immunized with MG-Bac, and the control group which received no vaccine.

Day	Group A		Group B		Control group	
	Fraction	%	Fraction	%	Fraction	%
0	0/29	0	0/30	0	0/18	0
7	0/27	0	0/28	0	0/18	0
27	0/27	0	0/25	0	0/17	0
34	5/28	18	1/26	3.8	0/15	0
45	14/26	54	0/26	0	0/16	0
53	15/26	58	1/26	3.8	0/14	0
83	6/23	26	0/25	0	0/14	0

The average titre values representing the antibody response elicited in the ostriches on the Schoeman farm following immunization with the poultry mycoplasma vaccines against MS and MG as obtained from the MG Antibody Test kit plates are shown in Figure 3.4.

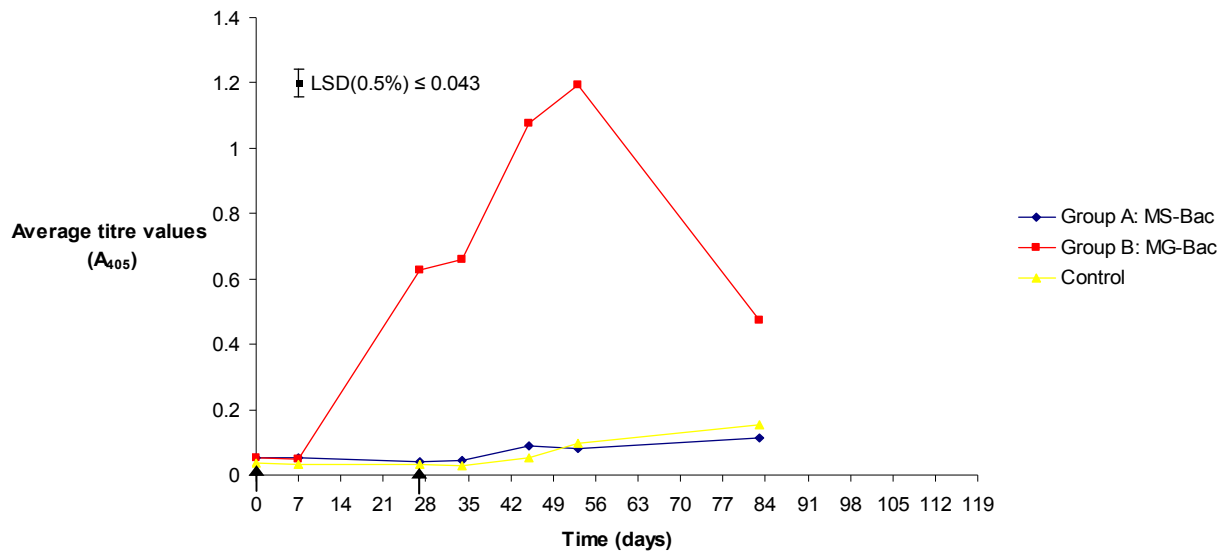


Figure 3.4 Antibody responses, as determined with MG Antibody Test kit plates, elicited in ostriches from the Schoeman farm after immunization with poultry mycoplasma vaccines. Groups A and B were vaccinated with 1 ml oil-free MS-Bac and MG-Bac respectively, and the control group received no vaccine. The days on which the initial (day 0) and second (day 27) immunizations took place, are indicated with arrows. The LSD value for this trial was 0.043.

The MG vaccine elicited an immune response in ostriches in group B, while no antibodies could be detected in serum samples from ostriches in group A, immunized with the MS vaccine when testing with the MG Antibody Test kit plates. As before, the available data points made it difficult to distinguish a clear primary and secondary antibody response, in all likelihood because the serum samples were not collected at the peak of the immune response. Ostriches in group B reached their peak of serum antibody production after the second vaccination (day 27) when 96% of birds responded with a titre higher than 0.2 (see Table 3.4, day 53). Although serum antibody levels dropped after 83 days, ostriches in group B still showed high levels of circulating antibody with 92% of birds maintaining a positive antibody response (see Table 3.4, day 83). No immune response was detected in ostriches in the control group and group A, immunized with MS vaccine.

Once again both vaccines elicited antibody responses in the immunized ostriches. Similarly as before, a comparison between the antibody responses of ostriches immunized with the MS and MG vaccine and measured with ELISAs against MS and MG respectively (comparison of group A in Figure 3.3 with group B in Figure 3.4) was not statistically comparable due to the fact that two different ELISAs were used for the detection of these antibody responses.

Table 3.4 Antibody responses detected in serum samples of ostriches from the Schmoeman farm as detected with the MG Antibody Test kit plates. Positive responses ($0.2 \leq \text{titre}$) are indicated as fractions and percentages for group A immunized with MS-Bac, group G immunized with MG-Bac, and the control group which received no vaccine.

Day	Group A		Group B		Control group	
	Fraction	%	Fraction	%	Fraction	%
0	0/29	0	0/30	0	0/18	0
7	0/27	0	0/28	0	0/18	0
27	0/27	0	22/25	88	0/17	0
34	0/28	0	23/26	89	0/15	0
45	0/26	0	24/26	92	0/16	0
53	1/26	3.8	25/26	96	2/14	14.3
83	1/23	4.3	23/25	92	3/14	21.4

3.3.2 Field challenge

Although there was generally a lower incidence of mycoplasma infections in ostriches during the autumn of 2008, symptoms associated with mycoplasma infections were still observed in the immunized birds once exposed to *Ms01*, *Ms02* and *Ms03* by field challenge. Immunized birds on the Schoeman farm showed a high incidence of symptoms associated with mycoplasma infection such as the appearance of mucus exudates from the nasal cavities and foaming eyes, and had notably poor growth rates. Birds from the Kwessie farm sporadically showed signs of mycoplasma infection, such as mucus exudates from the nasal cavities and foaming eyes, before the second immunization was administered. However, there was not such a large effect on the growth rate of the birds from the Kwessie farm. Unfortunately none of the findings of the visual inspections were properly documented due to the unmanageable large flock sizes on the respective farms.

3.4 Discussion

In chickens and turkeys, MS vaccines provide protection against MS infections and MG vaccines provide protection against MG infections. The fact that two separate vaccines have been developed for protection against these mycoplasmas indicates that the respective vaccines do not give sufficient cross protection against both MS and MG infections, and that a separate vaccine is required in order to elicit antibody responses against each of these mycoplasmas.

From the results obtained in this study, it can be concluded that poultry mycoplasma vaccines can also elicit strong primary antibody responses in ostriches, and even more rapid and greater secondary antibody responses, indicative of acquired memory. It was further found that the produced anti-MS and anti-MG antibodies were specific for MS and MG antigen respectively, and that cross-reactivity between the immunity raised against them is low. It is therefore unlikely that one of the two would provide protection against infections with both mycoplasmas. Furthermore, the antibody responses elicited against MS and

MG in ostriches did not provide protection against the ostrich-specific mycoplasmas *Ms01*, *Ms02* and *Ms03* as observed from the high infection rate of the ostriches with the ostrich-specific mycoplasmas *Ms01*, *Ms02* or *Ms03* after vaccination with MS or MG. Due to the distant phylogenetic relationship of MG to the ostrich-specific mycoplasmas *Ms01*, *Ms02* and *Ms03* it was not unexpected that MG vaccines do not give protection against these ostrich-specific mycoplasmas. However, MS is phylogenetically more closely related to the ostrich-specific mycoplasmas *Ms02* and *Ms03*, yet it would appear that MS vaccines also do not give protection against ostrich-specific mycoplasmas. However, it must be considered that it was not established which of the ostrich-specific mycoplasmas, *Ms01*, *Ms02* or *Ms03* was the causative organism that was responsible for the mycoplasma infection symptoms that these ostriches showed. Therefore, the lack of protection could also be attributed to the phylogenetic distance between MS and the ostrich-specific mycoplasma *Ms01*, with the result that even if MS vaccines could provide protection against its two closely related ostrich-specific mycoplasmas (*Ms02* and *Ms03*), *Ms01* could have been responsible for the observed mycoplasma infections.

A second possible explanation for lack of protection provided by MS could be the highly divergent antigenic properties of *Ms01*, *Ms02* and *Ms03*. Ostrich mycoplasma serological cross-reactivity tests were conducted at Onderstepoort to determine the incidence of cross-reactivity between the different strains of ostrich mycoplasmas and these results, which are unpublished (Gouws, personal communication) are shown in Table 4.3.

Table 4.3 Antibody titres obtained from serological cross-reactivity tests with the ostrich-specific mycoplasmas *Ms01*, *Ms02* and *Ms03*.

		Antibody		
		Ms01	Ms02	Ms03
Antigen	Ms01	1280	-	-
	Ms02	-	320	-
	Ms03	-	80	2560

This revealed that antibodies to *Ms01* did not recognize *Ms02* or *Ms03* and antibodies to *Ms03* did not recognize *Ms01* or *Ms02*. Antibodies to *Ms02* did not recognize *Ms01*, but did partially cross-react with *Ms03*. This further illustrates that the cross-reactivity between mycoplasmas appears to be low in general. *Ms02* and *Ms03* are phylogenetically more closely related to each other than they are to MS, yet they show little cross-reactivity toward each other. Therefore it is not surprising that MS vaccines do not give protection against any of the ostrich-specific mycoplasmas.

A third possible explanation could be the result of insufficient mucosal immunity elicited by the poultry vaccines in ostriches. In chickens, live vaccines administered by eye drop or orally have been found to induce protective mucosal immunity mediated by IgA antibodies, while injected inactivate vaccines lead to the production of high levels of serum antibodies (Blignaut, 1998). As ostrich mycoplasmas are pathogens of the respiratory tract of ostriches, the production of serum antibodies may be insufficient to provide protection against these pathogens of the respiratory tract.

An alternative to employing poultry mycoplasma vaccines, such as MS and MG vaccines, to control *Ms01*, *Ms02* and *Ms03* infections, is to develop specific vaccines against each of the ostrich-specific mycoplasmas separately. The possibility of developing a conventional vaccine such as a whole killed organism or live attenuated organism vaccine has to date been hindered by the poor *in vitro* cultivation properties of these mycoplasmas. The development of a DNA vaccine may therefore be an attractive alternative since this strategy does not require the large scale *in vitro* cultivation of ostrich mycoplasmas.

Chapter 4 – Identification, isolation, and site-directed mutagenesis of the *P100* vaccine candidate gene in the ostrich mycoplasma *Ms01*

4.1 Introduction

Mycoplasmas are members of the class *Mollicutes* (*mollis*, soft; *cutes*, skin) and are described to be the smallest known organisms capable of self-reproduction (Razin, 1985; Weisburg *et al.*, 1989). They originated by degenerate evolution from low G+C content Gram-positive bacteria by losing many non-essential genes, such as genes encoding proteins involved in several biosynthetic pathways, as well as cell wall synthesis. This degenerate mode of mycoplasma evolution ultimately resulted in their two most prominent features being (i) the complete lack of a cell wall, and (ii) their unusually small genome (580 - 1350 kb) with a G+C content of 23-41 mol%. The AT-richness of the mycoplasma genome has resulted in their use of an alternative genetic code, where the universal termination codon TGA, encodes tryptophan instead (Dubvig and Voelker, 1996; Razin *et al.*, 1998; Söll and RajBhandary, 2006).

Mycoplasmas are parasites of a wide range of animal hosts. Their restricted anabolic abilities render them completely reliant on their hosts for their nutrient requirements. Accordingly, the mycoplasma genome encodes a large number of transport proteins, the majority of which are ABC transporters, responsible for uptake of essential nutrients such as sterols, fatty acids, vitamins, amino acids, purines, pyrimidines from their host (Heinrich *et al.*, 1999; Prescott *et al.*, 2002). Although in many cases mycoplasmas form part of the normal flora of their hosts, a number of species have been shown to be pathogenic (Razin, 1985). Attachment of mycoplasmas to the cells of their host plays a key role in their pathogenesis and is, in fact, a prerequisite for mycoplasma infection to occur. In some species a specialized organelle or tip structure has been identified to mediate mycoplasma attachment to their host (Henrich *et al.*, 1993; Lockaby *et al.*, 1998). In mammals and birds, mycoplasmas often reside in the mucosal surfaces and are associated with diseases of the respiratory and urogenital tracts (Prescott *et al.*, 2002). Recently, three unique mycoplasmas were identified to infect ostriches in South Africa. These ostrich-specific pathogens, provisionally named *Ms01*, *Ms02* and *Ms03* are associated with respiratory disease in ostriches, ultimately resulting in large production losses and therefore posing a threat to the ostrich industry (Botes *et al.*, 2005).

Even though mycoplasma infections in ostriches can be controlled by following a certain set of bio-security guidelines to try and maintain infection free flocks, as well as by treatment with a narrow range of antibiotics (Kleven, 2008), there is currently no registered vaccine available to prevent mycoplasma infections in ostriches. Conventional approaches to mycoplasma vaccine development are hindered by their weak cultivation properties, and to date, attempts at developing a vaccine against ostrich-specific mycoplasmas have been unsuccessful. We have also found that vaccines against the poultry mycoplasmas, *M. gallisepticum* and *M. synoviae*, do not provide protection against any of the ostrich-specific mycoplasmas. These vaccines are therefore ruled out as an alternative to vaccine development

(unpublished results). The approach of DNA vaccine development is an attractive alternative as it obviates the requirement for large quantity *in vitro* cultivation of the pathogens (Gurunathan *et al.*, 2000). As a first step to DNA vaccine development, a vaccine candidate gene needs to be identified. Genes encoding membrane proteins, such as proteins involved in transport of nutrients across the plasma membrane, as well as proteins mediating attachment to host cells, have been shown to possess strong antigenic properties, and have therefore been proposed as good vaccine candidate genes (Garmory and Titball, 2007).

In order to identify suitable vaccine candidate genes, the whole genome of *Mso1* was sequenced using GS20 sequencing. To this end, *Mso1* gDNA was isolated by means of four different extraction methods. One gDNA preparation was subsequently used for whole-genome GS20 sequencing followed by assembly of the resulting contiguous sequences. A search was launched for suitable vaccine candidate genes in these contigs. The *P100* gene was identified in the genome of *Mso1*, cloned, and modified by changing the mycoplasma codon for tryptophan (TGA), to the universal codon for tryptophan (TGG), to prepare the gene for insertion into a suitable DNA vaccine vector.

4.2 Materials and Methods

4.2.1 Isolation of genomic DNA

Mso1 was cultivated on modified Chanock's medium in candle jars incubated at 37°C for approximately 15 days (cultivation was performed by Mr Johan Gouws, Onderstepoort Veterinary Institute, Pretoria). Colonies were usually visible 72 hours after inoculation of the plates, although this was found to vary. The mycoplasmas tended to penetrate the surface of the solid medium, resulting in a central nucleus that remained embedded in the medium, while the rest of the colony (peripheral zone) was removed by gentle scraping of the agar. The colonies of 50 plates were suspended in 100 ml of phosphate buffered saline (PBS). The gDNA was subsequently isolated from the above suspension. The modified Hempstead method, a modified phenol:chloroform method, and two DNA isolation kits were employed in an attempt to achieve optimum yields of gDNA for subsequent whole-genome GS20 sequencing.

4.2.1.1 Modified Hempstead method

The protocol for gDNA isolation as described by Hempstead (1990) was modified and adapted to optimize yield. Briefly, 20 ml *Mso1* culture was placed in a sterilized screw cap JA-20 tube followed by centrifugation at 27 200 x g for 50 min at 4°C (Beckman Model J2-21 Centrifuge, JA-20 rotor). The supernatant was decanted and the pellet resuspended in 10 ml concentrated TE buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0) followed by centrifugation at 12 100 x g for 40 min at 4°C. The supernatant was again decanted and the pellet resuspended in 1 ml concentrated TE buffer. The suspension was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 16 000 x g for 40 min at 4°C (Biofuge Fresco, Heraeus). Following centrifugation, the supernatant was decanted and the pellet resuspended in 100 µl concentrated TE buffer and the sample kept at -20°C overnight. A volume of 1 ml concentrated

TE buffer containing 1% (v/v) SDS, and 11 μ l proteinase K (20 mg/ml, Roche) was added to lysed cells, followed by 15-30 min incubation at 55°C. To the clear suspension, 100 μ l of 5 M potassium acetate solution was added, and the mixture kept on ice for 30 min. This was followed by centrifugation at 16 000 x g for 10 min at 4°C, after which the upper phase was removed and divided into two clean 2 ml microcentrifuge tubes. An equal volume of chloroform:isoamylalcohol (24:1) was added to each tube and mixed by inverting the tubes for 15 min, followed by centrifugation at 16 000 x g for 2 min at 4°C. This was repeated three times. Subsequently the upper phase was transferred to a clean 1.5 ml microcentrifuge tube, to which 1/10 volume sodium acetate (3 M, pH 4.8), as well as 2 volumes 95% (v/v) ethanol was added. DNA was allowed to precipitate overnight at -20°C. Subsequently the mixtures were centrifuged at 16 000 x g for 10 min at 4°C, after which the supernatants were decanted and the pellets washed in 70% (v/v) ethanol for 30 min at room temperature. The ethanol was decanted and the remaining pellets air-dried for 30 min at 37°C, after which the DNA was redissolved overnight in 50 μ l TE buffer.

4.2.1.2 Modified phenol:chloroform isolation method

The commonly used phenol:chloroform DNA extraction method was also employed for the isolation of gDNA, with certain adjustments to the protocol to optimize yield. In this case, 25 ml *Ms01* culture was measured into a sterilized screw cap JA-20 tube followed by centrifugation at 12 100 x g for 60 min at 4°C (Beckman Model J2-21 Centrifuge, JA-20 rotor). The supernatant was decanted and the remaining pellet washed in an equal volume (25 ml) PBS (pH 7.2), followed by centrifugation at 12 100 x g for 60 min at 4°C. The supernatant was subsequently decanted and the pellet resuspended in 6 ml TE buffer. A volume of 385 μ l of a 10% (v/v) SDS solution, and 240 μ l proteinase K (20 mg/ml) was added to the suspension, mixed, and incubated for 60 min at 55°C. Following incubation, an equal volume of phenol:chloroform (1:1) was added, and the suspension mixed by inverting the tube for 10 min, followed by centrifugation at 7000 x g for 5 min at room temperature. The upper phase was removed and transferred to a fresh sterilized screw cap JA-20 tube. An equal volume of phenol:chloroform was added and the suspension mixed, followed by centrifugation at 7000 x g for 5 min at room temperature. The upper phase was again removed and this time transferred to a clean 2 ml microcentrifuge tube to which 1/10 volume sodium acetate (3 M, pH 5.2), as well as 0.6 volume isopropanol (-20°C) was added. After thorough mixing, the sample was left overnight at room temperature to allow the DNA to precipitate. Subsequently the sample was centrifuged at 13 000 x g for 10 minutes at 4°C (Biofuge Fresco, Heraeus), after which the supernatant was decanted, and the pellet washed twice in 30 ml 70% (v/v) ethanol for 20 min at room temperature. The ethanol was decanted and the remaining pellets air-dried for 30 min at 37°C, after which the DNA was dissolved overnight in 50 μ l TE buffer.

4.2.1.3 DNA isolations with commercial kits

Genomic DNA was isolated from *Ms01* cultures using the protocol for the isolation of gDNA from Gram negative bacteria as described by the Wizard[®] Genomic DNA Purification Kit (Promega), as well as the ZR Fungal/Bacterial DNA Kit[™] (ZYMO Research) according to manufacturers' instructions.

4.2.1.4 Quantity and quality determination

After the isolation of gDNA from *Ms01* cultures by means of the four different extraction methods, the resulting gDNA preparations were analysed spectrophotometrically (NanoDrop, ND-1000) and their concentration (ng/μl) and purity (A_{260}/A_{230} and A_{260}/A_{280} ratios) determined in order to establish which extraction method produced gDNA most suitable for subsequent whole-genome GS20 sequencing.

4.2.1.5 Confirmation of *Ms01* identity

In order to confirm that the gDNA preparations isolated from the cultures received from Onderstepoort were indeed of *Ms01* origin, and were not contaminated by *Ms02* or *Ms03*, a standardized mycoplasma polymerase chain reaction (PCR) was carried out using the species specific primer pair with the forward primer (F) GPO3F and reverse primer (R) MYC 16R1541 (R) based on the 16S rRNA gene sequence of *Ms01* (Table 3.1) and this product was sequenced (Botes *et al.*, 2005).

Table 4.1 Nucleotide sequences of the species specific primer pair used to verify *Ms01* identity of gDNA preparation isolated from cultures prepared at and received from Onderstepoort.

Primer name	Primer sequence (5'→3')	bp-position	T _m (°C)
GPO3F (F)	5' -TGGGGAGCAAACAGGATTAGATACC-3'	745	78
MYC 16R1541 (R)	5' -TGCACCATCTGTCACTCTGTTAACCTC-3'	1017	79

Amplification reactions using the primer pair GPO3F and MYC 16R1541 (Table 3.1) were carried out in 50 μl volumes. Each reaction mixture contained 25 μl of 2 x Ready Mix reaction buffer (Kapa), 1.0 μl of each primer (20 pmol/μl), 2.5 μl gDNA (544 ng/μl), and 20.5 μl Milli-Q[®] water. Amplifications were performed in a Hybaid Px2 Thermal Cycler programmed to perform 35 cycles of 94°C (45 s), 55°C (45 s) and 72°C (1 min 30 s), followed by a final extension reaction for 6 min at 72°C. Amplified DNA was subsequently analyzed by gel electrophoresis. Ten microliters of the PCR product, mixed with 0.1 volume of gel loading buffer [50% (v/v) glycerol; 0.1% (v/v) bromophenol blue; 50 mM EDTA; 100 mM Tris-base, pH 8.0] were electrophoresed (100 V, ~60 min) on a 2% (w/v) agarose gel (Molecular Grade Agarose D1-LE, Whitehead Scientific) in 1 x TAE buffer (0.04 M Tris-acetate; 0.002 M EDTA, pH 8.0). Ethidium bromide (0.175 μg/ml) was included in the gel for ultraviolet (UV) visualization of the DNA. Following visualization, the PCR product obtained from the respective DNA preparations with the primer pair GPO3F and MYC 16R1541 were purified as template for sequencing reactions. To this end, the

remaining PCR product was electrophoresed (150 V, 2-3 hours) on a 0.5% (w/v) agarose gel in 1 x TAE buffer containing ethidium bromide (0.15 µg/ml) as previously described. Single DNA containing bands corresponding to a size of 750 bp were excised under UV light and purified using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturers' instructions. The purified DNA samples were each concentrated by centrifugal evaporation to 20 µl on a Savant Speedvac, after which 2 µl of the purified and concentrated product was analyzed by gel electrophoresis as previously described.

Sequencing reactions on the purified DNA templates were subsequently prepared using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Each 10 µl sequencing reaction consisted of 5 µl of 5x Sequencing buffer, 2 µl Terminator mix, 1 µl primer (GPO3F, 0.8 pmol/ µl), 0.5 µl purified and concentrated DNA, and 1.5 µl Milli-Q® water. Amplifications were performed in a Hybaid Px2 Thermal Cycler programmed to perform 35 cycles of 96°C (10 s), 52°C (30 s) and 60°C (4min), followed by a final extension reaction of 10 min at 60°C.

Sequencing PCR reaction products were analyzed with an ABI® 3100 Genetic Analyser (Applied Biosystems) at the DNA sequencing facility of the University of Stellenbosch. Resulting chromatograms were edited using Chromas (v 2.23, Technelysium, Pty., Ltd.) and the nucleotide sequences manually aligned with the known 16S rRNA gene sequence of *Ms01* in BioEdit (v 7.0.5.2, Hall).

4.2.2 Whole-genome GS20 sequencing of Ms01

Following the successful isolation of uncontaminated gDNA from *Ms01* cultures, the entire genome of *Ms01* was sequenced using the Roche 454 Sequencing System based on GS20 sequencing technology at InqabaBiotec in Pretoria (see section 2.12.1.1). Two 20 Mb runs of this sequencing were performed.

Short sequences of an average length of 100 bp were generated, which were assembled into larger contiguous sequences based on the overlap between the reads. This was done with a *de novo* shotgun sequence assembler program which also gave an estimate of the size and G+C content of the *Ms01* genome.

4.2.3 Identification of a vaccine candidate gene in Ms01 by bioinformatic analysis of the whole-genome GS20 sequencing data

In order to identify a gene encoding a membrane protein in *Ms01* to serve as a possible DNA vaccine candidate gene, the data obtained from the whole-genome GS20 sequencing of *Ms01* were subjected to bioinformatic analysis.

4.2.3.1 Similarity searches in the National Center for Biotechnology Information (NCBI) database

Firstly, similarity searches were performed on the individual contiguous sequences (contigs) spanning the entire *Ms01* genome using the Basic Local Alignment Search Tool (BLAST) engine available online on the NCBI website (<http://www.ncbi.nlm.nih.gov/blast>). Several BLAST search programs, each with a

different search strategy, are available. For this study, blastn, which compares a nucleotide query sequence with a nucleotide sequence database, as well as tblastx, which compares a translated nucleotide query sequence with a translated nucleotide sequence database, was used.

Each individual contig obtained from the whole-genome sequencing of *Ms01*, was analysed for similarity at nucleotide level using blastn with a specified wordsize of 15 bases. The significance of identified sequence similarities, also referred to as blast hits, was determined by the Expect (E)-value, as well as the bit score of the match. The E-value indicates the statistical significance of an alignment between the query sequence and a sequence in a database; the lower the E-value, the more likely it is that the alignment did not occur randomly, and therefore reflects a true sequence similarity. On the other hand, a bit score reflects the length of the alignment between a query sequence and a sequence in a database. In general, as in this study, hits with E-values higher than 0.1, and bit scores lower than 50, are not regarded to reflect statistically significant sequence similarity.

The results obtained from blastn searches with the individual contigs were screened for hits with genes encoding membrane proteins involved in membrane transport and/or host attachment. Contigs in which such genes were identified were further analysed for similarity at amino acid level using tblastx employing the specified mold mitochondrial genetic code.

4.2.3.2 Open reading frame identification using CLC Combined Workbench software

Following identification of contigs showing significant similarity with a suitable membrane protein within the NCBI database, these contigs were subjected to open reading frame (ORF) analysis in CLC Combined Workbench (v 4.0.1). For the purpose of our search, an ORF was assigned the initiation and termination codon ATG and TAA respectively (see section 2.5.6.2), with a minimum length of 100 codons. Resulting ORFs were confirmed by alignment and comparison to the ORFs of the identified homologous genes within the NCBI database.

4.2.3.3 Linkage of contiguous sequences by PCR

Following identification of two separate contigs; contig number 835 (1645 bp) and contig number 9312 (53326 bp), each containing a part of the same vaccine candidate gene in *Ms01*, the contigs were linked together by PCR.

For this, two primers were designed using Primer Designer (v 1.01); the forward primer on the 3' end of contig 835, and the reverse primer on the 5' end of contig 9312 (Figure 4.1).

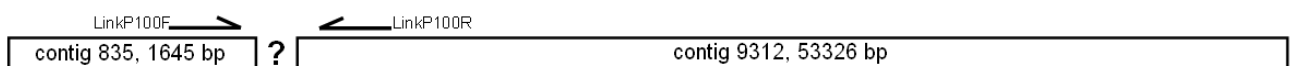


Figure 4.1 Schematic illustration of the primer position on contig 835 (1645 bp) and contig 9312 (53 326 bp) for subsequent linkage PCR.

The nucleotide sequences of the primers that were subsequently synthesized by the DNA Synthesis Laboratory, Department of Molecular and Cellular Biology, University of Cape Town, are shown in Table 4.2.

Table 4.2 Nucleotide sequences of the primers used for the linkage of contig 835 and contig 9312, each identified to contain part of a gene encoding a membrane protein in *Ms01*.

Primer name	Primer sequence (5'→3')	bp-position	T _m (°C)
LinkP100F	5' -GATCAATTAATGCATGACTATACATTCT-3'	1511-1539	71
LinkP100R	5' -CACCATATCAGTAAACACCTAATTTAGC-3'	29-57	73

Amplification reactions using the primer pair LinkP100F and LinkP100R (Table 4.2) were carried out in 50 µl volumes. Each reaction mixture contained 5 µl of 10 x Taq reaction buffer, 2 µl of 5 mM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP) (Bioline), 2.5 µl of each primer (20 pmol/µl), 3 µl of 25 mM MgCl₂, 0.5 µl of 5 U/µl Super-Therm Taq DNA polymerase (Southern Cross Biotechnology), 5 µl of 1:10 diluted purified DNA, and 29.5 µl Milli-Q[®]. Amplifications were performed in a Hybaid Px2 Thermal Cycler, programmed to perform 35 cycles of 94°C (45 s), 55°C (45 s) and 72°C (1 min 30 s), followed by a final extension reaction for 6 min at 72°C.

Amplified DNA was subsequently analyzed by gel electrophoresis and the remaining product purified as template for sequencing reactions as previously described. Following purification and centrifugal evaporation, the concentration of the PCR product was determined spectrophotometrically (NanoDrop, ND-1000).

Sequencing reactions on the purified DNA templates were prepared using the BigDye[®] Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Each sequencing reaction consisted of 5 µl of 5x Sequencing buffer, 2 µl Terminator mix, 1 µl of either of the primers (LinkP100F or LinkP100R, 0.8 pmol/µl), 3 ng purified and concentrated DNA, and Milli-Q[®] water to a final volume of 10 µl. Amplifications were performed in a Hybaid Px2 Thermal Cycler, programmed to perform 35 cycles of 96°C (10 s), 52°C (30 s) and 60°C (4 min), followed by a final extension reaction of 10 min at 60°C. Sequencing PCR reaction products were analyzed with an ABI[®] 3100 Genetic Analyser as previously described, and resulting chromatograms were edited using Chromas and the nucleotide sequences aligned with the 3' end of contig 835 and the 5' end of contig 9312 in BioEdit.

4.2.3.4 Revision on open reading frames in CLC Combined Workbench

Once contig 835 and contig 9312 were successfully linked together in one contig (54 973 bp), previously identified ORFs on the separate contigs were revised to confirm assigned ORF positions in CLC Combined Workbench as previously described.

4.2.3.5 Comparative genomics

Consensus promoter areas upstream of the predicted initiation codon, and conserved motifs related to the function of the proteins encoded by the *opp* genes were identified by manual alignment to the gene homologs of *M. hominis* and published ABC transporter consensus motifs (Henrich *et al.*, 1999; Weiner *et al.*, 2000; Garmory and Titball, 2004; Hopfe and Henrich, 2004; Davidson and Maloney, 2007).

4.2.4 Isolation of the *P100* gene of *Ms01* by PCR

Once the complete nucleotide sequence of the P100 vaccine candidate gene was determined, the gene was isolated from the gDNA of *Ms01* by PCR for subsequent cloning. Primers for the isolation of the *P100* gene were designed using Primer Designer (v 1.01). Endonuclease restriction sites were incorporated at the 5' end of each primer for later insertion of the gene into the vaccine expression vector pCI-neo (see Figure 2.2 section 2.11.2.1). Accordingly the choice of restriction sites was limited to endonucleases that recognize restriction sites within both the cloning vectors pGEM[®]-T Easy and pCI-neo, but with no restriction sites in the *P100* gene prior to or following modification. The primers, of which the sequences are shown in Table 4.3, were synthesized by Integrated DNA Technologies (IDT).

Table 4.3 Nucleotide sequences of the primers used for the isolation of the *P100* gene from the gDNA of *Ms01*. The endonuclease recognition sequences incorporated for ultimate cloning in pCI-neo are underlined, with the arrows indicating the respective restriction sites. The initiation codon (ATG) and the termination codon (TAA) of *P100* gene are indicated in bold.

Primer name	Primer sequence (5'→3')	bp-position	T _m (°C)	Endonuclease
P100_ampF	5' - <u>ACGCGT</u> ATG AAAAAAGCGCAAGACT-3'	1-20	68	<i>MluI</i>
P100_ampR	5' - <u>GTCGACTT</u> A ⁺ TTTATTGATTCTTCAATCAAC-3'	2802-2829	64	<i>AccI</i>

Amplification reactions using the primer pair P100_ampF and P100_ampR (Table 4.3) were carried out in 50 µl volumes. Each reaction mixture contained 10 µl of 5x reaction buffer, 1.5 µl of 10 mM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP) (KapaBiosystems), 1.5 µl of each primer (20 pmol/µl), 0.5 µl of 25 mM MgCl₂, 1 µl of 1 U/µl High Fidelity proofreading Taq DNA polymerase (KapaBiosystems), 200 ng purified DNA, and Milli-Q[®] water to a final volume of 50 µl. Amplifications were carried out in a Hybaid Px2 Thermal Cycler programmed to perform an initial denaturation step at 95°C (2 min), 25 cycles of 98°C (20 s), 50°C (15 s) and 68°C (1 min 30 s), followed by a final extension step for 5 min at 68°C. Ten microliters of amplified DNA was analyzed by gel electrophoresis as previously described. The remaining PCR product was purified for subsequent cloning using the GFX[™] PCR DNA and Gel Band Purification kit (GE Healthcare) according to the manufacturer's instructions and the concentration of the purified DNA determined spectrophotometrically (NanoDrop).

4.2.5 Cloning of the *P100* gene into the pGEM[®]-T Easy plasmid

The purified *P100* gene was cloned into the pGEM[®]-T Easy plasmid. Prior to cloning the blunt-ended PCR product produced by the exonuclease activity (proofreading) DNA polymerase was modified, creating adenosine overhangs to allow sticky-end cloning of the *P100* gene into the pGEM[®]-T Easy plasmid.

4.2.5.1 A-Tailing of blunt-ended PCR product for subsequent ligation with the pGEM[®]-T Easy cloning vector

The purified PCR product of the primer pair P100_ampF and P100_ampR (Table 4.3) was used as template for A-Tailing. A-Tailing was performed according to the protocol described in the pGEM[®]-T and pGEM[®]-T Easy Vector System kit (Promega). In short, the 10 µl reaction mixture contained 1 µl of 10x reaction buffer, 0.4 µl of 0.2 mM dATP (Bioline), 0.8 µl of 25 mM MgCl₂, 1 µl of 5 U/µl Taq DNA polymerase (Super-Therm), 6 µl purified blunt-ended PCR product, and 0.8 µl Milli-Q[®] water. The reaction was incubated at 70°C for 25 min, after which the DNA was purified by using the DNA Clean & Concentrator[™]-5 Kit (ZYMO Research) according to the manufacturer's instructions.

4.2.5.2 Transformation of JM-109 cells with recombinant pGEM[®]-T Easy plasmids

The purified DNA obtained after A-Tailing was subsequently cloned into the pGEM[®]-T Easy vectors using the pGEM[®]-T Easy Vector System Kit according to the manufacturer's instructions. Briefly, 2 µl purified DNA was used in a ligation reaction with 50 ng pGEM[®]-T Easy vector, in a reaction also containing 5 µl 2x Rapid Ligation buffer, 1 µl T₄ DNA ligase, and Milli-Q[®] water amounting to 10 µl. A positive control ligation reaction containing control insert supplied with the kit, and a background ligation reaction containing no insert, were also included as suggested by the manufacturer. The ligation reactions were kept at 4°C for 16 hours before being used in transformation reactions. Two microliters of each recombinant plasmid construct were introduced to 50 µl competent *Escherichia coli* (JM-109, 1 x 10⁷ cfu/µg DNA, Promega). The cells were kept on ice for 20 min, followed by heat shock in a water bath at 42°C for 45 s, after which the cells were put on ice for a further 2 min. Subsequently, 950 µl LB-medium was added to the cells, and the cells were allowed to grow at 37°C for 1 hour 30 min on an IKA[®] KS 260 Basic orbital shaker at 150 rpm. Transformed cultures were then plated out in duplicate (50 µl and 150 µl) on LB/agar plates (15 g/ml) containing ampicillin at 100 µg/ml (Sigma), 0.1 M IPTG at 160 µl/100 ml (Bioline) and 50 mg/ml X-Gal at 80 µl/100 ml (Bioline) and were examined for colony development after 16 hours of incubation at 37°C.

4.2.5.3 Confirmation of insert by diagnostic PCR

The blue/white screening system was used to identify positive clones containing the recombinant pGEM[®]-T Easy vector. Positive transformants were subsequently subjected to diagnostic PCR with T7

(5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAGAA-3') primers to verify the presence of insert in the white colonies. Each PCR reaction contained 1 µl of 10 x reaction buffer, 0.6 µl of 25 mM MgCl₂, 0.5 µl of each primer (20 pmol/µl), 0.4 µl of 5 mM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP) (Bioline), 0.1 µl Taq DNA polymerase (Super-Therm), and 6.9 µl Milli-Q[®] water. A sterilized toothpick was pricked into a colony of interest, and the tip of the toothpick was then placed into the PCR mixture and flicked 5 times to release some of the bacteria from the colony. The DNA contained in the colony therefore served as DNA source for the PCR. Two positive control samples of previously purified plasmid DNA samples containing 800 bp inserts were also included adding 1 µl of 1:1000 diluted plasmid DNA (100 ng/µl) to the PCR mix. Amplifications were carried out in a Hybaid Px2 Thermal Cycler programmed to perform an initial denaturation step at 94°C (5 min), 25 cycles of 94°C (30 s), 55°C (30 s) and 72°C (30 s), followed by a final extension step at 72°C for 7 min. Subsequently the entire 10 µl product of each reaction was electrophoresed on a 1% (w/v) agarose gel for detection as previously described.

4.2.5.4 Isolation of pGEM T-easy constructs

The confirmed positive colonies were inoculated in 14 ml Falcon tubes in 5 ml of LB-medium containing ampicillin (100 µg/ml) (Sigma) and incubated at 37°C for 16 hours on an orbital shaker set at 200 rpm. A volume of 2 ml of each inoculant was used for subsequent plasmid isolations using a Plasmix Miniprep kit (Talent) according to the manufacture's instructions, while 500 µl of each inoculant was stored in an equal volume 80% (v/v) glycerol at -80°C for future use. Following plasmid isolation, 2 µl of each 50 µl product was electrophoresed on a 1% (w/v) agarose gel for detection as previously described, and the concentration of each sample determined spectrophotometrically (NanoDrop).

4.2.5.5 Sequencing of plasmid inserts

The insert in the purified plasmid DNA was amplified during sequencing PCR. For this, six overlapping primers were designed using Primer Designer (v 1.01) (Figure 4.2).

These primers (Table 4.4) were used in conjunction with T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAGAA-3') in order to sequence the entire 2829 bp insert in eight separate sequencing reactions. Sequencing was performed using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Each sequencing reaction contained 4 µl Terminator mix, 3 µl primer (3.3 pmol/µl), and 3 µl plasmid DNA (92.5 ng/µl). Amplifications were carried out in a Hybaid Px2 Thermal Cycler programmed to perform 35 cycles of 96°C (10 s), 52°C (30 s), 60°C (4 min), followed by a final extension step at 60°C for 10 min. Sequencing PCR reaction products were analyzed with an ABI[®] 3100 Genetic Analyser at the DNA sequencing facility of the University of Stellenbosch. Resulting chromatograms were edited using Chromas and the nucleotide sequences manually aligned in BioEdit.

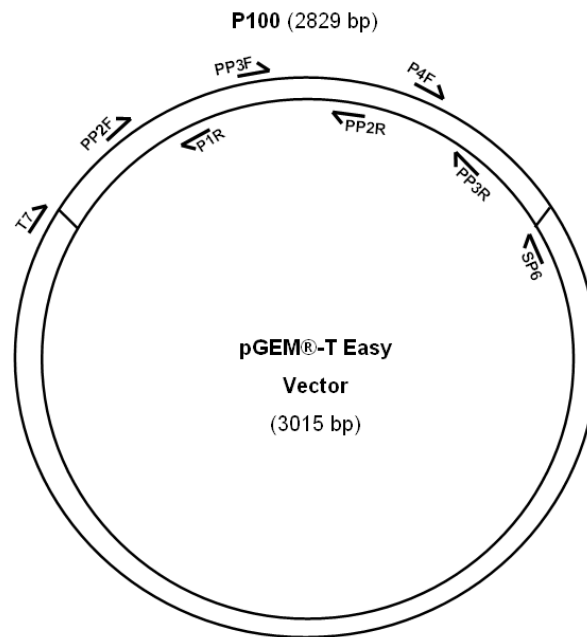


Figure 4.2 Schematic illustration of the *P100* gene (2829 bp) cloned into pGEM[®]-T Easy vector (3015 bp) to indicate the positions of the primers in the recombinant vector for subsequent sequencing of the *P100* gene.

4.2.6. Modification of the *P100* gene by site-directed mutagenesis

Following the successful cloning of the *P100* gene into the pGEM[®]-T Easy plasmid, the gene was modified by changing all the mycoplasma tryptophan codons (TGA) to universal tryptophan codons (TGG) by site-directed mutagenesis (SDM).

4.2.6.1 Primer design

Primers for the insertion of ten point mutations at specific sites within the *P100* gene (Figure 4.3) were designed using Primer Designer (v 1.01).

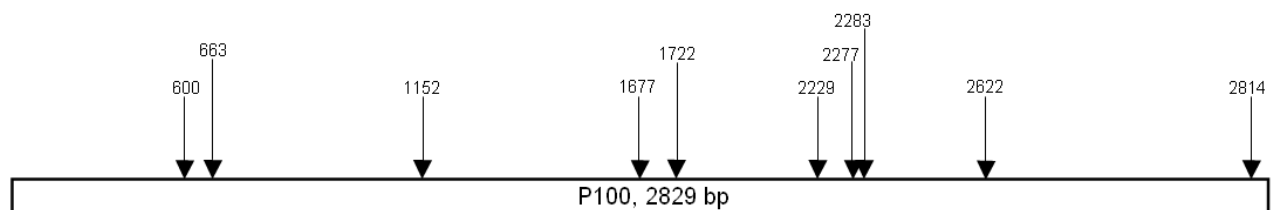


Figure 4.3 Schematic illustration of the position of the ten sites in the *P100* gene for which site-directed mutagenesis primers were designed.

The nucleotide sequences of the primers that were subsequently synthesized by IDT are shown in Table 4.5.

Table 4.5 Nucleotide sequences of the primers used for side-directed mutagenesis in the *P100* gene inserted in the pGEM[®]-T Easy plasmid. The underlined base in each primer represents the incorporated mutation; in the forward primers – TGA becomes TGG, and in the reverse primers – TCA becomes CCA. In the primer pair SDM P10F/R the termination codon is indicated in bold, and the *AccI* recognition sequence is underlined with the arrows indicating the restriction cut site.

Primer name	Primer sequence (5'→3')	bp-position	T _m (°C)
SDM P1F	5' -CTTAAAGACAATTTAAAATGGGTAAATTCAAAAGGTGAAG-3'	580-619	57.8
SDM P1R	5' -CTTCACCTTTTGAATTTACCCATTTTAAATTGTCTTTAAG-3'		
SDM P2F	5' -CTAAAGATTTCTATTATTCATGGCTAAGAACAAATCAAACAATTG-3'	641-685	58.7
SDM P2R	5' -CAATGTGTTGATTTGTCTTAGCCATGAATAATAGAAATCTTTAG-3'		
SDM P3F	5' -GCTAAATTAGGTGTTTACTGGTATGGTGTAAACAGCAAATAC-3'	1132-1172	61
SDM P3R	5' -CTATTTGCTGTTACACCATAACAGTAAACACCTAATTTAGC-3'		
SDM P4F	5' -CTTTATTACAAGCTGCAATTAAGTGAATACAGTAGCAGATGTAAGAAC-3'	1652-1700	62.4
SDM P4R	5' -GTTCTTACATCTGCTACTGTATTCCAGTTAATTGCAGCTTGTAAATAAAG-3'		
SDM P5F	5' -CAAACGGTGTTCAGAAGCTTGGTTGGCGAAATTAGCCGATGGTG-3'	1700-1744	68.2
SDM P5R	5' -CACCATCGGCTAATTTCCGCAACCAAGCTTCTGAAACACCGTTTG-3'		
SDM P6F	5' -GGAACAAGAAACGTTGGTTGGAGTTATGACTATAACTCAATAG-3'	2209-2251	61.6
SDM P6R	5' -CTATTGAGTTATAGTCATAACTCCAACCAACGTTTCTTGTTC-3'		
SDM P7&8F	5' -GTTATGATGGTTTATCATGGAATTGGCCATTATTCCCAACTC-3'	2258-2299	62.4
SDM P7&8R	5' -GAGTTGGGAATAATGGCCAATTCCATGATAAACCATCATAAC-3'		
SDM P9F	5' -CAATACTCAGCGTTTTCTGGAACCAATACGTAGCAGAC-3'	2602-2640	64.3
SDM P9R	5' -GTCTGCTACGTATTGGTTCCAGAAAACCGCTGAGTATTG-3'		
SDM P10F	5' -GACATGTATGTTGATTGGAGAATCAATAAA TAAGTCGAC -3'	2797-2835	60.1
SDM P10R	5' - <u>GTCGAC</u> TT ATTTATTGATTCTCCAATCAACATACATGTC-3'		

4.2.6.2 PCR based site-directed mutagenesis

PCR-based SDM reactions were carried out in accordance to the KAPAHiFi PCR protocol. Each reaction contained 10 µl of 5x reaction buffer, 1.5 µl of 10 mM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP) (KapaBiosystems), 1.5 µl of each complimentary mutagenic primer (10 pmol/µl), 0.5 µl of 25 mM MgCl₂, 1 µl of 1 U/µl High Fidelity Taq DNA polymerase (KapaBiosystems), 10 ng purified template DNA, and Milli-Q[®] water to a final volume of 50 µl. Amplifications were carried out in a Hybaid Px2 Thermal Cycler programmed to perform an initial denaturation step at 95°C (2 min), 15 cycles of 98°C (20 s), annealing at (T_m + 3°C) for 15 s and 68°C (1 min 30 s), followed by a final extension step for 5 min at 68°C.

In cases where the melting temperature of two mutagenic primer pairs did not differ by more than 4°C, and primers were not situated too close to one another, two primer pairs were used together in a SDM

PCR (SDM-PCR), thus allowing simultaneous modification of their respective sites. In cases where the melting temperature differed, the melting temperature that was used in the PCR was that of the primer pair possessing the lower melting temperature. Where two primer pairs were used, the PCR mix remained the same, with the only adjustment being the amount of Milli-Q[®] water added. The sequence in which the primer pairs or combinations of primer pairs were used were as follows: SDM P1 (F & R, $T_m = 57.8^\circ\text{C}$); SDM P4 (F & R, $T_m = 62.4^\circ\text{C}$) together with SDM P4 (F & R, $T_m = 62.4^\circ\text{C}$) and SDM P7&8 (F & R, $T_m = 62.4^\circ\text{C}$); SDM P3 (F & R, $T_m = 61^\circ\text{C}$) together with SDM P3 (F & R, $T_m = 61^\circ\text{C}$) and SDM P6 (F & R, $T_m = 61.6^\circ\text{C}$); SDM P2 (F & R, $T_m = 58.7^\circ\text{C}$) together with SDM P3 (F & R, $T_m = 61^\circ\text{C}$) and SDM P10 (F & R, $T_m = 60.1^\circ\text{C}$); and finally SDM P5 (F & R, $T_m = 68.2^\circ\text{C}$) together with SDM P5 (F & R, $T_m = 68.2^\circ\text{C}$) and SDM P9 (F & R, $T_m = 64.3^\circ\text{C}$).

4.2.6.3 DpnI treatment of PCR product

The SDM-PCR product was subsequently treated with DpnI to digest methylated wild-type DNA. The 50 μl reaction mixture contained 5 μl of 10x reaction buffer, 10 μl DNA (PCR product), 0.1 μl of 10 U/ μl Dpn I (Fermentas) and 34.9 μl Milli-Q[®] water. Digestion was carried out at 37°C for 2 hours, after which the enzyme was inactivated at 80°C for 20 min.

4.2.6.4 Agarose gel analysis

To assess the success of the SDM-PCR and digestion of methylated wild-type DNA, 2 μl supercoiled plasmid DNA (pre-PCR), 10 μl SDM-PCR product (post-PCR), and 18 μl DpnI treated SDM-PCR product (post-DpnI) respectively was electrophoresed on a 1% (w/v) agarose gel for visualization as previously described.

4.2.6.5 Isolation of modified recombinant pGEM T-easy constructs

The DpnI digestion products was subsequently purified by using the DNA Clean & ConcentratorTM-5 kit (ZYMO Research) according to manufacturers' instruction, and the vector constructs used directly in the transformation of competent JM109 cells as previously described.

Once again the blue/white screening system was used to identify positive clones containing the recombinant pGEM[®]-T Easy vector, and the positive transformants subjected to diagnostic PCR with T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAGAA-3') primers to verify the presence of insert as previously described. Confirmed positive clones were cultivated for subsequent plasmid isolations as previously described.

4.2.6.8 Sequencing of modified plasmid insert

In order to verify that a specific base was successfully modified, the insertion of the purified recombinant plasmid DNA was sequenced using the six overlapping primers described in Table 4.4, as well as T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAGAA-3') in order to

sequence the entire 2829 bp insert in eight separate sequencing reactions as previously described. Sequencing was performed using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit, and the products analyzed with an ABI[®] 3100 Genetic Analyser as previously described, and resulting chromatograms edited using Chromas and the nucleotide sequences manually aligned in BioEdit.

4.3 Results

4.3.1 Isolation of genomic DNA

4.3.1.1 Comparison of gDNA extraction methods

Genomic DNA was isolated successfully from *Ms01* cultures by the four different extraction methods. The concentration (ng/μl) and purity (A_{260}/A_{230} and A_{260}/A_{280} ratios) of the gDNA preparations obtained by means of the respective extraction methods are shown in Table 4.6.

Table 4.6 Comparison of the average gDNA yield obtained from the respective extraction methods determined by spectrophotometric analysis (NanoDrop, ND-1000).

Isolation method	Concentration (ng/μl)	μg	A_{260}/A_{280}	A_{260}/A_{230}
Hempstead	524.32	26.22	1.93	1.80
Phenol:chloroform	753.58	37.68	1.96	2.06
Wizard Genomic DNA Purification kit (Promega)	53.64	5.36	1.55	0.60
ZR Fungal/Bacterial DNA (ZYMO Research)	2.62	0.26	0.89	1.36

The phenol:chloroform extraction method yielded the highest concentration of DNA, with the Hempstead method giving a slightly lower yield, while the Wizard Genomic DNA Purification kit (Promega) yielded a low but adequate amount of DNA, and the ZR Fungal/Bacterial DNA kit (ZYMO Research) gave a less than satisfactory yield. The same trend was apparent when comparing the A_{260}/A_{280} and A_{260}/A_{230} ratios of the phenol:chloroform and Hempstead extractions methods. The Wizard Genomic DNA Purification kit gave lower A_{260}/A_{280} and A_{260}/A_{230} ratios, indicative of protein and organic solvent contamination respectively. Over and above its extremely low yield, the ZR Fungal/Bacterial DNA kit also gave a very low A_{260}/A_{280} ratio, indicative of major protein contamination, and a lower A_{260}/A_{230} ratio showing high organic solvent contamination.

4.3.1.2 Confirmation of *Ms01* identity

The identity of the mycoplasma from which gDNA was isolated was confirmed to be *Ms01* by the successful production of an amplification product of 750 bp, sequencing and subsequent alignment of the 750 bp product with the known 16S rRNA gene sequence of *Ms01*.

4.3.2 Whole-genome GS20 sequencing of *Ms01*

Using gDNA isolated by means of the Hempstead method, the entire genome of *Ms01* was successfully sequenced with 99.7% accuracy. *De novo* shotgun sequence assembly resulted in 64 large contiguous sequences (contigs) amounting to the estimated 700 kbp (693513 bp) genome size of *Ms01* with a G+C content of 27%. The contigs ranged in size from 491-86684 bp and are listed from smallest to largest in no fixed orientation in Table 4.7.

Table 4.7 Summary of the 64 large contigs obtained from whole-genome GS20 sequencing of *Ms01* using the Roche 454 Sequencing System.

500-1000 bp (29 contigs)			1000-10 000 bp (18 contigs)			>10 000 (17 contigs)		
Contig ID number	Contig length	Number of reads	Contig ID number	Contig length (bp)	Number of reads	Contig ID number	Contig length	Number of reads
9732	491	24	9330	1499	101	9373	11511	4159
9340	503	28	9390	1578	198	9372	13994	5105
9924	505	38	9405	1606	1107	9355	16063	5792
10610	520	28	835	1645	561	9391	16788	6195
9385	527	21	9337	1702	1256	4709	17166	6536
10199	529	25	9381	1989	687	4259	18387	6534
9656	535	22	9339	2145	749	9383	18643	7064
8968	542	31	9309	2263	834	9420	21331	7395
9913	548	23	9333	2263	858	9343	21528	7847
6122	550	25	9	3243	471	9423	29944	10868
9824	550	25	9409	5557	1977	9371	30066	10619
6749	557	19	9358	5874	1940	9310	32759	12524
9379	568	44	9360	7195	2578	9394	44778	15603
6767	605	22	1863	7364	2494	10065	49240	17434
10003	642	34	7839	7365	2661	9312	53326	17519
9336	657	36	9316	7607	2785	10066	68032	22897
3878	664	27	9387	8040	2898	9398	86684	29349
10592	665	28	6	9828	3325			
9318	701	231						
9542	707	32						
9885	726	39						
9406	767	40						
7865	791	41						
9914	793	38						
9317	820	213						
9401	848	262						
9319	874	575						
9356	887	634						
1224	888	36						

4.3.3 Identification of a vaccine candidate gene in Ms01 by bioinformatic analysis of whole-genome GS20 sequencing data

4.3.3.1 Identification of contigs in the genome of Ms01

Similarity searches using blastn with individual contigs in the NCBI database, led to the identification of two contigs in the *Ms01* genome, contig 835 (1645 bp) and contig 9312 (53 326 bp), which contained a number of genes encoding membrane proteins involved in membrane transport and/or host attachment. Similarity searches in contig 9312 using blastn and tblastx resulted in 1288 and 8095 hits respectively, while similar searches in contig 835 yielded 163 and 154 hits, respectively. Of these, the 5 most significant hits are indicated in Table 4.8. In particular, the well-documented oligopeptide permease system, the Opp operon, was further analyzed since the *oppA/P100* gene encodes a surface exposed substrate-binding protein in *M. hominis* and is predicted to show strong immunogenic properties when recognized by the host's immune system (Henrich *et al.*, 1993; Garmory and Titball, 2004; Nicolás *et al.*, 2007). Alignment of the contigs and their *M. hominis* Opp operon counterpart revealed that the *Ms01* *P100* gene analogue was situated on the 5' end of contig 9312, and on the 3' end of contig 835.

4.3.3.2 ORF analysis using CLC Combined Workbench software

ORF searches in contig 9312 resulted in a total of 49 ORFs of a minimum length of 100 codons of which 22 ORFs were in the positive direction, and 27 ORFs in the negative direction. In contig 835, an initiation codon was recognized at base position 545, but no corresponding termination codon could be identified; therefore no ORFs of a minimum length of 100 codons were identified. Since our interest lay in the 5' end of contig 9312, the first five 5' ORFs identified in contig 9312 are summarized in Table 4.9. Downstream of the *P100* gene, four ORFs with the highest similarity to genes encoding the core domains of the *M. hominis* oligopeptide permease (Opp) system were found. The second ORF started 17 bp downstream of the *P100* gene and encoded a putative protein with homologies to the integral membrane domain, OppB, of *M. hominis*. The third ORF followed 5 bp downstream of the *oppB* gene and encoded a putative protein with homologies to the integral membrane domain, OppC, of *M. hominis*. The fourth ORF started directly downstream of the *oppC* gene, with a single base overlap between the termination codon of the *oppC* gene and the initiation codon of the next ORF encoding a protein with homologies to the ATP-binding domain OppD. The fifth and final ORF showed an overlap of 14 bp at the 3' end of the *oppD* gene and showed homologies to the ATP-binding domain OppF. When comparing the length of the five ORFs identified at the 5' end of contig 9312 with those of the ORFs encoding the respective genes of the Opp operon in *M. hominis*, it was found that the lengths were very similar to the *oppBCDF* genes. However, the ORF encoding the *P100* gene in *Ms01* (1166 bp), was notably shorter than that of *M. hominis* (2885 bp), possibly indicating that the full gene had not been identified and that part of the *Ms01* *P100* gene was contained in contig 835.

Table 4.8 The five most significant hits obtained from BLAST searches on contig 9312 and 835 in the NCBI database; blastn; with a wordsize of 15, and tblastx employing the mold mitochondrial genetic code.

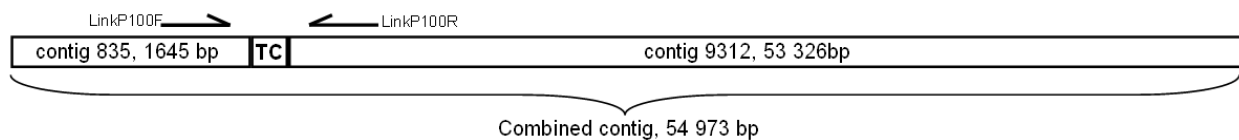
Query sequence	Search type	Accession number	Description	Bit score	E-value
Contig 9312	blastn	X99740.1	<i>M. hominis</i> P100, <i>oppB</i> , <i>oppC</i> , <i>oppD</i> , <i>oppF</i> genes (oligopeptide permease system)	1833	0
		CP001047.1	<i>M. arthritidis</i> 158L3-1, complete genome (peptide chain release factor 1; protoporphyrinogen oxidase)	1142	0
		CU179680.1	<i>M. agalactiae</i> PG2 chromosome, complete sequence (oligopeptide ABC transporter permease protein <i>oppC</i> and ATP-binding protein <i>oppD</i>)	711	0
		AE017245.1	<i>M. synoviae</i> 53, complete genome (oligopeptide ABC transporter ATP-binding protein)	587	2e-162
		AE017308.1	<i>M. mobile</i> 163K complete genome (protoporphyrinogen oxidase; peptide chain release factor 1)	580	3e-160
	tblastx	gb CP001047.1 	<i>M. arthritidis</i> 158L3-1, complete genome (peptide chain release factor 1; protoporphyrinogen oxidase)	888	0
		emb X99740.1 MHX99740	<i>M. hominis</i> P100, <i>oppB</i> , <i>oppC</i> , <i>oppD</i> , <i>oppF</i> genes (oligopeptide permease system)	719	0
		emb CU179680.1 	<i>M. agalactiae</i> PG2 chromosome, complete genome (oligopeptide ABC transporter permease protein <i>oppC</i> and ATP-binding protein <i>oppD</i>)	570	0
		gb AE017245.1 	<i>M. synoviae</i> 53, complete genome (oligopeptide ABC transporter ATP-binding protein)	519	0
		gb AE017243.1 	<i>M. hyopneumoniae</i> J, complete genome (putative lipoprotein)	279	0
Contig 835	blastn	X99740.1	<i>M. hominis</i> P100, <i>oppB</i> , <i>oppC</i> , <i>oppD</i> , <i>oppF</i> genes (oligopeptide permease system)	62.6	6e-06
		CP000123.1	<i>M. capricolum</i> subsp. <i>capricolum</i> ATCC 27343, complete genome (thioredoxin reductase)	44.6	0.001
		CP001047.1	<i>M. arthritidis</i> 158L3-1, complete genome (methylenetetrahydrofolate dehydrogenase)	41.0	0.013
		AL445563.1	<i>M. pulmonis</i> (strain UAB CTIP) complete genome; segment (ABC transporter ATP-binding protein)	41.0	0.013
		CU179680.1	<i>M. agalactiae</i> PG2 chromosome, complete sequence (putative transmembrane protein)	39.2	0.046
	tblastx	emb CU179680.1 	<i>M. agalactiae</i> PG2 chromosome, complete genome (conserved hypothetical protein; predicted lipoprotein)	99.6	1e-29
		emb X99740.1 MHX99740	<i>M. hominis</i> P100, <i>oppB</i> , <i>oppC</i> , <i>oppD</i> , <i>oppF</i> genes (oligopeptide permease system)	77.1	1e-29
		gb CP001047.1 	<i>M. arthritidis</i> 158L3-1, complete genome (hypothetical lipoprotein)	79.9	2e-22
		gb AE017244.1 	<i>M. hyopneumoniae</i> 7448, complete genome (lipoprotein)	64.3	8e-20
		gb AE017243.1 	<i>M. hyopneumoniae</i> J, complete genome (putative lipoprotein)	64.3	8e-20

Table 4.9 The first five ORF identified 5' in contig 9312.

Contig No.	Start position	End position	Length	Assigned gene analogue
9312	8	1726	1719	<i>P100</i>
	1744	2877	1134	<i>oppB</i>
	2883	4193	1311	<i>oppC</i>
	4193	5332	1140	<i>oppD</i>
	5319	7826	2508	<i>oppF</i>

4.3.3.3 Analysis of contiguous sequences by PCR

PCR amplification with the primer pair LinkP100F/R generated a 200 bp product, which upon sequencing and alignment with contig 835 and contig 9312 revealed firstly that the position of contig 835 was immediately upstream of contig 9312, and secondly that the two contigs were separated by two nucleotides only. Contigs 835 and 9312 could therefore be combined in a new contig of 54 971 bp in length (Figure 4.4).

**Figure 4.4** Schematic illustration of the combined contig following linkage of contig 9312 (53 326 bp) and contig 835 (1645 bp) by PCR with the primer pair LinkP100F and LinkP100R.

When ORF searches were repeated on the combined 54 973 bp contig, all the ORF identified in Table 4.9 remained the same, with the exception of the first ORF encoding the putative *P100* gene. Initial ORF analysis on the separate contigs indicated the first ORF to start at base position 8 on contig 9312. However, revision of this ORF revealed a new initiation codon at base position 545 in contig 835, resulting in an increase in the size of the first ORF assigned to the *P100* gene from 1719 bp to 2829 bp, corresponding in size with that of the ORF encoding the *P100* gene in *M. hominis* (2885 bp). No further ORFs were identified upstream of the *P100* gene and within 72 bp downstream of the *oppF* gene.

4.3.3.5 Identification of functional domains by comparative genomics

Analysis by comparative genomics revealed a putative ribosomal binding site (Shine-Dalgarno sequence) and two consensus promoter binding sites, designated the -35 and -10 box, 42 bp, 21 bp and 5 bp upstream of the translation initiation codon ATG of the *P100* gene in the combined 54 971 bp contig respectively (Figure 4.5 (A)). The length of the genes of the Opp operon of *Ms01* and *M. hominis* were

compared (Figure 4.5 (B)), and functionally important conserved motifs of the respective Opp domains were identified (Figure 4.5 (C-F)). At the N-terminal of the translated P100 polypeptide chain, a signal peptide II recognition site [(-4)-VAASC-(+1)] with a lipoprotein cysteine attachment site (₂₂LVAAACNSKSA₃₂) was identified. A characteristic oligopeptide-binding domain (₁₉₂YVLKDNLKW₂₀₀) was also identified on the translated P100 polypeptide chain confirming the substrate-binding function of the P100 protein. Furthermore, the highly conserved ATP-binding Walker A motif (₇₅₂GSGYDGLS₇₅₉), as well as the less conserved Walker B motif (₇₃₂INQGANGTRNVGWSYD₇₄₇), was identified on the translated P100 polypeptide chain, lending to the substrate-binding domain its unusual ecto-ATPase activity as first described for the OppA domain of *M. hominis* (Figure 4.5 (C)). A characteristic conserved membrane spanning domain was identified in each of the integral pore forming domains, OppB (₂₄₁IAKTKGLSSTQIFFKYVLR₂₅₉) and OppC (₃₂₅TAAKAIGASTKRQVFVHALP₃₄₄) (Figure 4.5 (D)). In each of the two ATP-binding domains, OppD and OppF, the highly conserved Walker A motif (₇₂GESGSGKS₇₈ and ₅₃GESGSGKT₆₀), the less conserved Walker B motif (₂₀₈VIIADEPTTALD₂₁₉ and ₆₉₅IIVADEPIASLD₇₀₆), as well as a highly conserved consensus signature sequence known as the C-motif (₁₈₈LSGGM₁₉₂ and ₆₇₅FSGGQ₆₇₉) were identified (Figure 4.5 (E-F)).

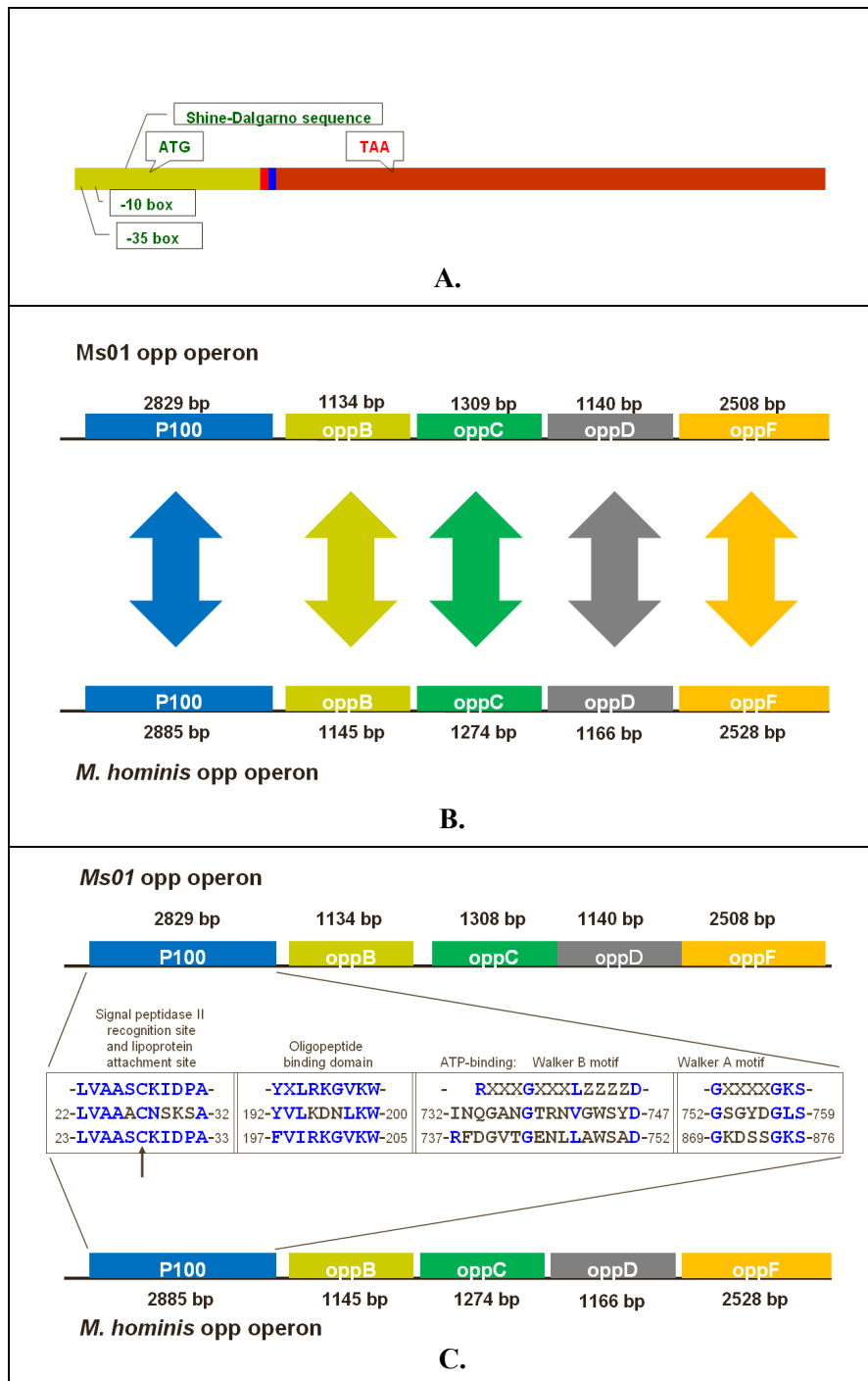


Figure 4.5 (A) Identification of a putative ribosomal binding site (Shine-Dalgarno sequence) and two consensus promoter binding sites (-10 and -35 box) upstream of translation initiation codon ATG of the *P100* gene. (B) Size (bp) comparison of the genes within the *Ms01* Opp operon and *M. hominis* Opp operon. (C) Identification of functionally important conserved motifs within the P100/oppA domain of *Ms01*.

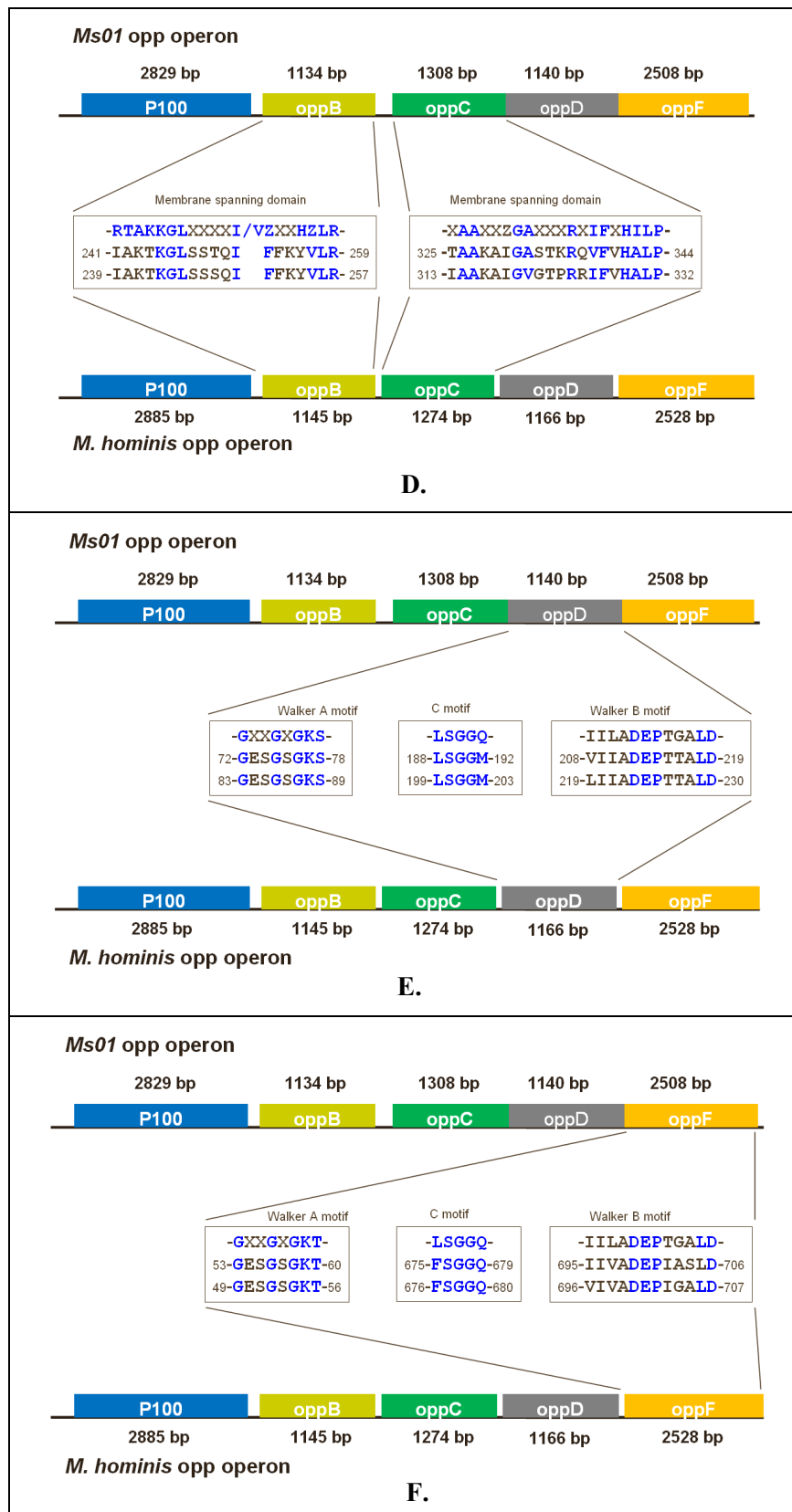


Figure 4.5 (continued) (D) Identification of functionally important conserved motifs within the integral membrane domains OppB and OppC of *Ms01*. (E and F) Identification of functionally important conserved motifs within the ATP-binding domains OppD and OppF of *Ms01* respectively.

4.3.4 Analysis of PCR amplification of the *P100* gene

The *P100* gene of *Ms01* was successfully isolated from gDNA by PCR using the primer pair P100_ampF and P100_ampR yielding a 2841 bp amplification product (2829 bp *P100* gene + 2 x 6 bp representing restriction enzyme cut sites) (Figure 4.6).

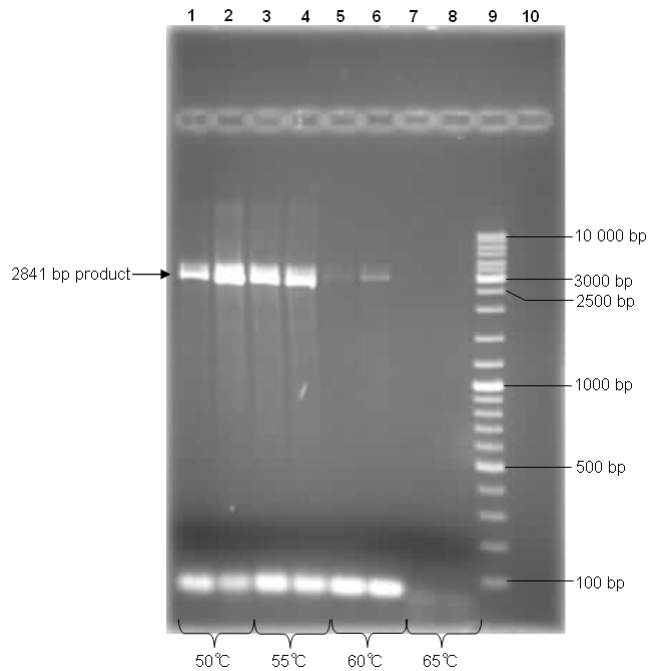


Figure 4.6 Image of the PCR product obtained with the primer pair P100_ampF and P100_ampR electrophoresed on a 2% (w/v) agarose gel. The gel contained ethidium bromide (0.175 µg/ml) for UV visualization of the DNA. Lane 1-8: 10 µl PCR product. Amplifications were performed and analyzed in duplicate and with an annealing temperature gradient (50°C -65°C). Lane 9: 5 µl DNA ladder mix (Fermentas). The bands in lanes 1 to 6 did not migrate as expected due to a reduced mobility on this gel (gel smiling).

A high-quality amplification product was obtained with annealing temperatures at 50°C and 55°C. An annealing temperature of 60°C yielded inadequate amplification product, while no amplification occurred at 65°C. Subsequently it was decided that an annealing temperature of 55°C was most suitable for amplifications with the primer pair P100_ampF and P100_ampR.

4.3.5 Cloning of *P100* gene into the pGEM[®]-T Easy plasmid

The *P100* gene of *Ms01* was successfully cloned into the pGEM[®]-T Easy plasmid using the pGEM[®]-T Easy cloning kit (Promega) resulting in ample white colonies. The presence of insert in white colonies was confirmed by use of diagnostic PCR with the primers T7 and SP6 resulting in a 2969 bp amplification product (2829 bp *P100* gene + 2 x 6 bp representing restriction enzyme cut sites + 128 bp plasmid DNA) (Figure 4.7).

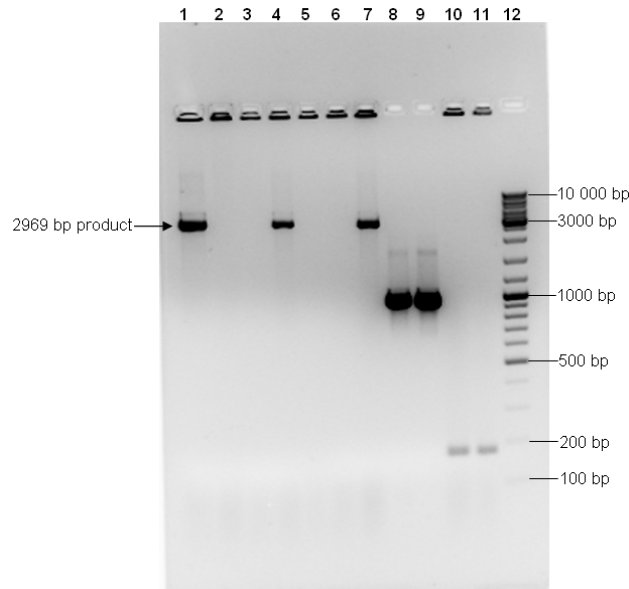


Figure 4.7 Image of the product obtained after diagnostic PCR with the primer pair T7 and SP6 electrophoresed on a 2% (w/v) agarose gel. The gel contained ethidium bromide (0.175 $\mu\text{g/ml}$) for UV visualization of the DNA. Lane 1-7: 10 μl PCR product using white colonies as template DNA. Lane 8-9: 10 μl PCR product (positive control). Lane 10-11: 10 μl PCR product using blue colonies as template DNA (negative control). Lane 12: 5 μl DNA ladder mix (Fermentas).

Plasmids containing the *P100* gene were successfully isolated from the bacteria, followed by sequencing to examine the inserted *P100* gene for point mutations (deletions, substitutions, insertions) that may have occurred during amplification. An inspection of the sequences of the *P100* gene of 8 clones in which the *P100* gene had been amplified with the non-proofreading DNA polymerase (Super-Therm), revealed that between 1 and 5 errors occurred per clone during amplification (0% error-free clones). Consequently, a proofreading DNA polymerase was used for amplification prior to cloning, where 8 clones containing the *P100* gene revealed that although errors did still occur in some clones, at least one point mutation-free clone could be identified (minimum of 12.5% error-free clones). A plasmid containing the error-free *P100* gene insert was selected for subsequent modification by site-directed mutagenesis of the *P100* gene.

4.3.6. Analysis of the *P100* gene after modification by site-directed mutagenesis

The progress of the PCR-based SDM and subsequent DpnI treatments was monitored by agarose gel electrophoresis. A typical result, in this instance the SDM of sites 5 and 9, is illustrated in Figure 4.8. Isolated recombinant plasmid, pGEM[®]-T Easy-*P100* gene construct was used as a supercoiled pre-SDM-PCR reference in lane 1. The SDM-PCR with one SDM primer pair produced a full plasmid copy (in which site 5 was corrected) which was unmethylated and not supercoiled, and therefore showed a lower mobility (lanes 2 and 3). In this instance, the supercoiled plasmid template is not visible in lanes 2 and 3 due to its low concentration. The SDM-PCR with two SDM primer pairs produced a full plasmid copy (in which sites 5 and 9 were corrected) and also a large and a small truncated PCR product (the product of

primers SDM P5R and SDM P9F, and primers SDM P5F and SDM P9R, respectively). The large and the small truncated PCR products are linear, blunt-ended and therefore not functional plasmids. The full plasmid copy is unmethylated and not supercoiled, and therefore showed a lower mobility (the faint bands in lanes 4 and 5 of an approximate size of 6000 bp). By contrast, the methylated template was supercoiled (which is not visible in lanes 4 and 5 due to its low concentration). The methylated supercoiled plasmid template was removed prior to transformation by DpnI treatment as the methylated plasmid template would be preferentially transformed over unmethylated SDM-PCR product. The SDM products after DpnI treatment are shown in lanes 6 to 9 respectively. The intensities of the bands are reduced due to the 5 fold dilution brought about by the DpnI treatment.

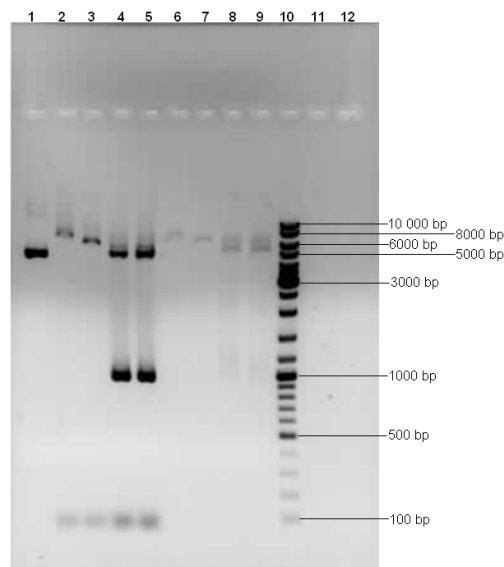


Figure 4.8 An image of a 1% (w/v) agarose gel showing the analysis of product following every step during site-directed mutagenesis of *P100* gene. Reactions were performed in duplicate. Lane 1: 2 μ l of a 100 ng/ μ l solution of plasmid containing *P100* gene insert (supercoiled) (pre-SDM PCR), lane 2-3: 10 μ l post-SDM PCR product with primer pair SDM P5F/R, lane 4-5: 10 μ l post-SDM PCR product with primer pairs SDM P5F/R (position 1722; T_m = 68.2°C) and SDM P9F/R (position 2622; T_m = 64.3°C), lane 6-7: 18 μ l of SDM PCR product (SDM P5F/R) post-DpnI-treatment, lane 8-9: 18 μ l of SDM PCR product (SDM P5F/R and SDM P9F/R) post-DpnI-treatment, lane 10: 5 μ l DNA ladder mix.

By employing SDM, the ten mycoplasmal codons for tryptophan (TGA) were modified to the universal codon for tryptophan (TGG), and the modifications confirmed by sequencing and subsequent alignment in BioEdit (Addendum C).

4.4 Discussion

The isolation of gDNA from *Ms01* cultures by means of the four different extraction methods yielded DNA with different degrees of purity. The phenol:chloroform extraction method yielded gDNA with the highest concentration and quality. However, in view of the fact that phenol contamination in the gDNA preparations may be problematic in GS20 sequencing, it was decided to use the gDNA isolated by means

of the Hempstead method for subsequent whole-genome GS20 sequencing, since it yielded gDNA of similar concentration and quality.

The entire genome of *Ms01* was sequenced successfully with 99.7% accuracy using GS20 sequencing technology. The estimated 700 kbp genome size of *Ms01* with a G+C content of 27 mol% is in agreement with the small size of mycoplasmal genomes ranging in size from 580 kbp (*M. genitalium*) to 1360 kbp (*M. penetrans*) and their characteristically low G+C content (23-41 mol%) (Nicolás *et al.*, 2007). It can be deduced that assembly of the contigs was largely successful as a high proportion of the contigs were of a size larger than 1000 bp. However, the undesirably large number of contigs generated may be attributed to the abundance of A and T-runs contained in the *Ms01* genome, as the assembler has difficulty in finding overlaps in the presence of poly-base runs exceeding a length of 8 bases, as well as when dealing with highly repetitive genomes (Leamon *et al.*, 2007).

During the search for a vaccine candidate gene in the *Ms01* genome data, no gene analogue encoding membrane proteins such as P1 in *M. pneumoniae*, MgPa in *M. genitalium*, or GapA in *M. gallisepticum*, could be identified. However, an analogue of the gene encoding the membrane protein P100 was identified. The *P100* gene forms part of the Opp operon encoding the well-documented oligopeptide permease (Opp) system. The identified *P100* gene analogue of *Ms01* was found to be contained partly on contig 835 (1645 bp) and contig 9312 (53 326 bp) respectively, and showed the highest sequence similarity to that of *M. hominis*. The Opp system of *M. hominis* contains a multifunctional P100 protein, which not only represents the substrate-binding OppA domain of the system, but is also involved in mycoplasma attachment to host cells (Henrich *et al.*, 1993, 1999; Hopfe and Henrich, 2004), therefore making the *P100* gene of *Ms01* a prime candidate for the development of a DNA vaccine.

Open reading frame searches on the two contigs, revealed that contig 9312 contained the major part of the *P100* gene on its 5' end, and that the rest of the gene was contained on the 3' end of 835. Consequently, a PCR strategy was followed to link these two contigs, followed by revision of identified open reading frames, which ultimately resulted in the identification of the entire *P100* gene, as well as the rest of the *opp* genes (*oppB*, *oppC*, *oppD* and *oppF*) of *Ms01*. Comparative genomics of the area upstream of the ATG translation initiation codon of the *P100* gene, led to the identification of the -10 and -35 consensus promoter binding sites, as well as a Shine-Dalgarno-like sequence, further confirming the assigned ORF of the P100 protein. Comparison of the Opp operon of *M. hominis* and the Opp operon of *Ms01*, revealed a clear correlation in gene length between the two species. The overlapping nature of the *opp* genes in the *Ms01* Opp operon is a common feature of many prokaryotes, bacteriophages, animal virus and mitochondrial genomes (Fukuda *et al.*, 1999). Although overlapping genes were initially thought to have emerged due to evolutionary pressure to minimize genome size, comparative genomics of the two mycoplasmas *M. pneumoniae* and *M. genitalium* reveal that overlapping genes, in many cases, arise due to the loss of a stop codon in either gene, resulting in elongation of the 3' of the genes' coding region. Furthermore, the overlapping genes of the *Ms01* Opp operon were found to be uni-directional, which is in

agreement with the majority of overlapping genes identified in the *M. pneumoniae* and *M. genitalium* genomes (Fukuda *et al.*, 1999).

Alignment of crucial conserved amino acid motifs of the *M. hominis* Opp operon and that of the *Ms01* Opp operon, revealed characteristic motifs related to the function of the individual *opp* genes, but also characteristic of ABC-transporters. The OppB and OppC domains contain motifs characteristic of integral membrane-spanning domains, while the OppD and OppF domains possess the typical highly conserved ATP-binding Walker A and Walker B motifs, as well as the highly conserved C-motif/linker peptide specific to the ABC superfamily. Together these motifs form the structure for ATP binding, lending to the ATPase domain the ability to bind and hydrolyse ATP to provide the energy for the transport of oligopeptides into the cell. The N-terminus of the P100 polypeptide chain contains a highly hydrophobic signal peptide II leader sequence which directs the translocation of the P100 protein from the cytoplasm to the plasma membrane. Within this sequence, a crucial cysteine residue was found, of which the SH-group is reportedly used to covalently link the P100 protein to an outer membrane lipoprotein, thereby ensuring that the P100 protein remains attached to the membrane of the mycoplasma after translocation (Henrich *et al.*, 1999). Furthermore, the P100 polypeptide chain contains a characteristic oligopeptide-binding domain which putatively binds oligopeptide substrates on the outside of the mycoplasma cell, and delivers them to the OppBC pore-forming complex of the permease system. An interesting finding is that the P100 polypeptide chain also possesses the highly conserved Walker A and Walker B motifs normally only found in the ATP-binding domains OppD and OppF. This was first described for the substrate-binding domain, OppA of *M. hominis*, where several functions for this ecto-ATPase activity of the substrate-binding domain were postulated; (i) protection from the cytolytic effect of extracellular ATP released by the colonized host cells, (ii) regulation of ecto-kinase substrate concentration, (iii) involvement in signal transduction, as well as (iv) possible involvement in cellular adhesion (Hopfe and Henrich, 2004). To our knowledge, this is only the second time that a mycoplasma substrate-binding domain has been identified to possess the highly conserved Walker A and Walker B motifs, shown to lend ecto-ATPase activity to the P100 of *M. hominis*.

The *P100* gene of *Ms01* was isolated from gDNA by PCR, exploring the use of proofreading and non-proofreading DNA polymerases. After successful cloning and sequencing of the *P100* gene in the pGEM[®]-T Easy plasmid, it was found that an abundance of point mutations was generated during amplification with non-proofreading enzyme. This indicated that the use of a proofreading DNA polymerase was a necessity during the isolation of the *P100* gene, as it resulted in notably less point mutations.

As the isolated and cloned *P100* gene of *Ms01* is to be used in a DNA vaccine, the ten mycoplasma tryptophan codons (TGA), encoding stop codons in the universal genetic code, were modified successfully by means of SDM to universal tryptophan codons (TGG) for expression in eukaryotes. The modified *P100* gene may now be cloned into a suitable eukaryotic expression vector such as pCI-neo,

after which vaccine trials in ostriches may be conducted to assess the efficacy of this gene in the successful application of a the DNA vaccine in combating mycoplasmas in ostriches. In addition, assembly of all the contigs and subsequent annotation of the entire genome of *Ms01*, will facilitate future identification of further vaccine candidate genes.

Chapter 5 – Conclusions and future perspectives

Three ostrich-specific mycoplasmas, *Ms01*, *Ms02* and *Ms03*, have been implicated in upper respiratory infection in feedlot ostriches. These infections place a considerable strain on production in the South African ostrich industry. Since there is currently no registered mycoplasma vaccine available for use in ostriches, the use of existing poultry mycoplasma vaccines to prevent mycoplasma infections in ostriches were suggested. From the results of the vaccine trials with MS and MG vaccines in ostriches conducted in this study, it is concluded that these poultry mycoplasma vaccines do not provide protection against ostrich-specific mycoplasma infecting ostriches in South Africa. Therefore, the approach of using poultry mycoplasma vaccines to prevent mycoplasma infections in ostriches is ineffective due to the lack of immunological cross-reactivity. This therefore emphasizes the importance of the development of a species specific vaccine against each of the ostrich-specific mycoplasmas *Ms01*, *Ms02* and *Ms03*.

However, conventional approaches to vaccine development against mycoplasmas are hindered by the weak cultivation properties of these ostrich mycoplasmas. Accordingly the DNA vaccine route was chosen for the development of a vaccine against the ostrich-specific mycoplasma *Ms01*. In order to identify a vaccine candidate gene in *Ms01*, the entire genome of *Ms01* was sequenced using the 454 Sequencing System based on GS20 sequencing technology. The resulting whole-genome sequencing data was subjected to bioinformatic analysis which resulted in the identification of the *P100* gene of *Ms01*. This gene was chosen as vaccine candidate as it was found to be an analogue of the *M. hominis* gene encoding the multifunctional substrate-binding and cytoadherence associated P100 protein. After the successful isolation and site-directed mutational modification of the *P100* gene of *Ms01*, the modified gene may be inserted into an appropriate DNA vaccine vector such as pCI-neo for subsequent use as a DNA vaccine in ostriches.

Vaccination trials with the resulting DNA vaccine, followed by field challenge with ostrich-specific mycoplasmas, will have to be conducted in future to assess the efficacy of this approach. To this end, an immunodiagnostic test, such as an ELISA, will have to be developed for assessment of immune responses elicited towards the *Ms01*-specific DNA vaccine in ostriches. Such assays will be carried out with ELISA plates coated with whole *Ms01* organism or P100 protein. The *P100* gene cloned in this study can be sub-cloned into a suitable prokaryotic expression vector for this purpose. Furthermore, the same labeled secondary antibody detection system used in this study, i.e. biotinylated rabbit anti-ostrich Ig antibodies, can also be used. If the DNA vaccine is found to provide protection in ostriches against *Ms01*, a similar approach may also be employed for the development of DNA vaccines against the other ostrich-specific mycoplasmas *Ms02* and *Ms03*. The whole-genome sequence data of *Ms01* and the approaches developed in this study may also be applied in searches for other potential vaccine candidate genes for the development of a DNA vaccine against *Ms01* in future. Thus, this study serves as a good platform for the future development of vaccines against the ostrich-specific mycoplasmas *Ms02* and *Ms03*.

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Addendum A Statistical analysis of the ELISA results using SAS

Kwessie (MS)

Vaccine trials with poultry mycoplasma vaccines in ostriches on the Kwessie farmstead: Statistical analysis of ELISA data, obtained with MS Antibody Test kits, using the Statistical Analysis System (SAS, Cary, NC) program (version 6.2).

Statistical analysis:

The General Linear Models (GLM) Procedure

Class Level Information										
Class	Levels	Values								
Trt	3	1	2	3						
Time	9	0	6	14	21	38	52	62	83	111

Number of Observations Read	1268
Number of Observations Used	1111

Dependent Variable: resp

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	26	36.81960471	1.41613864	65.57	<.0001
Error	1084	23.41261956	0.02159836		
Corrected Total	1110	60.23222427			

R-Squared	Coeff Var	Root MSE	Resp Mean
0.611294	144.5848	0.146964	0.101645

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	10.21842232	5.10921116	236.56	<.0001
Time	8	9.56305445	1.19538181	55.35	<.0001
Trt*Time	16	17.03812794	1.06488300	49.30	<.0001

The input data follows. On each page the data is arranged in three columns, each containing the ostrich identification number, treatment (Trt), time and response (Resp), read from top to bottom and left to right.

Ostrich	Trt	Time	Resp								
				360763	3	0	0				
360701	2	0	0.006	360764	3	0	0	360826	1	0	0.044
360702	2	0	0.026	360765	3	0	0.008	360828	1	0	0.035
360703	2	0	0.002	360766	3	0	0.011	360829	1	0	0.044
360704	2	0	0.008	360767	3	0	0.021	360830	1	0	0.042
360705	2	0	0.009	360768	3	0	0.039	360831	1	0	0.047
360706	2	0	0.006	360769	3	0	0.022	360832	1	0	0.058
360707	2	0	0.006	360770	3	0	0.012	360833	1	0	0.018
360708	2	0	0.008	360771	3	0	0.009	360834	1	0	0.037
360709	2	0	0.037	360772	3	0	0.026	360835	1	0	0.016
360710	2	0	0.026	360773	3	0	0.006	360836	1	0	0.03
360711	2	0	0.044	360774	3	0	0.052	360837	1	0	0.018
360712	2	0	0.024	360775	3	0	0.019	360838	1	0	0.031
360713	2	0	0.032	360776	3	0	0.033	360839	1	0	0.062
360714	2	0	0.038	360777	3	0	0.027	360840	1	0	0.015
360715	2	0	0.019	360778	3	0	0.021	360841	1	0	0.02
360716	2	0	0.043	360779	3	0	0.027	360701	2	6	0.023
360717	2	0	0.024	360780	3	0	0.027	360702	2	6	0.018
360718	2	0	0.028	360781	3	0	0.021	360703	2	6	0.029
360719	2	0	0.038	360782	3	0	0.025	360704	2	6	0.023
360720	2	0	0.022	360783	3	0	0.032	360705	2	6	0.032
360721	2	0	0.027	360784	3	0	0.014	360706	2	6	0.017
360722	2	0	0.043	360785	3	0	0.022	360707	2	6	0.019
360723	2	0	0.033	360786	3	0	0.023	360708	2	6	0.023
360724	2	0	0.021	360787	3	0	0.032	360709	2	6	0.019
360725	2	0	0.02	360788	3	0	0.024	360710	2	6	0.025
360726	2	0	0.029	360789	3	0	0.021	360711	2	6	0.026
360727	2	0	0.033	360790	3	0	0.019	360712	2	6	0.014
360728	2	0	0.034	360791	3	0	0.017	360713	2	6	0.024
360729	2	0	0.019	360792	3	0	0.016	360714	2	6	0.002
360730	2	0	0.034	360793	3	0	0.011	360715	2	6	0.005
360731	2	0	0.02	360794	3	0	0.02	360716	2	6	0.021
360732	2	0	0.01	360795	3	0	0.037	360717	2	6	0.016
360733	2	0	0.012	360796	3	0	0.017	360718	2	6	0.023
360734	2	0	0.03	360797	3	0	0.029	360719	2	6	0.022
360735	2	0	0.06	360798	3	0	0.042	360720	2	6	0.041
360736	2	0	0.034	360799	3	0	0.012	360721	2	6	0.04
360737	2	0	0.013	360800	3	0	0.023	360722	2	6	0.02
360738	2	0	0.031	360801	1	0	0.057	360723	2	6	0.015
360739	2	0	0.017	360802	1	0	0.026	360724	2	6	0.028
360740	2	0	0.022	360803	1	0	0.059	360725	2	6	0.009
360741	2	0	0.02	360804	1	0	0.013	360726	2	6	0.026
360742	2	0	0.032	360805	1	0	0.034	360727	2	6	0.011
360743	2	0	0.044	360806	1	0	0.049	360728	2	6	0.015
360744	2	0	0.03	360807	1	0	0.012	360729	2	6	0.014
360745	2	0	0.071	360808	1	0	0.015	360730	2	6	0.023
360746	2	0	0.034	360809	1	0	0.017	360731	2	6	0.047
360747	2	0	0.038	360810	1	0	0.043	360732	2	6	0.056
360748	2	0	0.022	360811	1	0	0.038	360733	2	6	0.027
360749	2	0	0.042	360812	1	0	0.034	360734	2	6	0.025
360750	2	0	0.026	360813	1	0	0.005	360735	2	6	0.019
360751	3	0	0.014	360814	1	0	0.025	360736	2	6	0.032
360752	3	0	0.01	360815	1	0	0.0320	360737	2	6	0.018
360753	3	0	0.016	360816	1	0	0.02	360738	2	6	0.012
360754	3	0	0.029	360817	1	0	0.026	360739	2	6	0.011
360755	3	0	0.003	360818	1	0	0.039	360740	2	6	0.027
360756	3	0	0	360819	1	0	0.036	360741	2	6	0.031
360757	3	0	0	360820	1	0	0.037	360742	2	6	0.027
360758	3	0	0	360821	1	0	0.042	360743	2	6	0.019
360759	3	0	0	360822	1	0	0.029	360744	2	6	0.02
360760	3	0	0.007	360823	1	0	0.053	360745	2	6	0.047
360761	3	0	0	360824	1	0	0.048	360746	2	6	0.026
360762	3	0	0	360825	1	0	0.032	360747	2	6	0.013

360748	2	6	0.03	360811	1	6	0.025				
360749	2	6	0.036	360812	1	6	0.057	360733	2	14	0.007
360750	2	6	0.013	360813	1	6	0.046	360735	2	14	0.005
360751	3	6	0.041	360814	1	6	0.031	360736	2	14	0.024
360752	3	6	0.023	360815	1	6	0.034	360737	2	14	0
360753	3	6	0.02	360816	1	6	0.012	360738	2	14	0
360754	3	6	0.029	360817	1	6	0.01	360739	2	14	0.007
360755	3	6	0.031	360818	1	6	0.022	360740	2	14	0.015
360756	3	6	0.017	360819	1	6	0.029	360741	2	14	0.008
360757	3	6	0.015	360820	1	6	0.019	360742	2	14	0.271
360758	3	6	0.014	360821	1	6	0.024	360743	2	14	0.025
360759	3	6	0.003	360822	1	6	0.027	360744	2	14	0.013
360760	3	6	0	360823	1	6	0.033	360745	2	14	0.092
360761	3	6	0.008	360824	1	6	0.025	360746	2	14	0.016
360762	3	6	0.01	360825	1	6	0.004	360747	2	14	0
360763	3	6	0.008	360826	1	6	0.016	360748	2	14	0.006
360764	3	6	0.012	360827	1	6	0.007	360749	2	14	0
360765	3	6	0.011	360828	1	6	0.012	360750	2	14	0.064
360766	3	6	0.013	360829	1	6	0.01	360751	3	14	0.009
360767	3	6	0.005	360830	1	6	.	360752	3	14	0
360768	3	6	0.028	360831	1	6	0.008	360753	3	14	0.002
360769	3	6	0.005	360832	1	6	.	360754	3	14	0.003
360770	3	6	0.036	360833	1	6	0.003	360755	3	14	0.019
360771	3	6	0.025	360834	1	6	0.005	360756	3	14	0.024
360772	3	6	0.021	360835	1	6	0.023	360757	3	14	0.024
360773	3	6	0.006	360836	1	6	0.043	360758	3	14	0.007
360774	3	6	0.074	360837	1	6	0.011	360759	3	14	0.021
360775	3	6	0.039	360838	1	6	0.02	360760	3	14	0.019
360776	3	6	0.011	360839	1	6	0.025	360761	3	14	0.005
360777	3	6	0.028	360840	1	6	0.014	360762	3	14	0.029
360778	3	6	0.018	360841	1	6	0.02	360763	3	14	0.013
360779	3	6	0.035	360701	2	14	0.006	360764	3	14	0.016
360780	3	6	0.032	360702	2	14	0.009	360765	3	14	0
360781	3	6	0.027	360703	2	14	0.067	360766	3	14	0.299
360782	3	6	0.017	360704	2	14	0.006	360767	3	14	0.018
360783	3	6	0.031	360705	2	14	0.055	360768	3	14	0.025
360784	3	6	0.017	360706	2	14	0.062	360769	3	14	0.26
360785	3	6	0.032	360707	2	14	0.015	360770	3	14	0.053
360786	3	6	0.023	360708	2	14	0.01	360771	3	14	0.032
360787	3	6	0.016	360709	2	14	0.061	360772	3	14	0.024
360788	3	6	0.03	360710	2	14	0.023	360773	3	14	0.056
360789	3	6	0.014	360711	2	14	0.032	360774	3	14	0.04
360790	3	6	0.02	360712	2	14	0.013	360775	3	14	0.034
360791	3	6	0.032	360713	2	14	0.047	360776	3	14	0.039
360792	3	6	0.005	360714	2	14	0.013	360777	3	14	0.141
360793	3	6	0.017	360715	2	14	0.031	360778	3	14	0.078
360794	3	6	0.025	360716	2	14	0.021	360779	3	14	0.028
360795	3	6	0.005	360717	2	14	0.04	360780	3	14	0.037
360796	3	6	0.01	360718	2	14	0.309	360781	3	14	0.056
360797	3	6	0.009	360719	2	14	0.006	360782	3	14	0.096
360798	3	6	0.031	360720	2	14	0.026	360783	3	14	0.048
360799	3	6	0.009	360721	2	14	0.005	360784	3	14	0.035
360800	3	6	0.017	360722	2	14	0.049	360785	3	14	0.027
360801	1	6	0.03	360723	2	14	0.014	360786	3	14	0.019
360802	1	6	0.022	360724	2	14	0.002	360787	3	14	0.028
360803	1	6	0.025	360725	2	14	0.058	360788	3	14	0.037
360804	1	6	0.013	360726	2	14	0.006	360789	3	14	0.036
360805	1	6	0.01	360727	2	14	0.004	360790	3	14	0.037
360806	1	6	0.025	360728	2	14	0.015	360791	3	14	0.03
360807	1	6	0.011	360729	2	14	0.003	360792	3	14	0.041
360808	1	6	0.017	360730	2	14	0.025	360793	3	14	0.034
360809	1	6	0.019	360731	2	14	0.008	360794	3	14	0.039
360810	1	6	0.015	360732	2	14	0.093	360795	3	14	0.036

360796	3	14	0.031	360718	2	21	0.351				
360797	3	14	0.036	360719	2	21	0.097	360781	3	21	0.036
360798	3	14	0.036	360720	2	21	0.044	360783	3	21	0.024
360799	3	14	0.057	360721	2	21	0.18	360784	3	21	0.016
360800	3	14	0.058	360722	2	21	0.14	360785	3	21	0.173
360801	1	14	0.05	360723	2	21	0.175	360786	3	21	0.004
360802	1	14	0	360724	2	21	0.096	360787	3	21	0.009
360803	1	14	0.034	360725	2	21	0.073	360788	3	21	0.025
360804	1	14	0	360726	2	21	0.095	360789	3	21	0.004
360805	1	14	0	360727	2	21	0.041	360790	3	21	0.035
360806	1	14	0	360728	2	21	0.05	360791	3	21	0.038
360807	1	14	0	360729	2	21	0.104	360792	3	21	0.047
360808	1	14	0	360730	2	21	0.041	360793	3	21	0.029
360809	1	14	0	360731	2	21	0.005	360794	3	21	0.048
360810	1	14	0	360732	2	21	1.116	360795	3	21	0.019
360811	1	14	0	360733	2	21	0.026	360796	3	21	0.02
360812	1	14	0.057	360734	2	21	0.004	360797	3	21	0.017
360813	1	14	0.011	360735	2	21	0	360798	3	21	0.018
360814	1	14	0.058	360736	2	21	0.016	360799	3	21	0.025
360815	1	14	0.027	360737	2	21	0	360800	3	21	0.046
360816	1	14	0.002	360738	2	21	0.253	360801	1	21	0.112
360817	1	14	0.005	360739	2	21	0.523	360802	1	21	0.059
360818	1	14	0.017	360740	2	21	0.165	360803	1	21	0.062
360819	1	14	0.033	360741	2	21	0.111	360804	1	21	0.039
360820	1	14	0.013	360742	2	21	0.608	360805	1	21	0.048
360821	1	14	0.01	360743	2	21	0.006	360806	1	21	0.056
360822	1	14	0.018	360744	2	21	0.014	360807	1	21	0.096
360823	1	14	0.012	360745	2	21	0.015	360808	1	21	0.042
360824	1	14	0.022	360746	2	21	0.092	360809	1	21	0.065
360825	1	14	0.032	360747	2	21	0.189	360810	1	21	0.071
360826	1	14	0.025	360748	2	21	0.009	360811	1	21	0.117
360827	1	14	0.009	360749	2	21	0.019	360812	1	21	0.047
360828	1	14	0.016	360750	2	21	0.034	360813	1	21	0.021
360829	1	14	0.005	360751	3	21	0.052	360814	1	21	0.008
360830	1	14	0.006	360752	3	21	0.047	360815	1	21	0.018
360831	1	14	0.003	360753	3	21	0.059	360816	1	21	0.01
360832	1	14	0.007	360754	3	21	0.036	360817	1	21	0.012
360833	1	14	0.009	360755	3	21	0.018	360818	1	21	0.011
360834	1	14	0.009	360756	3	21	0.061	360819	1	21	0.014
360835	1	14	0.005	360757	3	21	0.008	360820	1	21	0.01
360836	1	14	0.007	360758	3	21	0.05	360821	1	21	0.012
360837	1	14	0.011	360759	3	21	0.018	360822	1	21	0.02
360838	1	14	0.017	360760	3	21	0.033	360823	1	21	0.028
360839	1	14	0.01	360761	3	21	0.061	360824	1	21	0.016
360840	1	14	0.006	360762	3	21	0.064	360825	1	21	0.034
360841	1	14	0.013	360763	3	21	0.027	360826	1	21	0.017
360701	2	21	0.049	360764	3	21	0.025	360827	1	21	0.019
360702	2	21	0.131	360765	3	21	0.04	360828	1	21	0.016
360703	2	21	0.129	360766	3	21	0.185	360829	1	21	0.024
360704	2	21	0.023	360767	3	21	0.05	360830	1	21	0.028
360705	2	21	0.167	360768	3	21	0.019	360831	1	21	0.01
360706	2	21	0.062	360769	3	21	0.552	360832	1	21	0.025
360707	2	21	0.162	360770	3	21	0.017	360833	1	21	0.028
360708	2	21	0.013	360771	3	21	0.041	360834	1	21	0.024
360709	2	21	0.12	360772	3	21	0.012	360835	1	21	0.008
360710	2	21	0.176	360773	3	21	0.022	360836	1	21	0.024
360711	2	21	0.086	360774	3	21	0.036	360837	1	21	0.02
360712	2	21	0.162	360775	3	21	0.026	360838	1	21	0.013
360713	2	21	0.072	360776	3	21	0.055	360839	1	21	0.019
360714	2	21	0.03	360777	3	21	0.1	360840	1	21	0.015
360715	2	21	0.074	360778	3	21	0.017	360841	1	21	0.012
360716	2	21	0.018	360779	3	21	0.004	360701	2	38	0.037
360717	2	21	0.288	360780	3	21	0.216	360702	2	38	0.145

360703	2	38	0.036	360766	3	38	0.001				
360704	2	38	0.123	360767	3	38	0.007	360830	1	38	0.03
360705	2	38	0.053	360768	3	38	0.007	360831	1	38	0.012
360706	2	38	0.065	360769	3	38	0.01	360832	1	38	0.031
360707	2	38	0.01	360770	3	38	0.006	360833	1	38	0.053
360708	2	38	0.297	360771	3	38	0	360834	1	38	0.016
360709	2	38	0.021	360772	3	38	0	360835	1	38	0.073
360710	2	38	0.131	360773	3	38	0.004	360836	1	38	0.017
360711	2	38	0.106	360774	3	38	0.047	360837	1	38	0.001
360712	2	38	0.001	360775	3	38	0.018	360838	1	38	0.015
360713	2	38	0.006	360776	3	38	0.028	360839	1	38	0
360714	2	38	0.011	360777	3	38	0.026	360840	1	38	0.002
360715	2	38	0	360778	3	38	0.055	360841	1	38	0.005
360716	2	38	0	360779	3	38	0.035	360701	2	52	0.455
360717	2	38	0.328	360780	3	38	0.027	360702	2	52	0.93
360718	2	38	0.266	360781	3	38	0.014	360703	2	52	0.315
360719	2	38	0.052	360782	3	38	0.016	360704	2	52	0.777
360720	2	38	0.156	360783	3	38	0.036	360705	2	52	0.965
360721	2	38	0.134	360784	3	38	0.055	360706	2	52	1.222
360722	2	38	0.101	360785	3	38	0.112	360707	2	52	0.385
360723	2	38	0.087	360786	3	38	0.02	360708	2	52	0.649
360724	2	38	0.066	360787	3	38	0.034	360709	2	52	.
360725	2	38	0.062	360788	3	38	0.017	360710	2	52	.
360726	2	38	0.21	360789	3	38	0.007	360711	2	52	.
360727	2	38	0.083	360790	3	38	0.011	360712	2	52	1.191
360728	2	38	0.121	360791	3	38	0.006	360713	2	52	0.418
360729	2	38	0.068	360792	3	38	0.002	360714	2	52	0.504
360730	2	38	0.116	360793	3	38	0.012	360715	2	52	0.17
360731	2	38	0.027	360794	3	38	0.004	360716	2	52	.
360732	2	38	0.495	360795	3	38	0.014	360717	2	52	0.681
360733	2	38	0.045	360796	3	38	0.022	360718	2	52	1.153
360734	2	38	0.031	360797	3	38	0.024	360719	2	52	.
360735	2	38	0.004	360798	3	38	0.021	360720	2	52	0.339
360736	2	38	0.147	360799	3	38	0.008	360721	2	52	0.487
360737	2	38	0.012	360800	3	38	0.213	360722	2	52	0.558
360738	2	38	0.036	360801	1	38	0.036	360723	2	52	1.111
360739	2	38	0.053	360802	1	38	0.016	360724	2	52	.
360740	2	38	0.015	360803	1	38	0.039	360725	2	52	0.273
360741	2	38	0.017	360804	1	38	0.033	360726	2	52	0.377
360742	2	38	0.072	360805	1	38	0.027	360727	2	52	.
360743	2	38	0.11	360806	1	38	0.033	360728	2	52	0.518
360744	2	38	0.047	360807	1	38	0.027	360729	2	52	0.79
360745	2	38	0.004	360808	1	38	0.012	360730	2	52	0.989
360746	2	38	0.004	360809	1	38	0.024	360731	2	52	0.165
360747	2	38	0.073	360810	1	38	0.032	360732	2	52	1.089
360748	2	38	0.005	360811	1	38	0.024	360733	2	52	.
360749	2	38	0.003	360812	1	38	0.015	360734	2	52	0.721
360750	2	38	0.015	360813	1	38	0.01	360735	2	52	.
360751	3	38	0.045	360814	1	38	0.017	360736	2	52	1.387
360752	3	38	0.016	360815	1	38	0.007	360737	2	52	1.037
360753	3	38	0.024	360816	1	38	0.006	360738	2	52	1.126
360754	3	38	0.029	360817	1	38	0.024	360739	2	52	.
360755	3	38	0.068	360818	1	38	0.009	360740	2	52	0.179
360756	3	38	0.038	360819	1	38	0.008	360741	2	52	0.321
360757	3	38	0.017	360820	1	38	0.004	360742	2	52	1.171
360758	3	38	0.041	360821	1	38	0.005	360743	2	52	1.381
360759	3	38	0.013	360822	1	38	0.029	360744	2	52	0.426
360760	3	38	0.011	360823	1	38	0.031	360745	2	52	0.195
360761	3	38	0.02	360824	1	38	0.024	360746	2	52	2.056
360762	3	38	0.038	360825	1	38	0.031	360747	2	52	.
360763	3	38	0.004	360826	1	38	0.017	360748	2	52	.
360764	3	38	0.02	360827	1	38	0.031	360749	2	52	.
360765	3	38	0.023	360828	1	38	0.008	360750	2	52	0.516

360751	3	52	0.036	360814	1	52	0.014				
360752	3	52	0.02	360815	1	52	0.02	360736	2	62	1.186
360753	3	52	0.078	360816	1	52	0.031	360738	2	62	0.701
360754	3	52	0.049	360817	1	52	.	360739	2	62	.
360755	3	52	0.209	360818	1	52	0.035	360740	2	62	0.29
360756	3	52	0.031	360819	1	52	0.077	360741	2	62	0.236
360757	3	52	.	360820	1	52	.	360742	2	62	0.857
360758	3	52	0.033	360821	1	52	0.033	360743	2	62	1.156
360759	3	52	0.009	360822	1	52	0.091	360744	2	62	0.344
360760	3	52	0.025	360823	1	52	0.042	360745	2	62	0.366
360761	3	52	.	360824	1	52	0.05	360746	2	62	1.488
360762	3	52	0.028	360825	1	52	0.042	360747	2	62	.
360763	3	52	.	360826	1	52	0.065	360748	2	62	.
360764	3	52	0.042	360827	1	52	0.011	360749	2	62	.
360765	3	52	0.09	360828	1	52	0.017	360750	2	62	.
360766	3	52	0.013	360829	1	52	0.026	360751	3	62	0
360767	3	52	.	360830	1	52	.	360752	3	62	0
360768	3	52	0.072	360831	1	52	0.031	360753	3	62	0.002
360769	3	52	0.062	360832	1	52	0.024	360754	3	62	0.024
360770	3	52	0.007	360833	1	52	0.037	360755	3	62	0.047
360771	3	52	0.006	360834	1	52	.	360756	3	62	0.065
360772	3	52	0.012	360835	1	52	0.022	360757	3	62	.
360773	3	52	0.028	360836	1	52	.	360758	3	62	.
360774	3	52	.	360837	1	52	0.02	360759	3	62	0.029
360775	3	52	0.028	360838	1	52	0.042	360760	3	62	0.026
360776	3	52	0.01	360839	1	52	0.024	360761	3	62	.
360777	3	52	0.013	360840	1	52	0.054	360762	3	62	0.012
360778	3	52	0.038	360841	1	52	0.01	360763	3	62	.
360779	3	52	0.019	360701	2	62	0.15	360764	3	62	0.023
360780	3	52	.	360702	2	62	0.86	360765	3	62	0.012
360781	3	52	0.004	360703	2	62	0.481	360766	3	62	.
360782	3	52	0.001	360704	2	62	0.869	360767	3	62	.
360783	3	52	.	360705	2	62	1.033	360768	3	62	0.001
360784	3	52	0.012	360706	2	62	1.518	360769	3	62	0.041
360785	3	52	0.005	360707	2	62	0.733	360770	3	62	0.022
360786	3	52	0	360708	2	62	0.516	360771	3	62	0.034
360787	3	52	.	360709	2	62	.	360772	3	62	0.018
360788	3	52	0.001	360710	2	62	.	360773	3	62	0.011
360789	3	52	.	360711	2	62	.	360774	3	62	.
360790	3	52	.	360712	2	62	0.976	360775	3	62	0.009
360791	3	52	0	360713	2	62	0.363	360776	3	62	0.014
360792	3	52	0.008	360714	2	62	0.92	360777	3	62	0.019
360793	3	52	0.006	360715	2	62	0.136	360778	3	62	0.01
360794	3	52	0.037	360716	2	62	.	360779	3	62	0.011
360795	3	52	.	360717	2	62	0.583	360780	3	62	.
360796	3	52	0.023	360718	2	62	1.103	360781	3	62	0.003
360797	3	52	0.191	360719	2	62	.	360782	3	62	0.022
360798	3	52	0.005	360720	2	62	0.52	360783	3	62	.
360799	3	52	0.028	360721	2	62	0.485	360784	3	62	0.012
360800	3	52	0.014	360722	2	62	0.456	360785	3	62	0.048
360801	1	52	0.059	360723	2	62	1.308	360786	3	62	0.016
360802	1	52	.	360724	2	62	.	360787	3	62	.
360803	1	52	0.046	360725	2	62	0.107	360788	3	62	0.016
360804	1	52	.	360726	2	62	0.327	360789	3	62	.
360805	1	52	.	360727	2	62	.	360790	3	62	.
360806	1	52	0.035	360728	2	62	0.552	360791	3	62	0.022
360807	1	52	0.053	360729	2	62	1.282	360792	3	62	0.008
360808	1	52	0.043	360730	2	62	1.414	360793	3	62	0.009
360809	1	52	0.048	360731	2	62	0.322	360794	3	62	0.005
360810	1	52	0.023	360732	2	62	.	360795	3	62	.
360811	1	52	0.056	360733	2	62	0.424	360796	3	62	0.004
360812	1	52	0.054	360734	2	62	0.6	360797	3	62	0.002
360813	1	52	0.065	360735	2	62	0.574	360798	3	62	0.009

360799	3	62	0.02	360721	2	83	0.242				
360800	3	62	0.035	360722	2	83	0.041	360784	3	83	0.053
360801	1	62	.	360723	2	83	0.636	360786	3	83	0.033
360802	1	62	0.014	360724	2	83	0.095	360787	3	83	.
360803	1	62	.	360725	2	83	0.065	360788	3	83	0.044
360804	1	62	.	360726	2	83	.	360789	3	83	.
360805	1	62	.	360727	2	83	.	360790	3	83	.
360806	1	62	0.023	360728	2	83	0.302	360791	3	83	0.022
360807	1	62	.	360729	2	83	1.241	360792	3	83	.
360808	1	62	0.008	360730	2	83	.	360793	3	83	.
360809	1	62	0.049	360731	2	83	0.119	360794	3	83	0.068
360810	1	62	0.016	360732	2	83	0.878	360795	3	83	0.028
360811	1	62	0.088	360733	2	83	.	360796	3	83	0.08
360812	1	62	0.008	360734	2	83	0.243	360797	3	83	0.083
360813	1	62	0.008	360735	2	83	0.133	360798	3	83	.
360814	1	62	0.021	360736	2	83	1.062	360799	3	83	0.033
360815	1	62	0.015	360737	2	83	0.699	360800	3	83	.
360816	1	62	0.011	360738	2	83	0.147	360801	1	83	0.068
360817	1	62	.	360739	2	83	.	360802	1	83	.
360818	1	62	0.027	360740	2	83	0.123	360803	1	83	0.049
360819	1	62	0.008	360741	2	83	0.084	360804	1	83	.
360820	1	62	.	360742	2	83	0.313	360805	1	83	0.027
360821	1	62	0.022	360743	2	83	0.599	360806	1	83	0.096
360822	1	62	0.046	360744	2	83	0.148	360807	1	83	0.047
360823	1	62	0.031	360745	2	83	.	360808	1	83	0.044
360824	1	62	0.032	360746	2	83	0.294	360809	1	83	0.038
360825	1	62	0.019	360747	2	83	.	360810	1	83	0.089
360826	1	62	0.033	360748	2	83	0.391	360811	1	83	0.072
360827	1	62	0.028	360749	2	83	.	360812	1	83	0.054
360828	1	62	0.022	360750	2	83	0.058	360813	1	83	0.037
360829	1	62	0.031	360751	3	83	0.028	360814	1	83	0.068
360830	1	62	.	360752	3	83	.	360815	1	83	0
360831	1	62	.	360753	3	83	0.015	360816	1	83	0.042
360832	1	62	0.048	360754	3	83	0.048	360817	1	83	.
360833	1	62	0.041	360755	3	83	0.03	360818	1	83	.
360834	1	62	.	360756	3	83	0.033	360819	1	83	0.068
360835	1	62	0.041	360757	3	83	.	360820	1	83	.
360836	1	62	.	360758	3	83	.	360821	1	83	0
360837	1	62	0.04	360759	3	83	0.039	360822	1	83	0.051
360838	1	62	0.033	360760	3	83	0.036	360823	1	83	.
360839	1	62	0.046	360761	3	83	0.015	360824	1	83	0.067
360840	1	62	0.012	360762	3	83	0.058	360825	1	83	0.019
360841	1	62	0.022	360763	3	83	.	360826	1	83	0.027
360701	2	83	0.194	360764	3	83	0.029	360827	1	83	0.041
360702	2	83	0.518	360765	3	83	.	360828	1	83	0.029
360703	2	83	.	360766	3	83	.	360829	1	83	0.033
360704	2	83	0.465	360767	3	83	0.015	360830	1	83	0.031
360705	2	83	.	360768	3	83	0.018	360831	1	83	.
360706	2	83	0.998	360769	3	83	0.047	360832	1	83	0.034
360707	2	83	0.244	360770	3	83	0.071	360833	1	83	.
360708	2	83	0.287	360771	3	83	0.025	360834	1	83	.
360709	2	83	.	360772	3	83	0.031	360835	1	83	.
360710	2	83	.	360773	3	83	0.061	360836	1	83	.
360711	2	83	1.03	360774	3	83	.	360837	1	83	.
360712	2	83	0.612	360775	3	83	0.048	360838	1	83	.
360713	2	83	0.098	360776	3	83	0.064	360839	1	83	0.025
360714	2	83	0.588	360777	3	83	0.054	360840	1	83	0.024
360715	2	83	0.065	360778	3	83	0.058	360841	1	83	.
360716	2	83	.	360779	3	83	.	360701	2	111	.
360717	2	83	0.407	360780	3	83	.	360702	2	111	0.203
360718	2	83	0.533	360781	3	83	0.061	360703	2	111	.
360719	2	83	.	360782	3	83	0.048	360704	2	111	0.17
360720	2	83	0.277	360783	3	83	.	360705	2	111	0.235

360706	2	111	0.361	360753	3	111	0.029				
360707	2	111	0.091	360754	3	111	0.02	360800	3	111	0.111
360708	2	111	0.265	360755	3	111	0.031	360802	1	111	.
360709	2	111	.	360756	3	111	0.024	360803	1	111	0.016
360710	2	111	.	360757	3	111	0.02	360804	1	111	.
360711	2	111	0.623	360758	3	111	.	360805	1	111	0.007
360712	2	111	0.229	360759	3	111	0.043	360806	1	111	0.018
360713	2	111	0.01	360760	3	111	0.043	360807	1	111	0.061
360714	2	111	0.111	360761	3	111	0.015	360808	1	111	0.01
360715	2	111	0.032	360762	3	111	0.041	360809	1	111	0.07
360716	2	111	.	360763	3	111	.	360810	1	111	0.074
360717	2	111	0.178	360764	3	111	0.045	360811	1	111	0.037
360718	2	111	.	360765	3	111	.	360812	1	111	0.019
360719	2	111	.	360766	3	111	.	360813	1	111	0.05
360720	2	111	0.157	360767	3	111	0.041	360814	1	111	0.037
360721	2	111	0.103	360768	3	111	0.015	360815	1	111	0.034
360722	2	111	0.121	360769	3	111	0.038	360816	1	111	0.073
360723	2	111	0.285	360770	3	111	0.017	360817	1	111	.
360724	2	111	0.107	360771	3	111	0.024	360818	1	111	.
360725	2	111	0.022	360772	3	111	0.024	360819	1	111	0.031
360726	2	111	.	360773	3	111	0.115	360820	1	111	.
360727	2	111	.	360774	3	111	.	360821	1	111	0.031
360728	2	111	0.149	360775	3	111	0.047	360822	1	111	0.07
360729	2	111	0.495	360776	3	111	0.03	360823	1	111	0.037
360730	2	111	.	360777	3	111	0.028	360824	1	111	0.058
360731	2	111	0.122	360778	3	111	0.053	360825	1	111	0.027
360732	2	111	0.687	360779	3	111	.	360826	1	111	0.047
360733	2	111	0.122	360780	3	111	.	360827	1	111	0.028
360734	2	111	0.169	360781	3	111	0.011	360828	1	111	0.047
360735	2	111	0.228	360782	3	111	0.009	360829	1	111	0.048
360736	2	111	0.902	360783	3	111	.	360830	1	111	0.058
360737	2	111	0.327	360784	3	111	0.011	360831	1	111	.
360738	2	111	0.07	360785	3	111	0.011	360832	1	111	0.031
360739	2	111	.	360786	3	111	0.016	360833	1	111	.
360740	2	111	0.085	360787	3	111	0.008	360834	1	111	.
360741	2	111	0.029	360788	3	111	0.005	360835	1	111	0.071
360742	2	111	0.18	360789	3	111	.	360836	1	111	.
360743	2	111	0.414	360790	3	111	.	360837	1	111	0.049
360744	2	111	0.18	360791	3	111	0.015	360838	1	111	.
360745	2	111	.	360792	3	111	.	360839	1	111	0.007
360746	2	111	0.164	360793	3	111	0.008	360840	1	111	0.027
360747	2	111	.	360794	3	111	0.019	360841	1	111	.
360748	2	111	0.595	360795	3	111	.				
360749	2	111	.	360796	3	111	0.011				
360750	2	111	0.082	360797	3	111	0				
360751	3	111	.	360798	3	111	.				
360752	3	111	.	360799	3	111	0				

Kwessie (MG)

Vaccine trials with poultry mycoplasma vaccines in ostriches on the Kwessie farmstead: Statistical analysis of ELISA data, obtained with MG Antibody Test kits, using the Statistical Analysis System (SAS, Cary, NC) program (version 6.2).

Statistical analysis:**The General Linear Models (GLM) Procedure**

Class Level Information										
Class	Levels	Values								
Trt	3	1	2	3						
Time	9	0	6	14	21	38	52	62	83	111

Number of Observations Read	1269
Number of Observations Used	1110

Dependent Variable: Resp

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	26	163.7116441	6.2966017	121.53	<.0001
Error	1083	56.1119499	0.0518116		
Corrected Total	1109	219.8235940			

R-Squared	Coeff Var	Root MSE	Resp Mean
0.744741	85.04034	0.227622	0.267663

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	85.21597202	42.60798601	822.36	<.0001
Time	8	27.98438409	3.49804801	67.51	<.0001
Trt*Time	16	50.51128795	3.15695550	60.93	<.0001

The input data follows. On each page the data is arranged in three columns, each containing the ostrich identification number, treatment (Trt), time and response (Resp), read from top to bottom and left to right.

Ostrich	Trt	Time	Resp								
				360763	3	0	0.063				
360701	2	0	0.079	360764	3	0	0.08	360825	1	0	0.074
360702	2	0	0.084	360765	3	0	0.05	360828	1	0	0.091
360703	2	0	0.083	360766	3	0	0.066	360829	1	0	0.084
360704	2	0	0.084	360767	3	0	0.105	360830	1	0	0.054
360705	2	0	0.081	360768	3	0	0.108	360831	1	0	0.094
360706	2	0	0.065	360769	3	0	0.059	360832	1	0	0.062
360707	2	0	0.074	360770	3	0	0.078	360833	1	0	0.082
360708	2	0	0.066	360771	3	0	0.063	360834	1	0	0.086
360709	2	0	0.048	360772	3	0	0.07	360835	1	0	0.073
360710	2	0	0.053	360773	3	0	0.066	360836	1	0	0.075
360711	2	0	0.055	360774	3	0	0.11	360837	1	0	0.099
360712	2	0	0.073	360775	3	0	0.085	360838	1	0	0.082
360713	2	0	0.073	360776	3	0	0.115	360839	1	0	0.114
360714	2	0	0.101	360777	3	0	0.124	360840	1	0	0.072
360715	2	0	0.089	360778	3	0	0.065	360841	1	0	0.061
360716	2	0	0.100	360779	3	0	0.082	360701	2	6	0.063
360717	2	0	0.074	360780	3	0	0.106	360702	2	6	0.067
360718	2	0	0.068	360781	3	0	0.093	360703	2	6	0.085
360719	2	0	0.079	360782	3	0	0.088	360704	2	6	0.108
360720	2	0	0.091	360783	3	0	0.077	360705	2	6	0.093
360721	2	0	0.063	360784	3	0	0.05	360706	2	6	0.083
360722	2	0	0.087	360785	3	0	0.072	360707	2	6	0.079
360723	2	0	0.09	360786	3	0	0.083	360708	2	6	0.088
360724	2	0	0.078	360787	3	0	0.089	360709	2	6	0.084
360725	2	0	0.059	360788	3	0	0.067	360710	2	6	0.071
360726	2	0	0.078	360789	3	0	0.069	360711	2	6	0.087
360727	2	0	0.087	360790	3	0	0.063	360712	2	6	0.046
360728	2	0	0.098	360791	3	0	0.058	360713	2	6	0.069
360729	2	0	0.078	360792	3	0	0.076	360714	2	6	0.077
360730	2	0	0.089	360793	3	0	0.055	360715	2	6	0.117
360731	2	0	0.088	360794	3	0	0.087	360716	2	6	0.079
360732	2	0	0.066	360795	3	0	0.078	360717	2	6	0.061
360733	2	0	0.054	360796	3	0	0.056	360718	2	6	0.063
360734	2	0	0.076	360797	3	0	0.067	360719	2	6	0.091
360735	2	0	0.104	360798	3	0	0.077	360720	2	6	0.09
360736	2	0	0.066	360799	3	0	0.069	360721	2	6	0.083
360737	2	0	0.053	360800	3	0	0.053	360722	2	6	0.062
360738	2	0	0.082	360801	1	0	0.086	360723	2	6	0.042
360739	2	0	0.079	360802	1	0	0.055	360724	2	6	0.048
360740	2	0	0.084	360803	1	0	0.071	360725	2	6	0.077
360741	2	0	0.06	360804	1	0	0.058	360726	2	6	0.074
360742	2	0	0.093	360805	1	0	0.091	360727	2	6	0.077
360743	2	0	0.04	360806	1	0	0.071	360728	2	6	0.084
360744	2	0	0.044	360807	1	0	0.07	360729	2	6	0.065
360745	2	0	0.064	360808	1	0	0.061	360730	2	6	0.082
360746	2	0	0.067	360809	1	0	0.067	360731	2	6	0.078
360747	2	0	0.07	360810	1	0	0.063	360732	2	6	0.102
360748	2	0	0.082	360811	1	0	0.084	360733	2	6	0.076
360749	2	0	0.096	360812	1	0	0.061	360734	2	6	0.055
360750	2	0	0.079	360813	1	0	0.067	360735	2	6	0.03
360751	3	0	0.075	360814	1	0	0.062	360736	2	6	0.078
360752	3	0	0.058	360815	1	0	0.077	360737	2	6	0.061
360753	3	0	0.045	360816	1	0	0.081	360738	2	6	0.075
360754	3	0	0.049	360817	1	0	0.083	360739	2	6	0.093
360755	3	0	0.066	360818	1	0	0.087	360740	2	6	0.128
360756	3	0	0.066	360819	1	0	0.09	360741	2	6	0.081
360757	3	0	0.098	360820	1	0	0.076	360742	2	6	0.083
360758	3	0	0.093	360821	1	0	0.067	360743	2	6	0.058
360759	3	0	0.069	360822	1	0	0.067	360744	2	6	0.062
360760	3	0	0.078	360823	1	0	0.1	360745	2	6	0.105
360761	3	0	0.093	360824	1	0	0.081	360746	2	6	0.06
360762	3	0	0.095	360825	1	0	0.071	360747	2	6	0.072

360748	2	6	0.072	360811	1	6	0.067				
360749	2	6	0.071	360812	1	6	0.07	360734	2	14	0.022
360750	2	6	0.077	360813	1	6	0.063	360735	2	14	0.037
360751	3	6	0.106	360814	1	6	0.065	360736	2	14	0.046
360752	3	6	0.107	360815	1	6	0.056	360737	2	14	0.014
360753	3	6	0.105	360816	1	6	0.056	360738	2	14	0.039
360754	3	6	0.115	360817	1	6	0.032	360739	2	14	0.056
360755	3	6	0.059	360818	1	6	0.053	360740	2	14	0.048
360756	3	6	0.056	360819	1	6	0.07	360741	2	14	0.038
360757	3	6	0.067	360820	1	6	0.061	360742	2	14	0.04
360758	3	6	0.064	360821	1	6	0.065	360743	2	14	0.037
360759	3	6	0.074	360822	1	6	0.05	360744	2	14	0.033
360760	3	6	0.077	360823	1	6	0.057	360745	2	14	0.041
360761	3	6	0.119	360824	1	6	0.074	360746	2	14	0.048
360762	3	6	0.094	360825	1	6	0.079	360747	2	14	0.022
360763	3	6	0.066	360826	1	6	0.074	360748	2	14	0.048
360764	3	6	0.086	360827	1	6	0.091	360749	2	14	0.031
360765	3	6	0.086	360828	1	6	0.089	360750	2	14	0.047
360766	3	6	0.074	360829	1	6	0.091	360751	3	14	0.223
360767	3	6	0.122	360830	1	6	.	360752	3	14	0.066
360768	3	6	0.137	360831	1	6	0.08	360753	3	14	0.044
360769	3	6	0.065	360832	1	6	.	360754	3	14	0.075
360770	3	6	0.088	360833	1	6	0.075	360755	3	14	0.07
360771	3	6	0.069	360834	1	6	0.108	360756	3	14	0.102
360772	3	6	0.073	360835	1	6	0.09	360757	3	14	0.066
360773	3	6	0.065	360836	1	6	0.105	360758	3	14	0.218
360774	3	6	0.113	360837	1	6	0.041	360759	3	14	0.2
360775	3	6	0.087	360838	1	6	0.046	360760	3	14	0.03
360776	3	6	0.076	360839	1	6	0.075	360761	3	14	0.068
360777	3	6	0.125	360840	1	6	0.063	360762	3	14	0.8
360778	3	6	0.081	360841	1	6	0.062	360763	3	14	0.301
360779	3	6	0.115	360701	2	14	0.024	360764	3	14	0.035
360780	3	6	0.093	360702	2	14	0.057	360765	3	14	0.055
360781	3	6	0.074	360703	2	14	0.04	360766	3	14	0.609
360782	3	6	0.051	360704	2	14	0.052	360767	3	14	0.22
360783	3	6	0.053	360705	2	14	0.058	360768	3	14	0.121
360784	3	6	0.041	360706	2	14	0.036	360769	3	14	0.622
360785	3	6	0.041	360707	2	14	0.042	360770	3	14	0.293
360786	3	6	0.051	360708	2	14	0.03	360771	3	14	0.289
360787	3	6	0.056	360709	2	14	0.048	360772	3	14	0.078
360788	3	6	0.054	360710	2	14	0.062	360773	3	14	0.42
360789	3	6	0.052	360711	2	14	0.062	360774	3	14	0.202
360790	3	6	0.043	360712	2	14	0.04	360775	3	14	0.306
360791	3	6	0.071	360713	2	14	0.04	360776	3	14	0.313
360792	3	6	0.055	360714	2	14	0.062	360777	3	14	0.554
360793	3	6	0.066	360715	2	14	0.071	360778	3	14	0.21
360794	3	6	0.059	360716	2	14	0.014	360779	3	14	0.234
360795	3	6	0.058	360717	2	14	0	360780	3	14	0.469
360796	3	6	0.053	360718	2	14	0	360781	3	14	1.056
360797	3	6	0.06	360719	2	14	0	360782	3	14	0.956
360798	3	6	0.061	360720	2	14	0.012	360783	3	14	0.13
360799	3	6	0.046	360721	2	14	0.016	360784	3	14	0.116
360800	3	6	0.058	360722	2	14	0.013	360785	3	14	0.148
360801	1	6	0.061	360723	2	14	0.018	360786	3	14	0.213
360802	1	6	0.066	360724	2	14	0.012	360787	3	14	0.434
360803	1	6	0.09	360725	2	14	0.005	360788	3	14	0.212
360804	1	6	0.051	360726	2	14	0.002	360789	3	14	0.081
360805	1	6	0.059	360727	2	14	0.003	360790	3	14	0.369
360806	1	6	0.066	360728	2	14	0.025	360791	3	14	0.143
360807	1	6	0.074	360729	2	14	0.002	360792	3	14	0.226
360808	1	6	0.072	360730	2	14	0.006	360793	3	14	0.102
360809	1	6	0.075	360731	2	14	0.003	360794	3	14	0.211
360810	1	6	0.05	360732	2	14	0.041	360795	3	14	0.127

360796	3	14	0.148	360718	2	21	0.051				
360797	3	14	0.053	360719	2	21	0.073	360782	3	21	0.808
360798	3	14	0.171	360720	2	21	0.066	360783	3	21	1.485
360799	3	14	0.357	360721	2	21	0.05	360784	3	21	0.379
360800	3	14	0.22	360722	2	21	0.07	360785	3	21	0.574
360801	1	14	0.025	360723	2	21	0.097	360786	3	21	0.17
360802	1	14	0.045	360724	2	21	0.191	360787	3	21	1.516
360803	1	14	0.034	360725	2	21	0.053	360788	3	21	1.814
360804	1	14	0.039	360726	2	21	0.09	360789	3	21	0.709
360805	1	14	0.072	360727	2	21	0.117	360790	3	21	0.644
360806	1	14	0.066	360728	2	21	0.083	360791	3	21	0.59
360807	1	14	0.054	360729	2	21	0.074	360792	3	21	1.652
360808	1	14	0.038	360730	2	21	0.049	360793	3	21	0.148
360809	1	14	0.039	360731	2	21	0.063	360794	3	21	0.51
360810	1	14	0.035	360732	2	21	0.093	360795	3	21	0.119
360811	1	14	0.077	360733	2	21	0.111	360796	3	21	0.811
360812	1	14	0.043	360734	2	21	0.053	360797	3	21	0.062
360813	1	14	0.035	360735	2	21	0.083	360798	3	21	1.345
360814	1	14	0.038	360736	2	21	0.053	360799	3	21	0.668
360815	1	14	0.030	360737	2	21	0.067	360800	3	21	0.863
360816	1	14	0.04	360738	2	21	0.062	360801	1	21	0.077
360817	1	14	0.045	360739	2	21	0.069	360802	1	21	0.059
360818	1	14	0.047	360740	2	21	0.055	360803	1	21	0.037
360819	1	14	0.047	360741	2	21	0.081	360804	1	21	0.016
360820	1	14	0.057	360742	2	21	0.096	360805	1	21	0.013
360821	1	14	0.054	360743	2	21	0.067	360806	1	21	0.019
360822	1	14	0.045	360744	2	21	0.063	360807	1	21	0.03
360823	1	14	0.034	360745	2	21	0.048	360808	1	21	0.028
360824	1	14	0.044	360746	2	21	0.05	360809	1	21	0.013
360825	1	14	0.087	360747	2	21	0.063	360810	1	21	0.032
360826	1	14	0.057	360748	2	21	0.17	360811	1	21	0.035
360827	1	14	0.046	360749	2	21	0.071	360812	1	21	0.09
360828	1	14	0.037	360750	2	21	0.023	360813	1	21	0.021
360829	1	14	0.041	360751	3	21	1.256	360814	1	21	0.024
360830	1	14	0.039	360752	3	21	0.566	360815	1	21	0.049
360831	1	14	0.033	360753	3	21	0.489	360816	1	21	0.059
360832	1	14	0.036	360754	3	21	0.23	360817	1	21	0.071
360833	1	14	0.03	360755	3	21	1.459	360818	1	21	0.048
360834	1	14	0.038	360756	3	21	1.69	360819	1	21	0.05
360835	1	14	0.043	360757	3	21	0.918	360820	1	21	0.056
360836	1	14	0.049	360758	3	21	1.273	360821	1	21	0.049
360837	1	14	0	360759	3	21	0.977	360822	1	21	0.067
360838	1	14	0.003	360760	3	21	0.713	360823	1	21	0.057
360839	1	14	0.009	360761	3	21	0.411	360824	1	21	0.085
360840	1	14	0	360762	3	21	0.904	360825	1	21	0.072
360841	1	14	0	360763	3	21	0.545	360826	1	21	0.056
360701	2	21	0.02	360764	3	21	0.203	360827	1	21	0.048
360702	2	21	0.034	360765	3	21	0.195	360828	1	21	0.061
360703	2	21	0.034	360766	3	21	1.770	360829	1	21	0.066
360704	2	21	0.023	360767	3	21	1.189	360830	1	21	0.079
360705	2	21	0.018	360768	3	21	1.08	360831	1	21	0.063
360706	2	21	0.018	360769	3	21	1.436	360832	1	21	0.057
360707	2	21	0.033	360770	3	21	0.391	360833	1	21	0.054
360708	2	21	0.846	360771	3	21	1.251	360834	1	21	0.085
360709	2	21	0.126	360772	3	21	0.054	360835	1	21	0.045
360710	2	21	0.026	360773	3	21	0.622	360836	1	21	0.075
360711	2	21	0.036	360774	3	21	1.084	360837	1	21	0.058
360712	2	21	0.015	360775	3	21	1.008	360838	1	21	0.059
360713	2	21	0.027	360776	3	21	0.35	360839	1	21	0.091
360714	2	21	0.017	360777	3	21	0.647	360840	1	21	0.058
360715	2	21	0.033	360778	3	21	0.561	360841	1	21	0.055
360716	2	21	0.064	360779	3	21	0.401	360701	2	38	0.021
360717	2	21	0.074	360780	3	21	0.078	360702	2	38	0.023

360703	2	38	0.014	360766	3	38	0.601				
360704	2	38	0.031	360767	3	38	0.242	360830	1	38	0.032
360705	2	38	0.05	360768	3	38	0.881	360831	1	38	0.001
360706	2	38	0.048	360769	3	38	0.599	360832	1	38	0.033
360707	2	38	0.025	360770	3	38	0.515	360833	1	38	0.079
360708	2	38	0.031	360771	3	38	0.881	360834	1	38	0.059
360709	2	38	0.05	360772	3	38	0.008	360835	1	38	0.048
360710	2	38	0.047	360773	3	38	0.555	360836	1	38	0.046
360711	2	38	0.048	360774	3	38	0.525	360837	1	38	0.033
360712	2	38	0.067	360775	3	38	0.509	360838	1	38	0.042
360713	2	38	0.055	360776	3	38	0.153	360839	1	38	0.031
360714	2	38	0.031	360777	3	38	0.681	360840	1	38	0.033
360715	2	38	0.036	360778	3	38	0.788	360841	1	38	0.014
360716	2	38	0.033	360779	3	38	0.407	360701	2	52	0.067
360717	2	38	0.031	360780	3	38	1.077	360702	2	52	0.073
360718	2	38	0.099	360781	3	38	0.189	360703	2	52	0.107
360719	2	38	0.108	360782	3	38	0.671	360704	2	52	0.063
360720	2	38	0.063	360783	3	38	0.65	360705	2	52	0.037
360721	2	38	0.144	360784	3	38	0.805	360706	2	52	0.061
360722	2	38	0.057	360785	3	38	0.522	360707	2	52	0.021
360723	2	38	0.081	360786	3	38	0.298	360708	2	52	0.078
360724	2	38	0.1	360787	3	38	1.03	360709	2	52	.
360725	2	38	0.058	360788	3	38	1.293	360710	2	52	.
360726	2	38	0.055	360789	3	38	0.41	360711	2	52	.
360727	2	38	0.081	360790	3	38	0.839	360712	2	52	0.131
360728	2	38	0.062	360791	3	38	0.363	360713	2	52	0.035
360729	2	38	0.051	360792	3	38	1.102	360714	2	52	0.049
360730	2	38	0.037	360793	3	38	0.176	360715	2	52	0.037
360731	2	38	0.037	360794	3	38	0.105	360716	2	52	.
360732	2	38	0.059	360795	3	38	0.76	360717	2	52	0.084
360733	2	38	0.047	360796	3	38	0.527	360718	2	52	0.044
360734	2	38	0.061	360797	3	38	0.133	360719	2	52	.
360735	2	38	0.044	360798	3	38	0.635	360720	2	52	0.051
360736	2	38	0.053	360799	3	38	0.653	360721	2	52	0.049
360737	2	38	0.02	360800	3	38	0.025	360722	2	52	0.052
360738	2	38	0.03	360801	1	38	0.04	360723	2	52	0.079
360739	2	38	0.052	360802	1	38	0.623	360724	2	52	.
360740	2	38	0.049	360803	1	38	0.056	360725	2	52	0.033
360741	2	38	0.014	360804	1	38	0.034	360726	2	52	0.073
360742	2	38	0.001	360805	1	38	0.018	360727	2	52	.
360743	2	38	0.069	360806	1	38	0.066	360728	2	52	0.057
360744	2	38	0.012	360807	1	38	0.042	360729	2	52	0.056
360745	2	38	0.094	360808	1	38	0.031	360730	2	52	0.018
360746	2	38	0	360809	1	38	0.033	360731	2	52	0.069
360747	2	38	0	360810	1	38	0.017	360732	2	52	0.052
360748	2	38	0.001	360811	1	38	0.029	360733	2	52	.
360749	2	38	0.008	360812	1	38	0.048	360734	2	52	0.064
360750	2	38	0.004	360813	1	38	0.019	360735	2	52	.
360751	3	38	0.657	360814	1	38	0.029	360736	2	52	0.024
360752	3	38	1.231	360815	1	38	0.037	360737	2	52	0.014
360753	3	38	0.409	360816	1	38	0.025	360738	2	52	0.053
360754	3	38	0.102	360817	1	38	0.03	360739	2	52	.
360755	3	38	1.376	360818	1	38	0.031	360740	2	52	0.033
360756	3	38	0.886	360819	1	38	0.025	360741	2	52	0.069
360757	3	38	0.797	360820	1	38	0.042	360742	2	52	0.036
360758	3	38	1.536	360821	1	38	0.029	360743	2	52	0.048
360759	3	38	1.133	360822	1	38	0.038	360744	2	52	0
360760	3	38	1.201	360823	1	38	0.064	360745	2	52	0.011
360761	3	38	0.595	360824	1	38	0.02	360746	2	52	0.017
360762	3	38	1.518	360825	1	38	0.029	360747	2	52	.
360763	3	38	0.076	360826	1	38	0.02	360748	2	52	.
360764	3	38	0.735	360827	1	38	0.033	360749	2	52	.
360765	3	38	0.505	360828	1	38	0.016	360750	2	52	0

360751	3	52	0.505	360814	1	52	0.067				
360752	3	52	1.491	360815	1	52	0.054	360736	2	62	0.042
360753	3	52	1.485	360816	1	52	0.054	360738	2	62	0.83
360754	3	52	0.374	360817	1	52	.	360739	2	62	.
360755	3	52	0.052	360818	1	52	0.073	360740	2	62	0.041
360756	3	52	1.406	360819	1	52	0.058	360741	2	62	0.027
360757	3	52	.	360820	1	52	.	360742	2	62	0.09
360758	3	52	1.755	360821	1	52	0.058	360743	2	62	0.055
360759	3	52	1.026	360822	1	52	0.057	360744	2	62	0.056
360760	3	52	1.212	360823	1	52	0.12	360745	2	62	0.059
360761	3	52	.	360824	1	52	0.072	360746	2	62	0.048
360762	3	52	1.742	360825	1	52	0.11	360747	2	62	.
360763	3	52	.	360826	1	52	0.111	360748	2	62	.
360764	3	52	1.351	360827	1	52	0.058	360749	2	62	.
360765	3	52	0.031	360828	1	52	0.056	360750	2	62	.
360766	3	52	0.691	360829	1	52	0.083	360751	3	62	0.789
360767	3	52	.	360830	1	52	.	360752	3	62	1.331
360768	3	52	0.942	360831	1	52	0.061	360753	3	62	1.521
360769	3	52	1.854	360832	1	52	0.073	360754	3	62	0.768
360770	3	52	0.978	360833	1	52	0.101	360755	3	62	1.604
360771	3	52	1.402	360834	1	52	.	360756	3	62	1.52
360772	3	52	0.077	360835	1	52	0.06	360757	3	62	.
360773	3	52	0.865	360836	1	52	.	360758	3	62	.
360774	3	52	.	360837	1	52	0.054	360759	3	62	1.214
360775	3	52	1.27	360838	1	52	0.077	360760	3	62	1.343
360776	3	52	1.076	360839	1	52	0.089	360761	3	62	.
360777	3	52	1.211	360840	1	52	0.154	360762	3	62	1.897
360778	3	52	1.245	360841	1	52	0.056	360763	3	62	.
360779	3	52	1.823	360701	2	62	0.079	360764	3	62	1.622
360780	3	52	.	360702	2	62	0.052	360765	3	62	1.232
360781	3	52	0.574	360703	2	62	0.072	360766	3	62	.
360782	3	52	1.694	360704	2	62	0.085	360767	3	62	.
360783	3	52	.	360705	2	62	0.108	360768	3	62	1.016
360784	3	52	1.516	360706	2	62	0.041	360769	3	62	1.566
360785	3	52	1.284	360707	2	62	.	360770	3	62	0.842
360786	3	52	0.767	360708	2	62	0.064	360771	3	62	1.553
360787	3	52	.	360709	2	62	.	360772	3	62	0.175
360788	3	52	1.218	360710	2	62	.	360773	3	62	1.603
360789	3	52	.	360711	2	62	.	360774	3	62	.
360790	3	52	.	360712	2	62	0.06	360775	3	62	1.18
360791	3	52	1.373	360713	2	62	0.035	360776	3	62	1.13
360792	3	52	1.558	360714	2	62	0.029	360777	3	62	1.563
360793	3	52	1.311	360715	2	62	0.073	360778	3	62	1.495
360794	3	52	1.511	360716	2	62	.	360779	3	62	1.973
360795	3	52	.	360717	2	62	0.054	360780	3	62	.
360796	3	52	1.576	360718	2	62	0.037	360781	3	62	0.612
360797	3	52	1.399	360719	2	62	.	360782	3	62	1.515
360798	3	52	1.602	360720	2	62	0.042	360783	3	62	.
360799	3	52	1.987	360721	2	62	0.031	360784	3	62	1.602
360800	3	52	1.678	360722	2	62	0.037	360785	3	62	1.761
360801	1	52	0	360723	2	62	0.037	360786	3	62	0.932
360802	1	52	.	360724	2	62	.	360787	3	62	.
360803	1	52	0	360725	2	62	0.024	360788	3	62	1.046
360804	1	52	.	360726	2	62	0.034	360789	3	62	.
360805	1	52	.	360727	2	62	.	360790	3	62	.
360806	1	52	0	360728	2	62	0.024	360791	3	62	1.287
360807	1	52	0	360729	2	62	0.053	360792	3	62	1.551
360808	1	52	0	360730	2	62	0.038	360793	3	62	1.294
360809	1	52	0	360731	2	62	0.021	360794	3	62	1.058
360810	1	52	0	360732	2	62	.	360795	3	62	.
360811	1	52	0	360733	2	62	0.037	360796	3	62	1.338
360812	1	52	0	360734	2	62	0.027	360797	3	62	1.615
360813	1	52	0	360735	2	62	0.068	360798	3	62	0.989

360799	3	62	1.748	360721	2	83	0.099				
360800	3	62	1.573	360722	2	83	0.079	360784	3	83	0.858
360801	1	62	.	360723	2	83	0.052	360786	3	83	0.376
360802	1	62	0.059	360724	2	83	0.081	360787	3	83	.
360803	1	62	.	360725	2	83	0.065	360788	3	83	0.843
360804	1	62	.	360726	2	83	.	360789	3	83	.
360805	1	62	.	360727	2	83	.	360790	3	83	.
360806	1	62	0.048	360728	2	83	0.087	360791	3	83	0.634
360807	1	62	.	360729	2	83	0.119	360792	3	83	.
360808	1	62	0.147	360730	2	83	.	360793	3	83	.
360809	1	62	0.085	360731	2	83	0.063	360794	3	83	0.64
360810	1	62	0.051	360732	2	83	0.077	360795	3	83	0.845
360811	1	62	0.1	360733	2	83	.	360796	3	83	0.706
360812	1	62	0.049	360734	2	83	0.073	360797	3	83	1.016
360813	1	62	0.112	360735	2	83	0.156	360798	3	83	.
360814	1	62	0.114	360736	2	83	0.118	360799	3	83	1.274
360815	1	62	0.032	360737	2	83	0.085	360800	3	83	.
360816	1	62	0.031	360738	2	83	0.088	360801	1	83	0.095
360817	1	62	.	360739	2	83	.	360802	1	83	.
360818	1	62	0.026	360740	2	83	0.063	360803	1	83	0.101
360819	1	62	0.066	360741	2	83	0.06	360804	1	83	.
360820	1	62	.	360742	2	83	0.087	360805	1	83	0.069
360821	1	62	0.127	360743	2	83	0.058	360806	1	83	0.193
360822	1	62	0.082	360744	2	83	0.04	360807	1	83	0.082
360823	1	62	0.061	360745	2	83	.	360808	1	83	0.084
360824	1	62	0.081	360746	2	83	0.07	360809	1	83	0.132
360825	1	62	0.06	360747	2	83	.	360810	1	83	0.105
360826	1	62	0.092	360748	2	83	0.062	360811	1	83	0.113
360827	1	62	0.071	360749	2	83	.	360812	1	83	0.149
360828	1	62	0.031	360750	2	83	0.114	360813	1	83	0.079
360829	1	62	0.048	360751	3	83	0.338	360814	1	83	0.153
360830	1	62	.	360752	3	83	.	360815	1	83	0.111
360831	1	62	.	360753	3	83	0.408	360816	1	83	0.102
360832	1	62	0.071	360754	3	83	0.244	360817	1	83	.
360833	1	62	0.056	360755	3	83	0.573	360818	1	83	.
360834	1	62	.	360756	3	83	1.035	360819	1	83	0.156
360835	1	62	0.057	360757	3	83	.	360820	1	83	.
360836	1	62	.	360758	3	83	.	360821	1	83	0.087
360837	1	62	0.055	360759	3	83	0.277	360822	1	83	0.166
360838	1	62	0.056	360760	3	83	1.146	360823	1	83	.
360839	1	62	0.11	360761	3	83	1.062	360824	1	83	0.134
360840	1	62	0.094	360762	3	83	0.618	360825	1	83	0.107
360841	1	62	0.064	360763	3	83	.	360826	1	83	0.087
360701	2	83	0.088	360764	3	83	1.541	360827	1	83	0.074
360702	2	83	0.089	360765	3	83	.	360828	1	83	0.064
360703	2	83	.	360766	3	83	.	360829	1	83	0.072
360704	2	83	0.078	360767	3	83	1.372	360830	1	83	0.103
360705	2	83	.	360768	3	83	0.431	360831	1	83	.
360706	2	83	0.108	360769	3	83	1.384	360832	1	83	0.061
360707	2	83	0.054	360770	3	83	0.664	360833	1	83	.
360708	2	83	0.081	360771	3	83	1.197	360834	1	83	.
360709	2	83	.	360772	3	83	0.087	360835	1	83	.
360710	2	83	.	360773	3	83	1.006	360836	1	83	.
360711	2	83	0.076	360774	3	83	.	360837	1	83	.
360712	2	83	0.047	360775	3	83	0.382	360838	1	83	.
360713	2	83	0.056	360776	3	83	0.254	360839	1	83	0.076
360714	2	83	0.046	360777	3	83	1.05	360840	1	83	0.056
360715	2	83	0.064	360778	3	83	1.164	360841	1	83	.
360716	2	83	.	360779	3	83	.	360701	2	111	.
360717	2	83	0.054	360780	3	83	.	360702	2	111	0.08
360718	2	83	0.079	360781	3	83	0.393	360703	2	111	.
360719	2	83	.	360782	3	83	0.403	360704	2	111	0.054
360720	2	83	0.138	360783	3	83	.	360705	2	111	0.05

360706	2	111	0.057	360753	3	111	1.35				
360707	2	111	0.067	360754	3	111	0.489	360800	3	111	0.1
360708	2	111	0.055	360755	3	111	1.549	360802	1	111	.
360709	2	111	.	360756	3	111	1.288	360803	1	111	0.092
360710	2	111	.	360757	3	111	0.535	360804	1	111	.
360711	2	111	0.036	360758	3	111	.	360805	1	111	0.049
360712	2	111	0.05	360759	3	111	0.849	360806	1	111	0.045
360713	2	111	0.063	360760	3	111	1.752	360807	1	111	0.044
360714	2	111	0.06	360761	3	111	1.226	360808	1	111	0.038
360715	2	111	0.097	360762	3	111	1.513	360809	1	111	0.071
360716	2	111	.	360763	3	111	.	360810	1	111	0.066
360717	2	111	0.039	360764	3	111	1.771	360811	1	111	0.052
360718	2	111	.	360765	3	111	.	360812	1	111	0.039
360719	2	111	.	360766	3	111	.	360813	1	111	0.056
360720	2	111	0.011	360767	3	111	1.2	360814	1	111	0.063
360721	2	111	0.027	360768	3	111	0.826	360815	1	111	0.047
360722	2	111	0.017	360769	3	111	1.674	360816	1	111	0.049
360723	2	111	0.039	360770	3	111	0.661	360817	1	111	.
360724	2	111	0.061	360771	3	111	1.753	360818	1	111	.
360725	2	111	0.027	360772	3	111	0.081	360819	1	111	0.033
360726	2	111	.	360773	3	111	0.056	360820	1	111	.
360727	2	111	.	360774	3	111	.	360821	1	111	0.069
360728	2	111	0.078	360775	3	111	0.738	360822	1	111	0.044
360729	2	111	0.166	360776	3	111	1.382	360823	1	111	0.068
360730	2	111	.	360777	3	111	1.58	360824	1	111	0.078
360731	2	111	0.032	360778	3	111	1.31	360825	1	111	0.064
360732	2	111	0.05	360779	3	111	.	360826	1	111	0.067
360733	2	111	0.043	360780	3	111	.	360827	1	111	0.044
360734	2	111	0.048	360781	3	111	0.279	360828	1	111	0.062
360735	2	111	0.079	360782	3	111	0.76	360829	1	111	0.172
360736	2	111	0.061	360783	3	111	.	360830	1	111	0.081
360737	2	111	0.04	360784	3	111	1.157	360831	1	111	.
360738	2	111	0.056	360785	3	111	1.796	360832	1	111	0.068
360739	2	111	.	360786	3	111	0.589	360833	1	111	.
360740	2	111	0.061	360787	3	111	0.049	360834	1	111	.
360741	2	111	0.055	360788	3	111	0.968	360835	1	111	0.099
360742	2	111	0.064	360789	3	111	.	360836	1	111	.
360743	2	111	0.046	360790	3	111	.	360837	1	111	0.046
360744	2	111	0.058	360791	3	111	1.147	360838	1	111	.
360745	2	111	.	360792	3	111	.	360839	1	111	0.064
360746	2	111	0.051	360793	3	111	1.162	360840	1	111	0.057
360747	2	111	.	360794	3	111	1.115	360841	1	111	.
360748	2	111	0.053	360795	3	111	.				
360749	2	111	.	360796	3	111	1.454				
360750	2	111	0.065	360797	3	111	1.803				
360751	3	111	.	360798	3	111	.				
360752	3	111	.	360799	3	111	1.919				

Schoeman (MS)

Vaccine trials with poultry mycoplasma vaccines in ostriches on the Schoeman farmstead: Statistical analysis of ELISA data, obtained with MS Antibody Test kits, using the Statistical Analysis System (SAS, Cary, NC) program (version 6.2).

Statistical analysis:

The General Linear Models (GLM) Procedure

Class Level Information						
Class	Levels	Values				
Trt	3	1	2	3		
Time	7	0	7	27	34	45 53 83

Number of Observations Read	546
Number of Observations Used	484

Dependent Variable: resp

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	20	6.28050221	0.31402511	15.49	<.0001
Error	463	9.38741951	0.02027520		
Corrected Total	483	15.66792172			

R-Squared	Coeff Var	Root MSE	Resp Mean
0.400851	168.8652	0.142391	0.084322

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	2.08139789	1.04069894	51.33	<.0001
Time	6	1.78833006	0.29805501	14.70	<.0001
Trt*Time	12	2.410774426	0.20089786	9.91	<.0001

The input data follows. On each page the data is arranged in three columns, each containing the ostrich identification number, treatment (Trt), time and response (Resp), read from top to bottom and left to right.

Ostrich	Trt	Time	Resp									
				353717	3	0	0.009					
353655	1	0	0.035	353718	3	0	0.026	353703	3	7	0.005	
353656	1	0	0.038	353719	3	0	0.012	353704	3	7	0.017	
353657	1	0	0.041	353720	3	0	0.024	353705	3	7	0.02	
353658	1	0	0.034	353721	1	0	0.001	353706	3	7	0.020	
353659	1	0	0.03	353722	1	0	0.025	353707	3	7	0.014	
353660	1	0	0.013	353723	1	0	0.016	353708	3	7	0.014	
353661	2	0	0	353724	1	0	0.043	353709	3	7	0.033	
353662	2	0	0	353725	1	0	0.02	353710	3	7	0.036	
353663	2	0	0	353726	1	0	0.022	353711	3	7	0.021	
353664	2	0	0.026	353727	1	0	0.043	353712	3	7	0.015	
353665	2	0	0	353728	1	0	0.028	353713	3	7	0.015	
353666	2	0	0.029	353729	1	0	0.01	353714	3	7	0.034	
353667	2	0	0.033	353730	1	0	0.011	353715	3	7	0.036	
353668	2	0	0.05	353731	1	0	0	353716	3	7	0.023	
353669	2	0	0.031	353732	1	0	0.006	353717	3	7	0.022	
353670	2	0	0.031	353655	1	7	0.012	353718	3	7	0.029	
353671	2	0	.	353656	1	7	0.025	353719	3	7	0.034	
353672	2	0	0.023	353657	1	7	0	353720	3	7	0.02	
353673	2	0	0.018	353658	1	7	0.032	353721	1	7	0.022	
353674	2	0	0.021	353659	1	7	0.034	353722	1	7	0.028	
353675	2	0	0.009	353660	1	7	0.028	353723	1	7	0.006	
353676	2	0	0.040	353661	2	7	0.003	353724	1	7	0.011	
353677	2	0	0.033	353662	2	7	0	353725	1	7	0.025	
353678	2	0	0.04	353663	2	7	0.001	353726	1	7	0.021	
353679	2	0	0.06	353664	2	7	0	353727	1	7	0.02	
353680	2	0	0.026	353665	2	7	0.046	353728	1	7	0.018	
353681	2	0	0.066	353666	2	7	0.025	353729	1	7	0.016	
353682	2	0	0.043	353667	2	7	0.012	353730	1	7	0	
353683	2	0	0.046	353668	2	7	.	353731	1	7	0.006	
353684	2	0	0.044	353669	2	7	0.014	353732	1	7	0	
353685	2	0	0.047	353670	2	7	0.012	353655	1	27	0.013	
353686	2	0	0.057	353671	2	7	0.004	353656	1	27	0.058	
353687	2	0	0.021	353672	2	7	0	353657	1	27	0.07	
353688	2	0	0.03	353673	2	7	0.008	353658	1	27	0.014	
353689	2	0	0.021	353674	2	7	0	353659	1	27	.	
353690	2	0	0.019	353675	2	7	0.028	353660	1	27	0.004	
353691	3	0	0	353676	2	7	0.013	353661	2	27	.	
353692	3	0	0.028	353677	2	7	0.008	353662	2	27	0.021	
353693	3	0	0.026	353678	2	7	0.007	353663	2	27	0.007	
353694	3	0	0.04	353679	2	7	0.015	353664	2	27	0	
353695	3	0	0.048	353680	2	7	0.011	353665	2	27	.	
353696	3	0	0.04	353681	2	7	0.047	353666	2	27	0	
353697	3	0	0.042	353682	2	7	0.037	353667	2	27	0	
353698	3	0	0.023	353683	2	7	0.021	353668	2	27	0.006	
353699	3	0	0.02	353684	2	7	0.037	353669	2	27	0.024	
353700	3	0	0.037	353685	2	7	0.032	353670	2	27	0.026	
353701	3	0	0.024	353686	2	7	0.027	353671	2	27	0.049	
353702	3	0	0.014	353687	2	7	.	353672	2	27	0.028	
353703	3	0	0.033	353688	2	7	0.025	353673	2	27	0.131	
353704	3	0	0.033	353689	2	7	0.027	353674	2	27	0.022	
353705	3	0	0.022	353690	2	7	.	353675	2	27	0.064	
353706	3	0	0.033	353691	3	7	0	353676	2	27	0.055	
353707	3	0	0.025	353692	3	7	.	353677	2	27	.	
353708	3	0	0.04	353693	3	7	0.01	353678	2	27	0.027	
353709	3	0	0.049	353694	3	7	0.019	353679	2	27	0.013	
353710	3	0	0.026	353695	3	7	0.037	353680	2	27	0.033	
353711	3	0	0.016	353696	3	7	0.018	353681	2	27	0.008	
353712	3	0	0.014	353697	3	7	0.024	353682	2	27	0.091	
353713	3	0	0.036	353698	3	7	0.02	353683	2	27	0.18	
353714	3	0	0.012	353699	3	7	0.013	353684	2	27	0.045	
353715	3	0	0.018	353700	3	7	0.017	353685	2	27	0.036	
353716	3	0	0.009	353701	3	7	.	353686	2	27	0.025	

353687	2	27	0.068	353672	2	34	0.115				
353688	2	27	0.026	353673	2	34	0.207	353658	1	45	0.023
353689	2	27	0.114	353674	2	34	0.067	353659	1	45	.
353690	2	27	0.158	353675	2	34	0.072	353660	1	45	0.016
353691	3	27	0	353676	2	34	0.052	353661	2	45	0
353692	3	27	0.011	353677	2	34	.	353662	2	45	0.251
353693	3	27	0.023	353678	2	34	0.041	353663	2	45	0.834
353694	3	27	0.06	353679	2	34	0.044	353664	2	45	0.02
353695	3	27	0.047	353680	2	34	0.05	353665	2	45	.
353696	3	27	0.021	353681	2	34	0.021	353666	2	45	0.42
353697	3	27	.	353682	2	34	0.067	353667	2	45	0.11
353698	3	27	0.047	353683	2	34	0.209	353668	2	45	1.469
353699	3	27	0.039	353684	2	34	0.063	353669	2	45	0.032
353700	3	27	0.036	353685	2	34	0.136	353670	2	45	0.34
353701	3	27	.	353686	2	34	0.067	353671	2	45	0.068
353702	3	27	0.017	353687	2	34	0.181	353672	2	45	0.288
353703	3	27	0.007	353688	2	34	0.069	353673	2	45	0.729
353704	3	27	0.044	353689	2	34	0.043	353674	2	45	0.172
353705	3	27	0.015	353690	2	34	0.559	353675	2	45	0.047
353706	3	27	0.01	353691	3	34	0.024	353676	2	45	0.469
353707	3	27	0.029	353692	3	34	.	353677	2	45	.
353708	3	27	0.006	353693	3	34	0.031	353678	2	45	0.92
353709	3	27	.	353694	3	34	0.016	353679	2	45	0.047
353710	3	27	.	353695	3	34	0.037	353680	2	45	0.062
353711	3	27	0.005	353696	3	34	0.016	353681	2	45	0.031
353712	3	27	0.008	353697	3	34	.	353682	2	45	0.475
353713	3	27	0.038	353698	3	34	0.012	353683	2	45	1.015
353714	3	27	0	353699	3	34	0.026	353684	2	45	.
353715	3	27	0.01	353700	3	34	0.021	353685	2	45	0.568
353716	3	27	.	353701	3	34	.	353686	2	45	0.068
353717	3	27	0.062	353702	3	34	0.055	353687	2	45	0.541
353718	3	27	0.004	353703	3	34	0.006	353688	2	45	0.688
353719	3	27	0.041	353704	3	34	0.015	353689	2	45	0.088
353720	3	27	0.022	353705	3	34	0.01	353690	2	45	.
353721	1	27	0.076	353706	3	34	.	353691	3	45	0.031
353722	1	27	0	353707	3	34	0.01	353692	3	45	0.023
353723	1	27	0	353708	3	34	0.024	353693	3	45	0.005
353724	1	27	0	353709	3	34	0.039	353694	3	45	0.017
353725	1	27	0	353710	3	34	0.031	353695	3	45	0.041
353726	1	27	0.033	353711	3	34	0.041	353696	3	45	0.028
353727	1	27	0	353712	3	34	0.028	353697	3	45	.
353728	1	27	0.035	353713	3	34	0.048	353698	3	45	0.03
353729	1	27	0.003	353714	3	34	0.042	353699	3	45	0.06
353730	1	27	0	353715	3	34	0.04	353700	3	45	0.037
353731	1	27	0	353716	3	34	0.044	353701	3	45	.
353732	1	27	0	353717	3	34	0.268	353702	3	45	0.028
353655	1	34	0.045	353718	3	34	0.02	353703	3	45	0.005
353656	1	34	0.048	353719	3	34	0.04	353704	3	45	0.056
353657	1	34	.	353720	3	34	0.047	353705	3	45	.
353658	1	34	0.076	353721	1	34	0.02	353706	3	45	0.042
353659	1	34	0.024	353722	1	34	0.038	353707	3	45	0.021
353660	1	34	0.029	353723	1	34	0.017	353708	3	45	0.022
353661	2	34	0.039	353724	1	34	0.061	353709	3	45	0.074
353662	2	34	0.044	353725	1	34	.	353710	3	45	0.013
353663	2	34	0.861	353726	1	34	0.053	353711	3	45	0.015
353664	2	34	0.036	353727	1	34	0.042	353712	3	45	0.013
353665	2	34	.	353728	1	34	0.052	353713	3	45	0.029
353666	2	34	0.028	353729	1	34	0.033	353714	3	45	0.113
353667	2	34	0.044	353730	1	34	0.05	353715	3	45	0.09
353668	2	34	0.406	353731	1	34	.	353716	3	45	.
353669	2	34	0.037	353732	1	34	0.022	353717	3	45	0.06
353670	2	34	0.102	353655	1	45	0.012	353718	3	45	0.073
353671	2	34	0.102	353656	1	45	0.013	353719	3	45	0.021

353720	3	45	0.044	353700	3	53	0.087				
353721	1	45	0.004	353701	3	53	.	353680	2	83	0.057
353722	1	45	0.056	353702	3	53	0.006	353682	2	83	0.174
353723	1	45	0	353703	3	53	0.006	353683	2	83	0.111
353724	1	45	0.007	353704	3	53	0.011	353684	2	83	0.109
353725	1	45	.	353705	3	53	0.019	353685	2	83	.
353726	1	45	0.017	353706	3	53	.	353686	2	83	0.083
353727	1	45	0.029	353707	3	53	0.02	353687	2	83	1.221
353728	1	45	0.034	353708	3	53	0.011	353688	2	83	0.267
353729	1	45	0.013	353709	3	53	0.037	353689	2	83	0.045
353730	1	45	0	353710	3	53	0.007	353690	2	83	0.36
353731	1	45	0	353711	3	53	0.036	353691	3	83	0.049
353732	1	45	0	353712	3	53	0.008	353692	3	83	0.084
353655	1	53	0.01	353713	3	53	0.018	353693	3	83	0.06
353656	1	53	.	353714	3	53	0.009	353694	3	83	0.023
353657	1	53	.	353715	3	53	0.018	353695	3	83	.
353658	1	53	0.014	353716	3	53	0.036	353696	3	83	0.034
353659	1	53	.	353717	3	53	0.341	353697	3	83	.
353660	1	53	0.007	353718	3	53	0.011	353698	3	83	0.045
353661	2	53	0.217	353719	3	53	0.048	353699	3	83	0.084
353662	2	53	0.319	353720	3	53	0.064	353700	3	83	0.044
353663	2	53	0.91	353721	1	53	0.001	353701	3	83	.
353664	2	53	0.048	353722	1	53	0.016	353702	3	83	0.054
353665	2	53	.	353723	1	53	0.004	353703	3	83	0.036
353666	2	53	0.426	353724	1	53	0.023	353704	3	83	0.041
353667	2	53	0.129	353725	1	53	.	353705	3	83	0.046
353668	2	53	1.271	353726	1	53	0.021	353706	3	83	.
353669	2	53	0.486	353727	1	53	0.008	353707	3	83	0.088
353670	2	53	0.19	353728	1	53	0.021	353708	3	83	0.068
353671	2	53	0.139	353729	1	53	0.02	353709	3	83	0.059
353672	2	53	0.245	353730	1	53	0.024	353710	3	83	0.057
353673	2	53	1.08	353731	1	53	0.053	353711	3	83	0.037
353674	2	53	.	353732	1	53	0.055	353712	3	83	0.072
353675	2	53	0.156	353655	1	83	0.075	353713	3	83	0.097
353676	2	53	0.868	353656	1	83	.	353714	3	83	0.1
353677	2	53	.	353657	1	83	0.013	353715	3	83	.
353678	2	53	0.457	353658	1	83	0.016	353716	3	83	0.06
353679	2	53	0.101	353659	1	83	.	353717	3	83	0.074
353680	2	53	0.088	353660	1	83	0.015	353718	3	83	0.12
353681	2	53	0.076	353661	2	83	0.158	353719	3	83	0.154
353682	2	53	0.662	353662	2	83	0.25	353720	3	83	0.029
353683	2	53	0.454	353663	2	83	0.17	353721	1	83	0.049
353684	2	53	0.086	353664	2	83	.	353722	1	83	0.084
353685	2	53	0.257	353665	2	83	.	353723	1	83	0.073
353686	2	53	0.077	353666	2	83	0.376	353724	1	83	0.086
353687	2	53	0.836	353667	2	83	0.034	353725	1	83	.
353688	2	53	0.749	353668	2	83	0.313	353726	1	83	0.034
353689	2	53	0.064	353669	2	83	0.183	353727	1	83	0.155
353690	2	53	.	353670	2	83	0.177	353728	1	83	0.075
353691	3	53	0.03	353671	2	83	0.124	353729	1	83	0.075
353692	3	53	.	353672	2	83	.	353730	1	83	.
353693	3	53	0.046	353673	2	83	0.064	353731	1	83	0.097
353694	3	53	0.048	353674	2	83	.	353732	1	83	0.067
353695	3	53	0.079	353675	2	83	0.141				
353696	3	53	0.019	353676	2	83	.				
353697	3	53	.	353677	2	83	.				
353698	3	53	0.046	353678	2	83	0.121				
353699	3	53	0.041	353679	2	83	0.05				

Schoeman (MG)

Vaccine trials with poultry mycoplasma vaccines in ostriches on the Schoeman farmstead: Statistical analysis of ELISA data, obtained with MG Antibody Test kits, using the Statistical Analysis System (SAS, Cary, NC) program (version 6.2).

Statistical analysis:

The General Linear Models (GLM) Procedure

Class Level Information								
Class	Levels	Values						
Trt	3	1	2	3				
Time	7	0	7	27	34	45	53	83

Number of Observations Read	546
Number of Observations Used	484

Dependent Variable: Resp

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	20	62.94947986	3.14747399	72.86	<.0001
Error	463	20.00172520	0.04320027		
Corrected Total	483	82.95120507			

R-Squared	Coeff Var	Root MSE	Resp Mean
0.758874	80.35612	0.207847	0.258657

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	29.69179881	14.84589941	343.65	<.0001
Time	6	13.98058427	2.33009738	53.94	<.0001
Trt*Time	12	19.27709678	1.60642473	37.19	<.0001

The input data follows. On each page the data is arranged in three columns, each containing the ostrich identification number, treatment (Trt), time and response (Resp), read from top to bottom and left to right.

Ostrich	Trt	Time	Resp									
				353715	3	0	0.051		353698	3	7	0.027
353655	1	0	0.09	353716	3	0	0.072		353699	3	7	0.014
353656	1	0	0.022	353717	3	0	0.017		353700	3	7	0.021
353657	1	0	0.049	353718	3	0	0.098		353701	3	7	.
353658	1	0	0.007	353719	3	0	0.122		353702	3	7	0.011
353659	1	0	0.014	353720	3	0	0.088		353703	3	7	0.011
353660	1	0	0.015	353721	1	0	0.016		353704	3	7	0.004
353661	2	0	0.021	353722	1	0	0.041		353705	3	7	0.007
353662	2	0	0.016	353723	1	0	0.002		353706	3	7	0.076
353663	2	0	0.028	353724	1	0	0.026		353707	3	7	0.055
353664	2	0	0.098	353725	1	0	0.08		353708	3	7	0.094
353665	2	0	0.009	353726	1	0	0.056		353709	3	7	0.138
353666	2	0	0.047	353727	1	0	0.048		353710	3	7	0.047
353667	2	0	0.04	353728	1	0	0.039		353711	3	7	0.08
353668	2	0	0.022	353729	1	0	0.059		353712	3	7	0.056
353669	2	0	0.022	353730	1	0	0.055		353713	3	7	0.111
353670	2	0	0.009	353731	1	0	0.023		353714	3	7	0.061
353671	2	0	.	353732	1	0	0.035		353715	3	7	0.067
353672	2	0	0.003	353655	1	7	0.074		353716	3	7	0.073
353673	2	0	0.001	353656	1	7	0.029		353717	3	7	0.047
353674	2	0	0.026	353657	1	7	0.039		353718	3	7	0.068
353675	2	0	0	353658	1	7	0.023		353719	3	7	0.06
353676	2	0	0.062	353659	1	7	0.039		353720	3	7	0.073
353677	2	0	0.057	353660	1	7	0.024		353721	1	7	0.023
353678	2	0	0.08	353661	2	7	0.028		353722	1	7	0.014
353679	2	0	0.085	353662	2	7	0.042		353723	1	7	0.015
353680	2	0	0.061	353663	2	7	0.028		353724	1	7	0.018
353681	2	0	0.119	353664	2	7	0.035		353725	1	7	0.026
353682	2	0	0.064	353665	2	7	0.053		353726	1	7	0.028
353683	2	0	0.088	353666	2	7	0.105		353727	1	7	0.031
353684	2	0	0.093	353667	2	7	0.056		353728	1	7	0.062
353685	2	0	0.079	353668	2	7	.		353729	1	7	0.067
353686	2	0	0.116	353669	2	7	0.051		353730	1	7	0.024
353687	2	0	0.064	353670	2	7	0.035		353731	1	7	0.024
353688	2	0	0.061	353671	2	7	0.063		353732	1	7	0.045
353689	2	0	0.054	353672	2	7	0.026		353655	1	27	0.073
353690	2	0	0.05	353673	2	7	0.058		353656	1	27	0.079
353691	3	0	0	353674	2	7	0.024		353657	1	27	0.013
353692	3	0	0	353675	2	7	0.024		353658	1	27	0.028
353693	3	0	0.027	353676	2	7	0.038		353659	1	27	.
353694	3	0	0.047	353677	2	7	0.068		353660	1	27	0.027
353695	3	0	0.016	353678	2	7	0.047		353661	2	27	.
353696	3	0	0.015	353679	2	7	0.004		353662	2	27	0.001
353697	3	0	0.013	353680	2	7	0.033		353663	2	27	0
353698	3	0	0.012	353681	2	7	0.087		353664	2	27	0.02
353699	3	0	0	353682	2	7	0.044		353665	2	27	.
353700	3	0	0	353683	2	7	0.043		353666	2	27	0
353701	3	0	0	353684	2	7	0.099		353667	2	27	0.009
353702	3	0	0	353685	2	7	0.053		353668	2	27	0.035
353703	3	0	0.012	353686	2	7	0.081		353669	2	27	0
353704	3	0	0.09	353687	2	7	.		353670	2	27	0
353705	3	0	0.089	353688	2	7	0.09		353671	2	27	0
353706	3	0	0.081	353689	2	7	0.059		353672	2	27	0
353707	3	0	0.097	353690	2	7	.		353673	2	27	0
353708	3	0	0.12	353691	3	7	0.062		353674	2	27	0
353709	3	0	0.094	353692	3	7	.		353675	2	27	0.001
353710	3	0	0.043	353693	3	7	0.051		353676	2	27	0.058
353711	3	0	0.062	353694	3	7	0.042		353677	2	27	.
353712	3	0	0.055	353695	3	7	0.01		353678	2	27	0.076
353713	3	0	0.084	353696	3	7	0.015		353679	2	27	0.089
353714	3	0	0.075	353697	3	7	0.016		353680	2	27	0.07

353681	2	27	0.043	353666	2	34	0				
353682	2	27	0.061	353667	2	34	0	353730	1	34	0.055
353683	2	27	0.066	353668	2	34	0	353731	1	34	.
353684	2	27	0.142	353669	2	34	0.052	353732	1	34	0.014
353685	2	27	0.081	353670	2	34	0.059	353655	1	45	0.045
353686	2	27	0.054	353671	2	34	0	353656	1	45	0.041
353687	2	27	0.054	353672	2	34	0	353657	1	45	0.028
353688	2	27	0.084	353673	2	34	0	353658	1	45	0.074
353689	2	27	0.057	353674	2	34	0	353659	1	45	.
353690	2	27	0.054	353675	2	34	0	353660	1	45	0.06
353691	3	27	0.628	353676	2	34	0.048	353661	2	45	0.052
353692	3	27	0.194	353677	2	34	.	353662	2	45	0.183
353693	3	27	0.333	353678	2	34	0.053	353663	2	45	0.06
353694	3	27	0.616	353679	2	34	0.052	353664	2	45	0.103
353695	3	27	1.046	353680	2	34	0.087	353665	2	45	.
353696	3	27	0.597	353681	2	34	0.065	353666	2	45	0.082
353697	3	27	.	353682	2	34	0.065	353667	2	45	0.046
353698	3	27	0.873	353683	2	34	0.086	353668	2	45	0.087
353699	3	27	0.886	353684	2	34	0.191	353669	2	45	0.072
353700	3	27	0.125	353685	2	34	0.114	353670	2	45	0.132
353701	3	27	.	353686	2	34	0.089	353671	2	45	0.106
353702	3	27	0.133	353687	2	34	0.126	353672	2	45	0.093
353703	3	27	0.278	353688	2	34	0.056	353673	2	45	0.067
353704	3	27	0.846	353689	2	34	0.058	353674	2	45	0.169
353705	3	27	1.035	353690	2	34	0.03	353675	2	45	0.07
353706	3	27	0.97	353691	3	34	0.504	353676	2	45	0.097
353707	3	27	0.675	353692	3	34	.	353677	2	45	.
353708	3	27	0.282	353693	3	34	0.729	353678	2	45	0.102
353709	3	27	.	353694	3	34	0.819	353679	2	45	0.053
353710	3	27	.	353695	3	34	1.001	353680	2	45	0.07
353711	3	27	1.974	353696	3	34	0.877	353681	2	45	0.062
353712	3	27	0.346	353697	3	34	.	353682	2	45	0.072
353713	3	27	0.535	353698	3	34	0.815	353683	2	45	0.057
353714	3	27	0.803	353699	3	34	0.534	353684	2	45	.
353715	3	27	0.49	353700	3	34	0.219	353685	2	45	0.12
353716	3	27	.	353701	3	34	.	353686	2	45	0.05
353717	3	27	0.447	353702	3	34	0.562	353687	2	45	0.078
353718	3	27	0.369	353703	3	34	0.539	353688	2	45	0.104
353719	3	27	0.387	353704	3	34	0.188	353689	2	45	0.102
353720	3	27	0.772	353705	3	34	0.804	353690	2	45	.
353721	1	27	0.037	353706	3	34	.	353691	3	45	0.727
353722	1	27	0.035	353707	3	34	0.304	353692	3	45	1.11
353723	1	27	0.033	353708	3	34	0.042	353693	3	45	1.276
353724	1	27	0.016	353709	3	34	0	353694	3	45	1.482
353725	1	27	0.011	353710	3	34	0.790	353695	3	45	0.784
353726	1	27	0.009	353711	3	34	1.55	353696	3	45	1.327
353727	1	27	0.013	353712	3	34	0.547	353697	3	45	.
353728	1	27	0.015	353713	3	34	0.767	353698	3	45	0.59
353729	1	27	0.034	353714	3	34	0.84	353699	3	45	1.042
353730	1	27	0.017	353715	3	34	0.843	353700	3	45	1.429
353731	1	27	0.081	353716	3	34	1.266	353701	3	45	.
353732	1	27	0.001	353717	3	34	0.674	353702	3	45	1.698
353655	1	34	0.07	353718	3	34	0.643	353703	3	45	1.649
353656	1	34	0.009	353719	3	34	0.707	353704	3	45	0.629
353657	1	34	.	353720	3	34	0.573	353705	3	45	.
353658	1	34	0.042	353721	1	34	0.008	353706	3	45	0.721
353659	1	34	0.003	353722	1	34	0.054	353707	3	45	0.481
353660	1	34	0.009	353723	1	34	0.023	353708	3	45	1.231
353661	2	34	0	353724	1	34	0.021	353709	3	45	0.137
353662	2	34	0	353725	1	34	.	353710	3	45	1.061
353663	2	34	0.003	353726	1	34	0	353711	3	45	1.276
353664	2	34	0.003	353727	1	34	0.017	353712	3	45	1.745
353665	2	34	.	353728	1	34	0.087	353713	3	45	1.847

353714	3	45	0.02	353699	3	53	1.194				
353715	3	45	1.584	353700	3	53	1.322	353684	2	83	0.125
353716	3	45	.	353701	3	53	.	353686	2	83	0.105
353717	3	45	1.055	353702	3	53	1.304	353687	2	83	0.089
353718	3	45	1.022	353703	3	53	1.918	353688	2	83	0.147
353719	3	45	0.466	353704	3	53	0.699	353689	2	83	0.06
353720	3	45	1.572	353705	3	53	0.651	353690	2	83	0.119
353721	1	45	0.025	353706	3	53	.	353691	3	83	0.445
353722	1	45	0.079	353707	3	53	0.699	353692	3	83	0.315
353723	1	45	0.014	353708	3	53	1.377	353693	3	83	1.157
353724	1	45	0.029	353709	3	53	0.112	353694	3	83	0.433
353725	1	45	.	353710	3	53	1.277	353695	3	83	.
353726	1	45	0.025	353711	3	53	1.08	353696	3	83	0.3
353727	1	45	0.09	353712	3	53	1.436	353697	3	83	.
353728	1	45	0.128	353713	3	53	1.731	353698	3	83	0.441
353729	1	45	0.092	353714	3	53	1.308	353699	3	83	0.791
353730	1	45	0.039	353715	3	53	1.68	353700	3	83	0.307
353731	1	45	0.046	353716	3	53	1.669	353701	3	83	.
353732	1	45	0.047	353717	3	53	1.112	353702	3	83	0.962
353655	1	53	0.035	353718	3	53	1.351	353703	3	83	0.965
353656	1	53	.	353719	3	53	0.31	353704	3	83	0.462
353657	1	53	.	353720	3	53	1.513	353705	3	83	0.411
353658	1	53	0.07	353721	1	53	0.037	353706	3	83	.
353659	1	53	.	353722	1	53	0.086	353707	3	83	0.439
353660	1	53	0.068	353723	1	53	0.027	353708	3	83	0.247
353661	2	53	0.056	353724	1	53	0.103	353709	3	83	0.121
353662	2	53	0.125	353725	1	53	.	353710	3	83	0.76
353663	2	53	0.279	353726	1	53	0.121	353711	3	83	0.546
353664	2	53	0.095	353727	1	53	0.073	353712	3	83	0.344
353665	2	53	.	353728	1	53	0.423	353713	3	83	0.358
353666	2	53	0.036	353729	1	53	0	353714	3	83	0.465
353667	2	53	0.071	353730	1	53	0.015	353715	3	83	.
353668	2	53	0.047	353731	1	53	0.289	353716	3	83	0.455
353669	2	53	0.085	353732	1	53	0.044	353717	3	83	0.226
353670	2	53	0.072	353655	1	83	0.087	353718	3	83	0.336
353671	2	53	0.053	353656	1	83	.	353719	3	83	0.179
353672	2	53	0.031	353657	1	83	0.066	353720	3	83	0.336
353673	2	53	0.093	353658	1	83	0.068	353721	1	83	0.082
353674	2	53	.	353659	1	83	.	353722	1	83	0.089
353675	2	53	0.079	353660	1	83	0.46	353723	1	83	0.126
353676	2	53	0.07	353661	2	83	0.123	353724	1	83	0.196
353677	2	53	.	353662	2	83	0.217	353725	1	83	.
353678	2	53	0.107	353663	2	83	0.086	353726	1	83	0.061
353679	2	53	0.018	353664	2	83	.	353727	1	83	0.056
353680	2	53	0.048	353665	2	83	.	353728	1	83	0.25
353681	2	53	0.078	353666	2	83	0.094	353729	1	83	0.098
353682	2	53	0.085	353667	2	83	0.069	353730	1	83	.
353683	2	53	0.076	353668	2	83	0.108	353731	1	83	0.382
353684	2	53	0.052	353669	2	83	0.137	353732	1	83	0.131
353685	2	53	0.141	353670	2	83	0.114				
353686	2	53	0.047	353671	2	83	0.162				
353687	2	53	0.14	353672	2	83	.				
353688	2	53	0.079	353673	2	83	0.171				
353689	2	53	0.037	353674	2	83	.				
353690	2	53	.	353675	2	83	0.102				
353691	3	53	0.955	353676	2	83	.				
353692	3	53	.	353677	2	83	.				
353693	3	53	0.935	353678	2	83	0.067				
353694	3	53	1.365	353679	2	83	0.077				
353695	3	53	1.236	353680	2	83	0.072				
353696	3	53	1.132	353681	2	83	0.163				
353697	3	53	.	353682	2	83	0.1				
353698	3	53	1.638	353683	2	83	0.101				

Addendum B Nucleotide/amino acid sequence of the *P100* gene of *Ms01*

The nucleotide and translated amino acid sequences of the *P100* gene of *Ms01* before modification by SDM. Indicated on the sequence are the consensus translation promoter areas in green, the translation initiation and termination codons in blue, as well as the signal peptide II recognition site with the cystein lipoprotein attachment site in bold, and the highly conserved Walker A and B motifs in grey. Also indicated are the ten sites destined for modification by SDM in yellow.

1	TAG TGT ATT ATC GGT TTA TAA ATT ATT TAA TTT ATA ACA TAC ACA	45
	-35 box	-10 box
46	CAT TAG GAG AAA AAA ATG AAA AAA aGC GCA AGA CTT TTA TTA TTA	90
	SD-like sequence	Initiation codon
		Met Lys Lys Ser Ala Arg Leu Leu Leu Leu
91	GGT GCT TTA CCA TTA GCA GCC TTA GCA GCT CCA TTA GTT GCT GCG	135
	Gly Ala Leu Pro Leu Ala Ala Leu Ala Ala Pro	Signal peptide II
		Leu Val Ala Ala
136	GCA TGT AAT AGT AAA TCA GCC CCT TCG CAG AAC ACT GCT TTA GCT	180
	Ala Cys Asn Ser Lys Ser Ala Pro Ser Gln Asn Thr Ala Leu Ala	
181	AAA CAG CAG TTC GTT ACT GAA ATA AAC GCA ACA CCA ACA TTT GAT	225
	Lys Gln Gln Phe Val Thr Glu Ile Asn Ala Thr Pro Thr Phe Asp	
226	GCT TAT ACA TAT GAT AGT TCA GCT TCA TAT GGT GGA TAT TCT TCA	270
	Ala Tyr Thr Tyr Asp Ser Ser Ala Ser Tyr Gly Gly Tyr Ser Ser	
271	AAT GCT AGC TAC CAA CAC ACA TCA GGT ATG TTA GTT AGA GAA CAA	315
	Asn Ala Ser Tyr Gln His Thr Ser Gly Met Leu Val Arg Glu Gln	
316	GGT GTT AAT GAA ATT CAA ATT GAT ACA GTG ACC TCA GAC ACT GGA	360
	Gly Val Asn Glu Ile Gln Ile Asp Thr Val Thr Ser Asp Thr Gly	
361	AAA GTT TCA AAC TAT ATT ACT AAA CCA GCT TTC TCA AAA TAT ACA	405
	Lys Val Ser Asn Tyr Ile Thr Lys Pro Ala Phe Ser Lys Tyr Thr	
406	TTA TCA TTA GCA AAA GCT GTA GTT TTA ACT TTA ACA GAT GGC ACA	450
	Leu Ser Leu Ala Lys Ala Val Val Leu Thr Leu Thr Asp Gly Thr	
451	GTT GTA GTT TAC GAT AAT GAT GAT GCT GAA GTT GTT CCT GCA CCA	495
	Val Val Val Tyr Asp Asn Asp Asp Ala Glu Val Val Pro Ala Pro	
496	GAT TTA ACT TAT GTA GAT GCT GCA GGT GAA ACT AAA AAA GCT TAT	540
	Asp Leu Thr Tyr Val Asp Ala Ala Gly Glu Thr Lys Lys Ala Tyr	
541	TCA TCA GCA TAT CAA AGA TTA AGT TCA GCA AAT TCA AAA TCA ATT	585
	Ser Ser Ala Tyr Gln Arg Leu Ser Ser Ala Asn Ser Lys Ser Ile	
586	AAT AGT CAA GAA TTT GCA GAA AAC TTG AAA AAA GCT AAA ACA TTA	630
	Asn Ser Gln Glu Phe Ala Glu Asn Leu Lys Lys Ala Lys Thr Leu	
631	CAA TAT GTA CTT AAA GAC AAT TTA AAA TGA GTA AAT TCA AAA GGT	675
	Gln Tyr Val Leu Lys Asp Asn Leu Lys End Val Asn Ser Lys Gly	
676	GAA GAA ACT AAA TAT CAA ATT GTT CCT AAA GAT TTC TAT TAT TCA	720
	Glu Glu Thr Lys Tyr Gln Ile Val Pro Lys Asp Phe Tyr Tyr Ser	
	Oligopeptide binding domain	site 1

1666	GGT ACA GGA GAT GCT TAT ATT TAC GGA ACA GGT TTA AGT TTT AGA	1710
	Gly Thr Gly Asp Ala Tyr Ile Tyr Gly Thr Gly Leu Ser Phe Arg	
	site 4	
1711	ACT TTA TTA CAA GCT GCA ATT AAC TGA AAT ACA GTA GCA GAT GTA	1755
	Thr Leu Leu Gln Ala Ala Ile Asn End Asn Thr Val Ala Asp Val	
	site 5	
1756	AGA ACA AAC GGT GTT TCA GAA GCT TGA TTG GCG AAA TTA GCC GAT	1800
	Arg Thr Asn Gly Val Ser Glu Ala End Leu Ala Lys Leu Ala Asp	
1801	GGT GGT AAT ATT GGT GGA AAA GAC CAA GAA TCA TCA GCA GAA AAA	1845
	Gly Gly Asn Ile Gly Gly Lys Asp Gln Glu Ser Ser Ala Glu Lys	
1846	aCA CCA TTT GAT GTA AAA GAT AAA ATT AAT GCA TTG AAA GCT GTA	1890
	Thr Pro Phe Asp Val Lys Asp Lys Ile Asn Ala Leu Lys Ala Val	
1891	AAT AAA GAT AAA CAA TTA GTG GAC TTC GGT GGC AAT TTA GGA AAA	1935
	Asn Lys Asp Lys Gln Leu Val Asp Phe Gly Gly Asn Leu Gly Lys	
1936	GAT CTA AAC CCA TCA GAA AAC GAT GCT GCT GTT AGA GAC AGA TCT	1980
	Asp Leu Asn Pro Ser Glu Asn Asp Ala Ala Val Arg Asp Arg Ser	
1981	AAT GTC AAC GAC AAA ATA AAA TCA GCT GGT TAT GAA AAA ATT AAA	2025
	Asn Val Asn Asp Lys Ile Lys Ser Ala Gly Tyr Glu Lys Ile Lys	
2026	GAA GCT GTA AAA GCA TTA TTA GAT GAG TTT GAA AGA ACA CAT CAA	2070
	Glu Ala Val Lys Ala Leu Leu Asp Glu Phe Glu Arg Thr His Gln	
2071	AAT GTT AGA CCG GCA GAT GGT AAA TAT AGA TTC ACT TCA TTC TAT	2115
	Asn Val Arg Pro Ala Asp Gly Lys Tyr Arg Phe Thr Ser Phe Tyr	
2116	CCA TTT ATT AAT CAA TCA AAA GAA TTT GGT GAA TCA TTA AAA TTT	2160
	Pro Phe Ile Asn Gln Ser Lys Glu Phe Gly Glu Ser Leu Lys Phe	
2161	GTT AAA GAG GCT ATA GAA GGA TTA GAT TCT AGA ATT CAA TTA GAT	2205
	Val Lys Glu Ala Ile Glu Gly Leu Asp Ser Arg Ile Gln Leu Asp	
2206	TTA GTA TTC TTT ACT GAT AAT AAA GAT CCT AAT TAT GTT GCA TAT	2250
	Leu Val Phe Phe Thr Asp Asn Lys Asp Pro Asn Tyr Val Ala Tyr	
	Walker B motif	site 6
2251	ATA AAC CAA GGA GCA AAT GGA ACA AGA AAC GTT GGT TGA AGT TAT	2295
	Ile Asn Gln Gly Ala Asn Gly Thr Arg Asn Val Gly End Ser Tyr	
	Walker A motif	site 7
2296	GAC TAT AAC TCA ATA GGT TCA GGT TAT GAT GGT TTA TCA TGA AAT	2340
	Asp Tyr Asn Ser Ile Gly Ser Gly Tyr Asp Gly Leu Ser End Asn	
	site 8	
2341	TGA CCA TTA TTC CCA ACT CTA ATT AAA ATT GGT GTT GAA AAA GAT	2385
	End Pro Leu Phe Pro Thr Leu Ile Lys Ile Gly Val Glu Lys Asp	
2386	AGT CAT CCA GAA TTT GCT ACT GCA TTT CCA AGA ATC GCT AAA TTA	2430
	Ser His Pro Glu Phe Ala Thr Ala Phe Pro Arg Ile Ala Lys Leu	
2431	GCA GAA GAT TTA TTA GCT TAT CAA GAA CAA CCA GGT CAC GAA TTT	2475
	Ala Glu Asp Leu Leu Ala Tyr Gln Glu Gln Pro Gly His Glu Phe	
2476	GTA TCT TCA GTA CCA TTT AAA GAA TTA TAC AAA GTA GAA CCA AGA	2520
	Val Ser Ser Val Pro Phe Lys Glu Leu Tyr Lys Val Glu Pro Arg	
2521	AGA TAC ACA GTA TTG CCT ACT CTA TTA GCT TCA AAT GTT ACA AAA	2565
	Arg Tyr Thr Val Leu Pro Thr Leu Leu Ala Ser Asn Val Thr Lys	
2566	AAT TCT GTA ACA GAT AAA TAT GAG CTT GTT TTA ACA GAA AAA AAT	2610
	Asn Ser Val Thr Asp Lys Tyr Glu Leu Val Leu Thr Glu Lys Asn	

Addendum C Alignment of the *P100* gene in *Ms01* after SDM

Alignments done in BioEdit of the *P100* gene in *Ms01* as obtained by sequencing after the modification of each of the ten TGA codons to TGG codons by site-directed mutagenesis.

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      10      20      30      40      50      60      70      80      90      100
ms01 P100  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 1  ATCAAAAAAGCGCAAGACTTTTATTATTAGGTGCTTTACCATTAGCAGCCTTAGCAGCTCCATTAGTTGCTGCGGCATGTAATAGTAAATCAG
SDM site 2  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 3  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 4  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 5  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 6  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 7&8 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 9  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 10 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |

      110     120     130     140     150     160     170     180     190     200
ms01 P100  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 1  CCCTTCGCAGAACACTGCTTTAGCTAAACAGCAGTTCGTTACTGAAATAAACGCCAACCAACATTGATGCTTATACATATGATAGTTCAGCTTCATA
SDM site 2  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 3  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 4  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 5  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 6  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 7&8 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 9  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 10 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |

      210     220     230     240     250     260     270     280     290     300
ms01 P100  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 1  TGGTGGATATTCCTCAAAATGCTAGCTACCAACACACATCAGGTATGTTAGTTAGAGAACAGGTGTTAATGAAATTCAAATTGATACAGTGACCTCAGAC
SDM site 2  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 3  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 4  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 5  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 6  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 7&8 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 9  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 10 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |

      310     320     330     340     350     360     370     380     390     400
ms01 P100  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 1  ACTGGAAAAGTTTCAAACCTATATTACTAAACCCAGCTTCTCAAATATACATTATCATTAGCAAAAGCTGTAGTTTAACTTTAACAGATGGCACAGTTG
SDM site 2  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 3  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 4  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 5  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 6  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 7&8 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 9  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 10 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |

      410     420     430     440     450     460     470     480     490     500
ms01 P100  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 1  TAGTTTACGATAATGATGATGCTGAAGTTGTTCCCTGCACCAGATTTAACTTATGTAGATGCTGCAGGTGAAACTAAAAAAGCTTATTTCATCAGCATATCA
SDM site 2  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 3  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 4  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 5  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 6  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 7&8 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 9  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 10 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |

      510     520     530     540     550     560     570     580     590     600
ms01 P100  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 1  AAGATTAAAGTTCAGCAAAATCAAATCAATTAATAGTCAAGAATTTGCAGAAAACCTGAAAAAAGCTAAAACATTACAATATGTACTTAAAGCAAAATTA
SDM site 2  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 3  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 4  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 5  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 6  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 7&8 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 9  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 10 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |

      610     620     630     640     650     660     670     680     690     700
ms01 P100  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 1  AAATGAGTAAATTCAAAAGGTGAAGAAACTAAATATCAAATTTGTTCTTAAAGATTTCTATTATTTCATGACTAAGAACAAATCAAACAATGGTAAATGTTTC
SDM site 2  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 3  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 4  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 5  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 6  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 7&8 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 9  . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |
SDM site 10 . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . | . . . . . |

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      710      720      730      740      750      760      770      780      790      800
ms01 P100  GTCATGATGAAGAAAAGTGGAGGTTTCAGAAACAATTAGACAATGAAGTTAGAGATGCATTAGCAAGACCCTAACAGTCGTATTATACAGATACAAGTGA
SDM site 1  .....
SDM site 2  .....
SDM site 3  .....
SDM site 4  .....
SDM site 5  .....
SDM site 6  .....
SDM site 7&8 .....
SDM site 9  .....
SDM site 10 .....

      810      820      830      840      850      860      870      880      890      900
ms01 P100  AACTCAAATGAATATGTTTTAAAAATCTTTGGTTTAGATACAGTAAAAATTAATGAAGAAAGTGAATTCGTTAAAAAGTTGCTCCAAGTGCAAATTTA
SDM site 1  .....
SDM site 2  .....
SDM site 3  .....
SDM site 4  .....
SDM site 5  .....
SDM site 6  .....
SDM site 7&8 .....
SDM site 9  .....
SDM site 10 .....

      910      920      930      940      950      960      970      980      990      1000
ms01 P100  GGAGATGTAAACAGCTGTAACCTTCCAAGGATTAACAGGTGAAGGTGCTAAAGTTCAAATGAATCAATTTTTGATCAATTAATGCATGACTATACATTC
SDM site 1  .....
SDM site 2  .....
SDM site 3  .....
SDM site 4  .....
SDM site 5  .....
SDM site 6  .....
SDM site 7&8 .....
SDM site 9  .....
SDM site 10 .....

      1010     1020     1030     1040     1050     1060     1070     1080     1090     1100
ms01 P100  ATCCAGCTCCATCACATACATTTGATGATATGAATGCCAACAAATGGTTACAAATTAACTAATACCAAGGCGATGTAACATGATAAAGTTCTGCACATAGA
SDM site 1  .....
SDM site 2  .....
SDM site 3  .....
SDM site 4  .....
SDM site 5  .....
SDM site 6  .....
SDM site 7&8 .....
SDM site 9  .....
SDM site 10 .....

      1110     1120     1130     1140     1150     1160     1170     1180     1190     1200
ms01 P100  AACTAAATCAAAAAGCAATGGATAAAGTAAATTAACCTGCTAAATTAGGTGTTTACTGATATGGTGTAAACAGCAAAATAGTACATTTGATTCAGGACCATAC
SDM site 1  .....
SDM site 2  .....
SDM site 3  .....
SDM site 4  .....
SDM site 5  .....
SDM site 6  .....
SDM site 7&8 .....
SDM site 9  .....
SDM site 10 .....

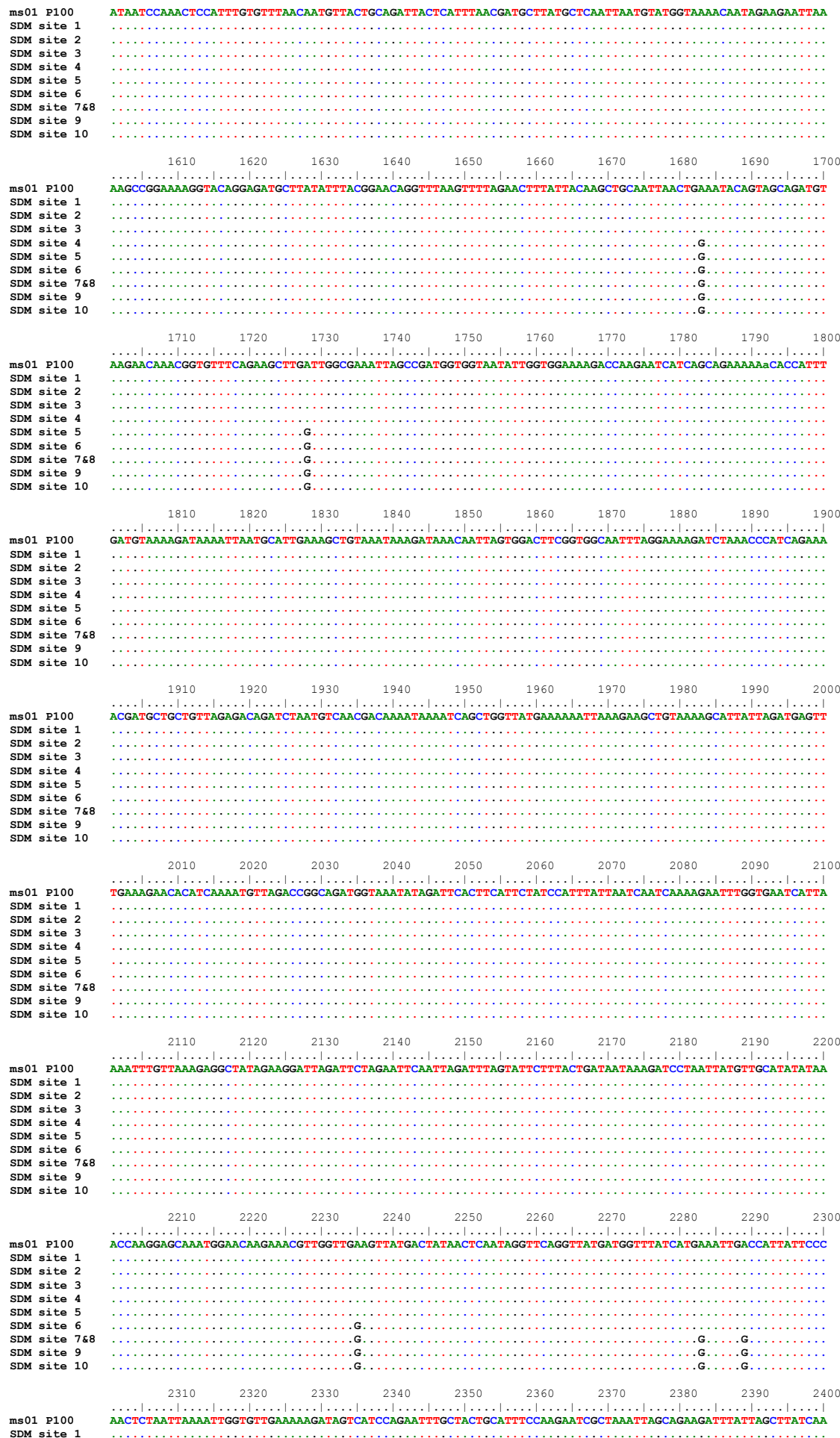
      1210     1220     1230     1240     1250     1260     1270     1280     1290     1300
ms01 P100  TAGGCACAGGCTTTGTAAGTGGTCAATCAGAAATATTTAAAAGAAATACATCCTTTGCAGAAAAGCCTTTGCAGAAATCAAAAATACAGTTAATGAAG
SDM site 1  .....
SDM site 2  .....
SDM site 3  .....
SDM site 4  .....
SDM site 5  .....
SDM site 6  .....
SDM site 7&8 .....
SDM site 9  .....
SDM site 10 .....

      1310     1320     1330     1340     1350     1360     1370     1380     1390     1400
ms01 P100  TTATTAACAATATCAACAAAAACCTTAAGCCCTGAAGAAATTTAATACAAACATCTTTAACTTATATAGACAAGGTACTACATCAACTACTCCATATTC
SDM site 1  .....
SDM site 2  .....
SDM site 3  .....
SDM site 4  .....
SDM site 5  .....
SDM site 6  .....
SDM site 7&8 .....
SDM site 9  .....
SDM site 10 .....

      1410     1420     1430     1440     1450     1460     1470     1480     1490     1500
ms01 P100  ATCATTACTGAAGCTCAAAAAACAATCGTTAACCAAGACCCCAAGGATTTGGTATTAGATTATTCAAAGAGAAAATACTAATTACAGCTCCTTATGAT
SDM site 1  .....
SDM site 2  .....
SDM site 3  .....
SDM site 4  .....
SDM site 5  .....
SDM site 6  .....
SDM site 7&8 .....
SDM site 9  .....
SDM site 10 .....

      1510     1520     1530     1540     1550     1560     1570     1580     1590     1600

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SDM site 2 .....
SDM site 3 .....
SDM site 4 .....
SDM site 5 .....
SDM site 6 .....
SDM site 7&8 .....
SDM site 9 .....
SDM site 10 .....

      2410      2420      2430      2440      2450      2460      2470      2480      2490      2500
ms01 P100 GAAACACCAGGTCACGAATTGTTATCTTCAGTACCATTTAAAGAATTATACAAAGTAGAACCAAGAAGATACACAGTATTGCCCTACTCTATTAGCTTCAA
SDM site 1 .....
SDM site 2 .....
SDM site 3 .....
SDM site 4 .....
SDM site 5 .....
SDM site 6 .....
SDM site 7&8 .....
SDM site 9 .....
SDM site 10 .....

      2510      2520      2530      2540      2550      2560      2570      2580      2590      2600
ms01 P100 ATGTTACAAAAATTCTGTAAACAGATAAATATGAGCTTGTTTTAACAGAAAAAATAGACCAATACCTTTATAAACCCACAAGGTAATAAGCAAGTAACTGA
SDM site 1 .....
SDM site 2 .....
SDM site 3 .....
SDM site 4 .....
SDM site 5 .....
SDM site 6 .....
SDM site 7&8 .....
SDM site 9 .....
SDM site 10 .....

      2610      2620      2630      2640      2650      2660      2670      2680      2690      2700
ms01 P100 TATTTATCAATACTCAGCGTTTTCTGAAACCAATACGTAGCAGACAAAACAAATGATTATTTAACTGAATTAATGGAGAACTAACACATTTTTAGGT
SDM site 1 .....
SDM site 2 .....
SDM site 3 .....
SDM site 4 .....
SDM site 5 .....
SDM site 6 .....
SDM site 7&8 .....
SDM site 9 .....G.....
SDM site 10 .....G.....

      2710      2720      2730      2740      2750      2760      2770      2780      2790      2800
ms01 P100 ATTGAATATTCATCAGCAACTATAACAAAAGCAAAGATTCAATTTGTTAACGTTTTAGTACAAAAGGTTATGTAGCACCTTACACAGTAAATTAATAGTG
SDM site 1 .....
SDM site 2 .....
SDM site 3 .....
SDM site 4 .....
SDM site 5 .....
SDM site 6 .....
SDM site 7&8 .....
SDM site 9 .....
SDM site 10 .....

      2810      2820      2830      2840
ms01 P100 TTGACATGTATGTTGATTGAAGAATCAATRAATAA
SDM site 1 .....GTCGAC
SDM site 2 .....GTCGAC
SDM site 3 .....GTCGAC
SDM site 4 .....GTCGAC
SDM site 5 .....GTCGAC
SDM site 6 .....GTCGAC
SDM site 7&8 .....GTCGAC
SDM site 9 .....GTCGAC
SDM site 10 .....G.....GTCGAC

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