# Preliminary investigations into the use of DNA vaccines to elicit protective immune responses against the ostrich mycoplasma *Ms01*

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## Abstract

Mycoplasmas are evolutionarily advanced prokaryotes which have acquired the elite status of "next generation" bacterial pathogens and necessitate new paradigms in completely understanding their disease potential. The recurrent failure to eradicate mycoplasmal diseases from humans and animals through the use of antibiotics and other techniques have led to the conclusion that the most promising approach would be the development of an effective vaccine with which to control mycoplasma infections. The identification of three species of *Mycoplasma* which infect South African ostriches, causing huge losses to the South African ostrich industry each year, has thus prompted a search for new vaccination strategies with which to control and eradicate them. This study investigated the use of three potential DNA vaccines, utilizing the adherence-associated *Ms01* OppA protein as antigenic determinant to generate antibody responses against the ostrich-infecting *Ms01* organism. A vaccine trial in which the antigenic potential of the pCIneo, VR1012 and VR1020 DNA vaccines were evaluated in ostriches, necessitated the development of an enzyme-linked immunosorbent assay (ELISA) for serological analysis. To this end, the *Ms01 oppA* gene was isolated, cloned into a prokaryotic expression vector and expressed as a recombinant GST-fusion product in *Escherichia coli*. The successful expression and purification of the recombinant protein enabled its subsequent utilization as antigen in the generation of an ELISA. The ELISA displayed a high signal to background ratio. Using this ELISA, it could be shown that ostriches already possessed antibodies to the *Ms01* organism prior to vaccination, a probable result of previous exposure. The expected antibody response pattern could not be detected in ostriches in response to the vaccinations, and therefore no final conclusion as to the immunostimulatory capabilities of the DNA vaccines could be drawn. Further vaccination trials in which ostriches that do not possess immunity to ostrich mycoplasmas, are required in order to obtain conclusive results.

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## Opsomming

Mikoplasmas is evolusionêr gevorderde prokariote wat as nuwe generasie bakteriese patogene beskou word en nuwe paradigmas word benodig om hulle siekte potensiaal te verstaan. Die herhaaldelike mislukking om mikoplasmatiese siektes van mense en diere deur die gebruik van antibiotika en ander tegnieke het tot die gevolgtrekking gelei dat die mees belowende benadering tot die beheer van mikoplasma infeksies, die ontwikkeling van 'n effektiewe entstof is. Die identifisering van drie spesies van *Mycoplasma* wat Suid-Afrikaanse volstruise infekteer, en groot verliese in die Suid-Afrikaanse volstruisbedryf veroorsaak, het tot 'n soektog na nuwe entstof strategieë gelei waarmee hulle beheer en uitgewis kan word. Hierdie studie het die gebruik van drie potensiële DNA entstowwe ondersoek, wat die selaanhegtings-geassosieerde *Ms01* OppA proteïen as antigeniese determinant benut, om antiliggaam response teen die volstruis infekterende *Ms01* organisme te genereer. 'n Entstof proef is onderneem waarin die antigeniese potensiaal van die pCIneo, VR1012 en VR1020 DNA entstowwe geëvalueer is in volstruise, en het die ontwikkeling van 'n ELISA vir die serologiese analise daarvan genoodsaak. Vir die ontwikkeling van die ELISA, is die *Ms01 oppA* gene geïsoleer, gekloneer in 'n prokariotiese ekspressie vektor en uitgedruk as 'n rekombinante GST fusie produk in *Escherichia coli*. Die suksesvolle uitdrukking en suiwering van die rekombinante proteïen het die daaropvolgende gebruik daarvan as antigeen in die ontwikkeling van die ELISA moontlik gemaak. Die ELISA het 'n groot sein tot agtergrond verhouding getoon. Die resultate wat met hierdie ELISA verkry is, het getoon dat die volstruise in hierdie inentingsproef blykbaar reeds voor immunisering antiliggame teen die *Ms01* organisme besit het, wat op vorige kontak met die organisme dui. Die verwagte antiliggaam responspatroon kon dus nie in hierdie volstruise waargeneem word nie, en om dié rede kon geen finale afleiding oor die immuunstimulerende vermoëns van die DNA entstowwe gemaak word nie. Verdere inentingsproewe van volstruise wat nie vorige immuniteit teen volstruis mikoplasmas besit nie, word benodig om deurslaggewende resultate te verkry.

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**Dedicated to:**

My mother, Wilhelmina

and

My father, Herman

For their love, support and encouragement, and

For giving me the opportunities they didn’t have.

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## Abbreviations

| | |
|---|---|
| ABC transporters | ATP-binding cassette transporters |
| ABTS | 2,2'-azino-bis(3ethylbenzthiazoline-6-sulphonic acid) |
| BSA | Bovine serum albumin |
| CpG | Cytosine-phosphate-guanine |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme-linked Immunosorbent Assay |
| GST | Glutathione S-transferase |
| HI | Hemagglutination inhibition |
| IBV | Infectious bronchitis virus |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| kDa | kiloDalton |
| LB | Luria-Bertani |
| MHC | Major histocompatibility complex |
| NDV | Newcastle disease virus |
| ODN | Oligodeoxynucleotide |
| $Poly^T$ | Polythymine |
| $Poly^A$ | Polyadenine |
| PPLO | Pleuropneumonia-like organisms |
| RE | Restriction endonuclease |

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| | |
|---|---|
| SDS-PAGE | Sodium-dodecylsulphate polyacrylamide gel electrophoresis |
| SPA | Serum plate agglutination |
| TLR9 | Toll-like receptor 9 |
| TNF | Tumor necrosis factor |

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# Table of Contents

**Chapter 1 - Introduction** ........ 1

**Chapter 2 - Literature review** ........ 3

**2.1 The Ostrich** ........ 3

**2.2 The South African Ostrich Industry** ........ 4

**2.3 History of the ostrich industry** ........ 4

**2.4 Mycoplasmas** ........ 5

**2.4.1 Phylogeny, Taxonomy and Evolution of Mycoplasmas** ........ 5

**2.4.2 History of mycoplasmas** ........ 6

**2.4.3 Distribution of Mycoplasmas** ........ 7

**2.4.4 Molecular biology of mycoplasmas** ........ 9

**2.4.5 Cell size, morphology and reproduction** ........ 9

**2.4.6 Genome structure and organization** ........ 9

**2.4.7 Protein production** ........ 11

**2.4.8 Metabolism** ........ 13

**2.4.9 *In vitro* culture** ........ 14

**2.4.10 Membrane characteristics** ........ 14

**2.5 ATP-binding cassette transporters** ........ 15

**2.5.1 Structure of OppA** ........ 17

**2.5.2 Function** ........ 18

**2.6 The importance of mycoplasmas in disease** ........ 18

**2.7 Pathogenicity of mycoplasmas** ........ 19

**2.7.1 Transmission and entry into the host** ........ 19

**2.7.2 Target tissues** ........ 20

**2.7.3 Motility** ........ 20

**2.7.3 Host cell interactions** ........ 20

**2.7.4 Host evasion** ........ 21

Stellenbosch University http://scholar.sun.ac.za



**2.7.5 Damage to the host** ......23

**2.7.6 Epidemiology** ......24

**2.7.7 Clinical Signs** ......24

**2.7.8 The effect of concurrent infections** ......26

**2.7.9 Factors contributing to avian mycoplasma infections** ......27

**2.7.10 Diagnosis** ......27

**2.7.11 Prevention and Control** ......27

**2.8 DNA Vaccines** ...... 28

**2.8.1 History** ......29

**2.8.2 Composition** ......31

**2.8.3 Administration** ......33

**2.8.4 Optimization of DNA vaccines** ......34

**2.8.5 Immune response generated** ......35

**2.8.6 Advantages of DNA vaccines** ......36

**Chapter 3 - Cloning, expression and purification of the *Ms01* OppA protein** ...... 38

**3.1 Introduction** ...... 38

**3.2 Materials and Methods** ...... 39

**3.2.1 Cloning of *oppA* gene into pGEM-T easy vector** ......39

**3.2.3 Expression of the recombinant *Ms01* OppA protein in *E. coli* BL21(DE3) pLysS cells using the pET14b and pGEX-4T-1 prokaryotic expression systems** ......46

**3.2.4 Protein Purification** ......48

**3.3 Results** ...... 49

**3.3.1 Primer design, PCR amplification and agarose gel electrophoresis of the *oppA* gene** ......49

**3.3.2 Cloning the *Ms01 oppA* gene into the pGEM®-T easy vector** ......49

**2.3.3 Cloning of the *Ms01 oppA* gene into the pET14b and pGEX -4T-1 vectors** ......50

**3.3.4 Expression of the recombinant *Ms01 oppA* gene in *E. coli* BL21(DE3) pLysS cells using the pET14b prokaryotic expression system** ......54

**3.3.5 Expression of the recombinant *Ms01 oppA* gene in *E. coli* BL21(DE3) pLysS cells using the pGEX-4T-1 prokaryotic expression system** ......55

Stellenbosch University http://scholar.sun.ac.za



**3.3.6 Purification of the recombinant OppA protein** ........................................................................55

**3.4 Discussion** ........................................................................................................................ 56

**4. An evaluation of the antibody responses elicited in ostriches by the pCIneo, VR1012 and VR1020 DNA vaccines** ........................................................................................................................ 64

**4.1 Introduction** ........................................................................................................................ 64

**4.2 Materials and Methods** ........................................................................................................ 67

**4.2.1 Vaccine preparation** ........................................................................................................67

**4.2.2 Vaccination trial** ........................................................................................................67

**4.2.3 Antigen preparation** ........................................................................................................67

**4.2.4 Secondary antibody purification and biotinylation** ...................................................68

**4.2.5 Enzyme-linked immunosorbent assay (ELISA)** ...........................................................68

**4.3 Results**........................................................................................................................ 69

**4.3.1 Optimization studies**........................................................................................................69

**4.3.2 Serum analysis of the pCIneo, VR1012 and VR10120 DNA vaccine trials** ...........................74

**4.4 Discussion** ........................................................................................................................ 84

**Chapter 5 - Conclusions and future perspectives** ........................................................................ 88

**Addendum A** ........................................................................................................................ 96

**Addendum B** ........................................................................................................................101

**Addendum C** ........................................................................................................................102

**Addendum D** ........................................................................................................................103

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# Chapter 1 - Introduction

The South African ostrich industry dominates the world ostrich trade, supplying an estimated 60% of the global ostrich produce. With 15 tanneries, eleven export approved abattoirs and investment activities exceeding R2.1 billion, the ostrich industry provides direct employment for around 20 000 workers in the production and processing sectors of South Africa [The South African Business Chamber, 2004; Cooper *et al.,* 2007; Thompson *et al.,* 2008]. Each year, around 230 000 slaughter birds are produced by the country's 558 export registered farms and, according to the South African Ostrich Business Chamber, income generated from export of ostrich products amounts to more than R1 billion annually [The South African Business Chamber, 2004; Cooper *et al.,* 2007]. As a result of this, the ostrich industry is one of the top twenty agro-based industries in South Africa and thus of significant economic importance to the country [The South African Business Chamber].

The high profit potential of ostrich farming is brought about by the assortment of products which may be obtained from a single bird [Jefferey, 1996]. The South African ostrich industry generates 45% of its total income from ostrich meat, 45% from ostrich skin and 10% from ostrich feathers [Cooper *et al.,* 2007]. The vast majority (90%) of leather and meat is exported, making the South African ostrich industry heavily reliant upon the export of ostrich leather and meat products to trade partners in Europe, USA and Japan [Thompson *et al.,* 2008]. Ostrich leather is a fashionable product utilized in the making of clothes, boots and upholstery. Four square meters of hide may be produced from a single adult bird, with one hide making three pairs of boots [Jefferey, 1996]. Ostrich feathers are popular in the fashion industry, but are also utilized for the production of cleaning products and other items. Ostrich meat, due to its low fat (<2%), cholesterol and calorie content, but high protein content, is considered to be the healthiest of the red meats and has, in recent years, grown in popularity due to increasing numbers of health conscious consumers [The South African Business Chamber, 2004; Jefferey, 1996]. Approximately 4 000 tonnes of ostrich meat is exported every year to the European Union, whose market is responsible for 60-70% of the global ostrich meat consumption [Cooper *et al.,* 2007; Thompson *et al.,* 2008].

The advantageous natural conditions, in addition to its historic advantage, should enable South Africa to maintain its leading role in the global ostrich trade market. However, recent bans (August 2004 to September 2005 and again July 2006 to November 2006, as well as from July 2011 to December 2011) on ostrich meat exports, due to avian influenza outbreaks, have highlighted the requirement for effective disease control measures [Thompson *et al.,* 2008]. A ban on ostrich meat exports may result in losses of at least R50 million per month during peak season, making job losses inevitable. In addition, the South African ostrich industry is already suffering losses of up to 30% as a result of respiratory disease in feedlot ostriches [Pretorius, 2009]. The recent isolation of three ostrich-specific mycoplasmas; *Ms01*, *Ms02 and Ms03*, which cause respiratory disease in South African ostriches has thus, in addition to causing dramatic production losses, raised concerns regarding the transmission of these mycoplasmas to other countries

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through contaminated meat products, which may consequently place constraints on the export of ostrich products, resulting in a significant impact on the economy of the country [Botes *et al.,* 2005].

A limited range of antibiotics, together with strict biosecurity practices, are currently the only measures aimed at the control of ostrich mycoplasma infections [Pretorius, 2009]. Although other avian mycoplasma vaccines have been employed as an attempted control measure, none have proven even slightly effective, as shown in a study by Pretorius [2009]. Needless to say, mycoplasma infections still occur at unacceptable frequencies in South African ostriches. Accordingly, the South African ostrich industry has launched an investigation into vaccination strategies with which to combat ostrich mycoplasma infections in South Africa. The investigation includes conventional whole-organism vaccine development studies, undertaken at Onderstepoort Veterinary Institute in Pretoria, as well as the more novel DNA vaccine development strategy described in this thesis.

For these reasons, the objectives of this study were:

- To produce a recombinant *Ms01* OppA protein by cloning the *Ms01 oppA* gene into a prokaryotic expression vector, expressing the gene in *Escherichia coli* and purifying the expressed protein.

- To generate two enzyme-linked immunosorbent assays (ELISAs), in which ELISA plates were coated with recombinant *Ms01* OppA protein and the *Ms01* whole organism, respectively, for serological analysis of immune responses to *Ms01* vaccines in ostriches.

- To conduct a vaccination trial in which the ability of DNA vaccines, consisting of the *Ms01 oppA* gene cloned into the pCIneo, VR1012 and VR1020 vaccine vectors respectively, to elicit antibody responses against the *Ms01* organism in ostriches could be evaluated.

Chapter 2 of this thesis provides a literature review of the phylogeny, taxonomy, evolution, distribution, molecular biology, morphology and pathogenesis of mycoplasmas. An overview of the utilization of DNA vaccines as well as a brief overview of the *Ms01* OppA protein concludes the literature review. Chapter 3 describes the cloning, expression and purification of the *Ms01* OppA protein. A description of the DNA vaccination trial undertaken and the ELISAs used to analyze the samples taken during the trial is provided in Chapter 4. Chapter 5 contains the conclusions drawn from this study as well as future perspectives. The thesis is concluded with a list of references followed by addenda which include the nucleotide sequence of the *Ms01* OppA protein and the raw data of the ELISA results.

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# Chapter 2 - Literature review

## 2.1 The Ostrich

Ostriches are part of a group of flightless birds known as ratites [Al-Nasser *et al.,* 2003]. Other members of this group include the emu, the cassowary, the rhea, the kiwi and the extinct moa [Al-Nasser *et al.,* 2003; Sales, 2009]. Ratites are characterized by their smooth, broad, bowl-shaped sternum or breastbone which lacks a keel [Al-Nasser *et al.,* 2003]. A keel is an adaptation for the attachment of breast or flight muscles in other birds.

Ostriches, which are currently the largest living birds on the planet, can range between 1.7 and 2.7 m in height and can reach adult body weights of between 70 and 130 kg [Cooper *et al.,* 2007; Sales, 2009]. Ostrich eggs can weigh up to 2 kg, but are the smallest egg of any bird, relative to the size of its parent [Adams and Revell, 2008]. Female ostriches are grayish-brown, while males attain a black and white plumage when they reach the age of two [Cooper *et al.,* 2007].

Ostriches, which can be seen in Figure 2.1, are classified under the class: Aves, family: *Struthionidae*, species: *Struthio camelus* [Al-Nasser *et al.,* 2003]. There are four living subspecies of ostrich [Sales, 2009]. These include *Struthio camelus australis,* which can be found south of the Zambezi and Kunene rivers in Southern Africa, *Struthio camelus molybdophanes*, from Somalia and Ethiopia, *Struthio camelus camelus*, found along the west coast of the Red Sea and the Sahara desert and *Struthio camelus massaicus*, found in Tanzania and eastern Kenya [Sales, 2009]. The Arabian ostrich, which used to be another subspecies of ostrich, *Struthio camelus syriacus,* was hunted to extinction between 1945 and 1966 [Al-Nasser *et al.,* 2003; Sales, 2009]. There is, however, a hybrid of *Struthio camelus australis* and *Struthio camelus syriacus* in existence, known as *Struthio camelus* var. *domesticus* [Al-Nasser *et al.,* 2003].

**Figure 2.1** A photograph of ostriches farmed in South Africa [Southern Africa direct , 2008].

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Ostriches are native to the savannas of Africa and can be found both north and south of the equatorial forest zone [Sales, 2009]. These birds, which can live up to 75 years, have a pair of powerful legs that allow them to reach speeds of up to 70 kilometers per hour [Sales, 2009; Adams and Revell, 2008]. While foraging for food, ostriches can range over an area with a radius of up to 20 kilometers in their native habitat.

## 2.2 The South African Ostrich Industry

The Western Cape Province currently represents 70-80% of the South African ostrich industry, with the main production and processing sites being located in the semi-arid Klein Karoo region [Cooper *et al.,* 2007; Thompson *et al.,* 2008]. Fifteen to twenty percent of slaughter ostriches are, however, also reared in the Eastern Cape Province [Cooper *et al.,* 2007].

## 2.3 History of the ostrich industry

Ostrich farming was first initiated in 1866, when the first eggs of domesticated ostriches were hatched in South Africa [Sales, 2009; Douglas, 1906]. Ostriches had long been hunted mainly for their feathers, but at around 1859 it became apparent that wild ostriches would soon become extinct if buyers in Europe were to depend solely upon ostriches being hunted in remote parts of Africa [Sales, 2009]. This resulted in the idea of farming with ostriches and in turn, led to the first ostriches being domesticated.

In 1880, South Africa was exporting 73 965 kg of feathers, of which only one-eighth was obtained from wild birds [Douglas, 1906]. A census done in 1891 reported that the number of tame birds in the country amounted to 154 880. By 1904, this figure had more than doubled to 357 970 and in 1910 the South African ostrich industry was booming with 764 736 breeding birds, producing 336 854 kg of feathers annually [Sales, 2009; Douglas, 1906]. While attempts had been made to initiate the farming of ostriches in New Zealand, Egypt, California, South America and Australasia, these efforts eventually failed. The Cape colony, to which ostrich farming was practically confined at this stage, remained to be a continuous success from the beginning. The ostrich industry, however, did not remain free of problems. Excessive supply of feathers, changing fashions, poorly coordinated marketing and the disruption of export brought on by World War I (1914-1918) resulted in a rapid collapse of the ostrich industry in the second decade of the twentieth century [The South African Business Chamber, 2004]. Thus, by 1930 only 23 000 farmed ostriches were left in South Africa. The ostrich industry, however, gradually recovered after World War II (1939-1945) and expanded to include the production of leather [South Invest, 2011]. A one-channel cooperative marketing system was launched in South Africa in 1959 and in 1964 the first abattoir was established in the country [The South African Business Chamber, 2004]. This was followed by the erection of a tannery in 1970. The turn of the century brought with it a growing consumption of ostrich meat and previously low prices steadily increased. In 2003, it was estimated that there were just under 500 000 commercially bred ostriches in the world, of which about 350 000 were in South Africa [South Invest, 2011]. Presently South Africa continues to dominate the global ostrich market.

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## 2.4 Mycoplasmas

### 2.4.1 Phylogeny, Taxonomy and Evolution of Mycoplasmas

*Mycoplasmas* are a genus of bacteria which fall within the class *Mollicutes* [Rottem, 2003; Bradbury, 2005]. The name *Mollicutes* refers to the fact that these organisms are surrounded only by a thin trilaminar membrane and lack the conventional cell wall which normally surrounds other bacterial cells [Bradbury, 2005]. The word *Mollicutis* is derived from the latin words '*mollis'* which means 'soft' and '*cutis*' which means 'skin'.

*Mollicutes* are divided into five phylogenetic units, based upon their 16S rRNA sequences, with the best studied genera found in the orders *Entomoplasmatales* (*Entomoplasma*, *Spiroplasma*, *Mesoplasma*), *Anaeroplasmatales* (*Asteroleplasma*, *Anaeroplasma*), *Acholeplasmatales* (*Acholeplasma*) and *Mycoplasmatales* (*Mycoplasma, Ureaplasma*) [Razin, 1998; Prescott *et al.,* 2002]. Although they were initially thought to be the primitive forerunners of the more complex pathogens, *Mollicutes* are now known to be phylogenetically related to Gram positive bacteria and are thought to have developed from these organisms by means of genome reduction [Rottem, 2003; Bradbury, 2005; Prescott *et al.,* 2002]. A phylogenetic scheme, based upon the 16S rRNA sequences of the *Mollicutes*, which was suggested by Maniloff [cited by Bradbury, 2005] indicates that *Mollicutes* diverged from *Streptococci* around 600 million years ago, with the loss of some nonessential genes, including those involved in cell wall synthesis [Razin, 1998; Bradbury, 2005]. Around 500 million years ago, *Mollicutes* split into two branches, the AAA branch and the SEM branch. The AAA branch consists of *Anaeroplasma*, *Acholeplasma* and *Asteroleplasma*, while the SEM branch comprises the *Spiroplasma*, *Entomoplasma*, and *Mycoplasma* [Razin, 1998]. Maniloff [cited by Bradbury, 2005] goes on to suggest that during the degenerate evolution of these two branches, genome reduction occurred independently, and that the *Acholeplasmas* and *Anaeroplasmas* were the first to evolve by reductive evolution. He further states that the *Spiroplasmas* originated during an early split from the *Acholeplasma*s, and that *Mycoplasmas* and *Ureaplasmas* evolved from the *Spiroplasmas*. The conversion of the UGA codon from a stop codon to a tryptophan codon is thought to have occurred during the early evolution of the SEM branch.

The term 'mycoplasma' was initially used to signify an intimate connection between plant-invading fungi or other microorganisms and their host cells by A.B. Frank in 1889, and then again by Jakob Erikson in 1897 [Krass *et al.,* 1973]. However, in 1929 it was used for the first time as a reference to the bovine pleuropneumonia organism by Nowak, who wrote in a paper on the morphology, nature, and life cycle of the organism: "the term *Mycoplasma peripneumoniae* seems to agree better with the nature and the morphology of the microbe" [Krass and Gardner, 1973; Hayflick and Chanock, 1965]. This followed the initial use of the term *Asterococcus mycoides*, designated by the Linnean system in 1910, but which was discarded due to its use for a genus of algae in 1908. Two other terms, *Micromyces* and *Coccobacillus*, had also previously been suggested, but were discarded for the same reason [Hayflick and Chanock, 1965].

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Nowak later explained about his chosen name for the bovine pleuropneumonia organism: "The name mycoplasma seems suited both to its unusual protoplasmic nature and also to its remarkable mycelial morphology," [cited by Krass and Gardner, 1973]. The legitimacy of the term 'mycoplasma' was then subsequently confirmed in separate papers by Edward and Freundt in 1955 and by the Editorial Board of the International Committee on Nomenclature of Bacteria prior to that. Thus, the term mycoplasma was taxonomically approved, interestingly, without particular reference to its prior use by Frank or Edward in mycology. However, this does not disqualify its legitimacy.

The International Committee on Systematic Bacteriology, Subcommittee on Taxonomy of Mollicutes has set out the minimum standards for classification as a *Mycoplasma* [Minion, 2002]. Although classification is problematic due to the few physiological and biochemical properties that can be used for differentiation, characteristics such as permanent lack of cell walls, cell size, genome size, colony formation and G+C content are often used. Biochemical characteristics such as fermentation of glucose or arginine, optimal growth temperature, morphology, serology and host origin are also considered. Phylogenetic analyses using 16S rRNA sequences are now, however, the defining phylogenetic tool used in the classification of *Mollicutes*.

In 2005, Botes *et al*. [2005] identified three novel species of *Mycoplasma* through the use of 16S rRNA sequencing. Until then, the poultry mycoplasmas, specifically *Mycoplasma gallisepticum*, was thought to be responsible for the huge losses caused by ostrich mycoplasmosis. Phylogenetic analysis indicated that the newly identified mycoplasmas fall within the Hominis group and that they are phylogenitically distant from one another, as can be seen in Figure 2.2. Since *Mycoplasmas* are usually named according to the host they inhabit, these *Mycoplasmas* should be named *Mycoplasma struthiolus*, in reference to their host *Struthio camelus*. However, due to the fact that these *Mycoplasmas* have not yet formally been characterized, they are temporarily referred to as *Ms01*, *Ms02* and *Ms03*.

**2.4.2 History of mycoplasmas**

While Louis Pasteur was the first to recognize the mycoplasmas as a microbial entity, he was unable to culture these organisms in nutrient broth or to examine them under a microscope [Hayflick and Chanock, 1965]. It was Nocard and Roux who, in 1898, first succeeded in cultivating the microbial agent associated with contagious bovine pleuropneumonia on a medium similar to that used to grow bacteria [Bradbury, 2005; Hayflick and Chanock, 1965; Bigland, 1969]. By 1910, the morphology of the pleuropneumonia organism (PPO) had been described and before long, microorganisms with similar properties were being isolated from other sources and referred to as pleuropneumonia-like organisms (PPLO) [Hayflick and Chanock, 1965]. It was soon found that PPOs were filterable through Berkefeld V filters and in 1929, with the aid of filtration studies using the Gradocol membrane, the size of the minimal units required for reproduction was estimated to be between 125 to 150 µm. Resultantly, despite the knowledge that this microbe, now referred to as *Mycoplasma mycoides* subspecies *mycoides*, could multiply in a cell-free

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medium, it was deemed to be a virus for 25 years [Bradbury, 2005; Hayflick and Chanock, 1965; Bigland, 1969]. That same year, Julien Nowak was the first to refer to the PPO as a *Mycoplasma* (*peripneumonia*).

The first reported isolation of an avian mycoplasma is believed to have occurred in 1936, when Nelson was investigating different causes of 'infectious coryza' and found cocco-bacilliform bodies in a mild respiratory disease condition of chickens [Bigland, 1969]. Chronic respiratory disease in avians was documented for the first time in Alberta in 1952 and involved six flocks of chickens and two flocks of turkeys, of which the number of chicken flocks affected had risen to ninety, by 1953. The first isolation of *M. gallisepticum* occurred in 1952 while Markam and Wong were investigating chronic respiratory disease in chickens, and between 1953 and 1957 Crawley and Faley isolated the microbe, developed the hemagglutination inhibition test, and made the earliest suggestions on how to control chronic respiratory disease in poultry.

Thus, the existence of a new group of saprophytic and parasitic microorganisms, distinguished from viruses, bacteria and rickettsias by their unusual properties, had officially been documented (following the isolation of mycoplasmas from various sources, including humans, animals and sewage), and in 1989, almost one hundred years after the first known species of mycoplasma was isolated, a filterable virus-like agent found in AIDS and other patients, was found to be a strain of *Mycoplasma fermentans* by Lo *et al*. [cited by Bradbury, 2005; Hayflick and Chanock, 1965; Bigland, 1969].

Interestingly, not long ago many rickettsias were reassigned from the genera *Eperythrozoon* and *Haemobartonella* to the genus *Mycoplasma* [cited by Bradbury, 2005]. This move was based upon the 16S rRNA similarity shared by the organisms. Another interesting fact is that already in 1983, Whitcomb and Bove [cited by Bradbury, 2005] remarked about the abovementioned organisms: 'although they have been classified as rickettsiae, ultrastructural studies have clearly established their mycoplasma-like nature' [cited by Bradbury, 2005].

### 2.4.3 Distribution of Mycoplasmas

Mycoplasmas are globally distributed and are considered to be principal pathogens in animal production units in each country of the world [Minion, 2002]. Although most mycoplasmas only thrive within a narrow host range, they are known to inhabit plants, fish, insects, reptiles, birds and mammals [Prescott, *et al.,* 2002; Minion, 2002, Dubvig, K., and Voelker, 1996]. Notably, one article stated that "It appears as though the main factor for adding an animal or plant to the list of hosts is the willingness of a mycoplasmologist to invest the effort and funds required to isolate and taxonomically characterize the mycoplasmas from the tested host" [Razin *et al.,* 1998].

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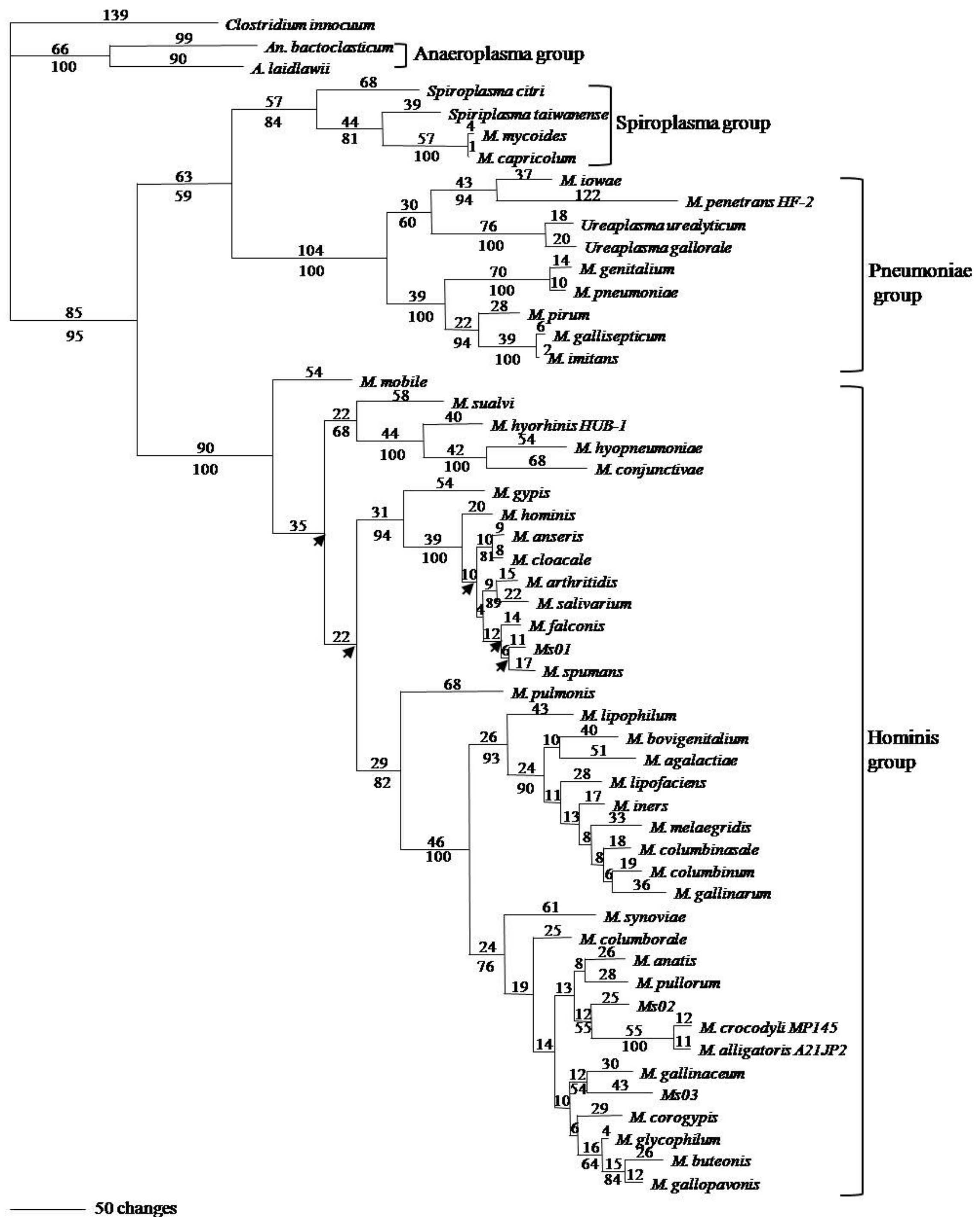


**Figure 2.2** A phylogenetic tree of mycoplasma species based on a parsimony analysis of 16S rRNA gene sequences. This tree represents one of eight of the shortest trees retrieved in a heuristic search (CI = 0.381, RI = 0.696). The branches that collapse in the strict consensus tree are indicated with arrows. Branch lengths and bootstrap values are indicated above and below the line respectively.

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### 2.4.4 Molecular biology of mycoplasmas

A mycoplasma cell essentially consists of three simple, yet unique structural organelles which have intrigued membrane and cellular biologists for several years and more recently attracted the attention of molecular biologists [Bradbury, 2005]. These organelles include a cell membrane, a circular double-stranded DNA molecule and ribosomes. Molecular biologists have been particularly fascinated with the mycoplasmas, because the loss of non-essential genes by these organisms is thought to hold the key to the minimal gene requirements capable of supporting life.

### 2.4.5 Cell size, morphology and reproduction

With diameters ranging between 200 and 800 nm in length, mycoplasmas are the smallest free-living, self-replicating organisms known to man [Razin *et al.,* 1998; Rottem, 2003; Bradbury, 2005; Zuo, *et al.,* 2009]. Due to their lack of a cell wall, the dominating shape of a mycoplasma cell is that of a sphere [Razin *et al.,* 1998; Rottem, 2003]. Several mycoplasmas are, however, known to display a variety of morphologies which may include flask-like cells with terminal tip structures, as well as filamentous and ring forms [Prescott, *et al.,* 2002; Razin *et al.,* 1998; Rottem, 2003]. Mycoplasmas grow optimally at 37°C and typically form small, circular, smooth colonies with fairly flat edges and a denser elevation in the middle, which has been likened to the shape of a fried egg [Kleven, 2008].

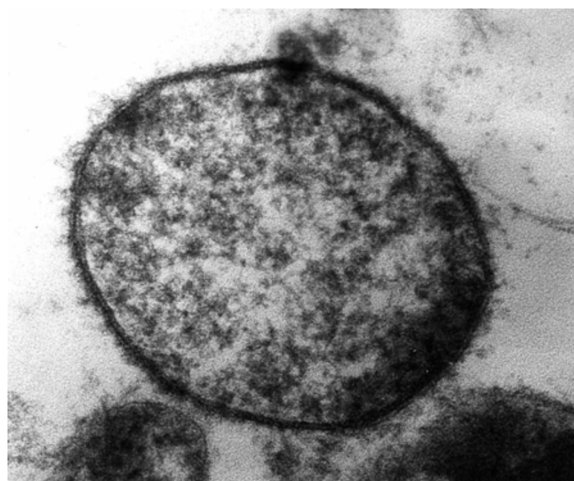

**Figure 2.3** An electron micrograph of an individual mycoplasma cell [Bradbury, 2005].

### 2.4.6 Genome structure and organization

2.4.6.1 Genome size

Mycoplasmas have the smallest reported genomes of any self-replicating living organism, with sizes ranging from 580 kb in *M. genitalium* to 1380 kb in *M. mycoides* subsp. *mycoides* [Dubvig and Voelker, 1996; Razin *et al.,* 1998; Rottem, 2003]. The striking differences between the genome sizes of

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mycoplasmas, and that of other microorganisms, are illustrated in a comparison between the genome sizes of *Mycoplasma genitalium* (580 kb), *Haemophilus influenza* (1 830 kb) and *Escherichia coli* (4 720 kb) [Bradbury, 2005]. The tiny genomes of mycoplasmas are thought to be the result of a loss of many dispensable genes, which accounts for their parasitic lifestyle, as explained later in this chapter. The considerable variation observed in the genome sizes of mycoplasmas, not only within the same genus, but between different strains of the same species, is thought to be the result of repetitive DNA elements which are scattered throughout the chromosome of mycoplasma cells [Dubvig and Voelker, 1996]. These repetitive elements are thought to consist of either insertion elements or segments of genes encoding major surface antigens, as discussed later in the thesis [Razin *et al.,* 1998; Dubvig and Voelker, 1996].

2.4.6.2 Base composition and codon usage

Mycoplasma genomes have a G+C base composition of between 23-40%, with less than 35% of G+C base pairs comprising the genomes of most species [Kleven, 2008; Dubvig and Voelker, 1996]. This low G+C content is thought to be the eventual consequence of ineffective uracil-DNA glycosylation activity in the mycoplasmas, which gradually led to G+C base pairs being replaced by A+T base pairs [Dubvig and Voelker, 1996]. This extreme bias in base composition has resulted in a similar bias in codon usage. As such, 90% of codons in most mycoplasmas have an A or T in the third nucleotide position. Considering the above, it should not be surprising that mycoplasmas employ an alternative genetic code. The TGA codon, which serves as a stop codon in most organisms, codes for tryptophan in the mycoplasma genome, instead of the TGG codon utilized in the "universal" genetic code. This adaptation in codon usage has resulted in obvious practical implications when cloned mycoplasmal genes are expressed in heterologous systems. Specifically, the TGA codons (encoding tryptophan) in mycoplasmas will be read as termination codons in *E. coli* and other organisms which employ the universal genetic code, thus resulting in the expression of truncated gene products.

Codon bias is, however, not restricted to the third nucleotide position and may also occur in the first and second positions, which results in a significant effect on the amino acid composition [Dubvig and Voelker, 1996]. Accordingly, mycoplasmas have less CCN, GGN, CGN and GCN codons compared to *E. coli*, which has a G+C base composition of around 50%, and consequently express proteins with fewer Pro, Gly, Arg and Ala residues than the latter. Likewise, mycoplasmas have an elevated proportion of TTY, AAN, ATN and TAY codons, and therefore a greater abundance of Lys, Asn, Phe, Ile and Tyr residues. Conservative amino acid substitutions resulting from a change in G-C base pairs to A-T base pairs make the bias in the amino acid composition of mycoplasmal proteins especially obvious. The fact that mycoplasmas frequently have Lys residues (codons AAG and AAA), in the amino acid positions where other organisms normally have Arg residues (codons AGG, CGN and AGA) in conserved proteins, serves as a good illustration of this point.

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### 2.4.7 Protein production

2.4.7.1 Transcription

As a result of their small genomes, mycoplasmas lack several genes which regulate gene expression [Beneina, 2002]. Mycoplasmas generally possess RNA polymerase promoter areas similar to the -10 (Pribnow box) and -35 consensus sequence of promotors recognized by the vegetative sigma factor $\sigma^A$ [Dubvig and Voelker, 1996]. The -10 consensus sequence in *E. coli* and *Bacillus subtilis* is TATAAT, whereas in the *Ms01 oppA* gene this sequence, which was identified 21 bp upstream of the translation initiation codon, is TAACAT [Pretorius, 2009; Dubvig and Voelker, 1996]. Similarly the -35 consensus sequence in *E. coli* and *B. subtilis* is TTGACA, whereas the corresponding sequence, identified 42 bp upstream of the translation initiation codon, is TCGGTT in *Ms01*.

While most mycoplasmal transcription signals resemble the classical eubactorial ones, mycoplasmal RNA polymerases share a peculiar resistance to rifamycin with *Clostridium acidiurici*, *C. innocuum*, and *C. ramosum*; their phylogenetic relatives [Razin *et al.,* 1998; Rottem, 2003]. The core RNA polymerase is encoded by the conserved genes *rpoA* (α subunit), *rpoB* (β subunit) and *rpoC* (β' subunit), and sequencing analysis of the *rpoB* gene of *M. gallisepticum* indicate that minor changes in the amino acid sequences in the RIF region (region responsible for rifamycin binding) of the β subunit may confer rifamycin resistance.

With regards to the direction of transcription, significant consistency has been observed, with only 15% of proposed open reading frames (ORF) in mycoplasmas being transcribed against the usual direction of transcription [Razin *et al.,* 1998]. The termination factor Rho appears to be absent from all mycoplasmal genomes analyzed thus far, indicating that termination of transcription may occur independently of this factor. Similar to other bacteria, however, a stem-loop structure, composed of characteristic terminator sequences in which short, interrupted palindromic regions are followed by a run of uracil residues, serves to stop transcription in mycoplasmas.

2.4.7.2 Translation

The transcription of most mycoplasmas' messenger RNA (mRNA) resembles that of the Gram-positive bacteria, with the exception of the TGA codon which encodes tryptophan in mycoplasmas and serves as a stop codon in other bacteria [Dubvig and Voelker, 1996]. Nucleotide sequence analysis indicate that the coding regions of most mycoplasmal genes begin with an ATG start codon, while TTG and GTG serve as alternative start codons. This is a trait shared by most prokaryotes, since better interaction occurs between the ATG initiation codon and the initiation transcript RNA (tRNA) than with other initiation codons [Pretorius, 2009].

Most mycoplasmal mRNAs contain a ribosome-binding site, usually situated four to ten bases upstream of the translation initiation codon, which is comparable to the Shine-Delgarno sequence of Gram positive

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bacteria [Dubvig and Voelker, 1996]. This sequence, situated five bases upstream of the translation initiation codon of the *oppA* gene, was identified to be 5'-TAGGAGAA-3' in *Ms01* [Pretorius, 2009]. A characteristic mycoplasmal ribosome-binding site is usually composed of a subset of around five bases of the sequence 5'-AGAAAGGAGG-3', to which the 16S ribosomal RNA (rRNA) anneals [Dubvig and Voelker, 1996]. Interestingly, however, the 3' end of mycoplasma 16S rRNA molecules is seven nucleotides longer than characteristic Gram negative 16S rRNA molecules and four nucleotides longer than typical Gram positive 16S rRNA molecules, probably suggesting that mycoplasmas require a Shine-Delgarno sequence with stronger complimentarity to 16S rRNA, than other bacteria. Nevertheless, not all mycoplasmal species were found to contain a Shine-Delgarno-like sequence, which indicates that alternative sequences may function as ribosomal binding sites in many mycoplasmas [Razin *et al.,* 1998]. Indeed, the *tuf* gene of *M. genitalium*, which lacks a Shine-Dalgarno sequence, yet is highly expressed, was found to contain a novel ribosome-binding site which is thought to anneal to a highly conserved region of the 16S rRNA molecule corresponding to nucleotides 1082–1093 of the 16S rRNA of *E. coli* [Dubvig and Voelker, 1996].

Usually there is one amino acyl-tRNA synthetase for each amino acid of the 20 activating enzymes typically present in eubacteria. Mycoplasmas appear to lack only glutaminyl-tRNA synthetase [Razin *et al.,* 1998]. This is a trait mycoplasmas share with Gram positive bacteria, in which the $tRNA^{Glu}$ is initially charged with glutamate, which is subsequently converted into glutamine by an aminotranferase enzyme. An aminotransferase enzyme has, however, not yet been identified in mycoplasmas, but genes encoding the elongation factors, for example *fus*, *efp*, *tsf*, and *tuf*, have been identified. Six of the eight codon family boxes of *M. mycoides* and *M. capricolum* were found to be read by single isoacceptor tRNA with an unmodified uridine in the first position of the anticodon (wobble position).

Mycoplasmas only possess one peptide release factor (RF), specifically RF1, which recognizes the TAG and TAA stop codons [Razin *et al.,* 1998]. Since the TGA codon is used to encode tryptophan in mycoplasmas, the use of RF2, which recognizes TGA and TAA stop codons, is not required by these organisms. In addition, considering their A+T-biased genome, it is not surprising that a preference to the use of TAA stop codons rather than that of TAG codons has been observed in mycoplasmas with a very low G+C genome composition. Likewise, the *oppA* gene of *Ms01* is terminated by the TAA stop codon.

2.4.7.3 Post-translational modification

Due to their lack of a cell wall, mycoplasmas do not have a periplasmic space and proteins that are not cytoplasmic are either secreted or membrane bound [Dubvig and Voelker, 1996]. Mycoplasma proteins are directed into a secretory pathway for transport across the membrane by typical eubacterial signal peptide sequences ((-4)-VAASC-(+1)) [Heinrich, 1999]. Mycoplasmas, however, possess an extremely high number of lipoproteins when compared to other bacteria [Dubvig and Voelker, 1996]. Lipoprotein processing often involves cleavage of the signal peptide sequence N-terminal to a cysteine residue to which

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a diacylglycerol moiety of glycerophospholipid has been transferred to the sulfhydryl group [Razin *et al.,* 1998; Dubvig and Voelker, 1996]. The prolipoprotein (lipoprotein precursor) is cleaved N-terminal to the modified cysteine by a signal peptidase II enzyme, resulting in the cysteine becoming the first amino acid of the processed protein. Thus, many mycoplasmal lipoproteins have a consensus signal peptidase II cleavage site, followed by a cysteine residue, including the *oppA* gene of *Ms01*, in which a signal peptidase II recognition site and lipoprotein attachment site ($_{22}$LVAAACNSKSA$_{32}$) has also been identified [Pretorius, 2009].

Isoprenylation and phosphorylation are additional examples of protein modifications which occur in mycoplasmas [Dubvig and Voelker, 1996]. Although the mechanism of isoprenylation and the function of modified proteins are not yet known, phosphorylation of cytoadherence accessory proteins by ATP-dependent serine-threonine kinases are thought to regulate activities such as cytoadherence, cell division and gliding motility.

### 2.4.8 Metabolism

The small genomes of mycoplasmas have availed them limited coding capacity, and consequently precludes several metabolic activities typically present in most bacteria, which accounts for these organsisms' parasitic way of life [Razin *et al.,* 1998; Prescott *et al.,* 2002; Dubvig and Voelker, 1996]. Genome analyses have indicated that mycoplasmal genomes encode numerous proteins associated with metabolite transport and catabolic activities, whereas few anabolic proteins are coded for. This indicates that mycoplasmal metabolic activities are primarily associated with energy production rather than the provision of substrates for biosynthetic pathways [Razin *et al.,* 1998]. Therefore, although these organisms do perform complex protein synthesis, they have to rely on their host (or growth medium) for the provision of several essential nutrients, including fatty acids, vitamins, amino acids, sterols, purines and pyramidines [Bradbury, 2005; 17, 18].

Mycoplasmas may be divided into fermentative and nonfermentative species [Razin *et al.,* 1998; Prescott *et al.,* 2002]. Fermentative mycoplasmas generate ATP through glycolysis and the pyruvate dehydrogenase pathway, with lactic acid production, whereas most nonfermentative species, such as *M. hominis* and *M. arthritidis*, as well as some fermentative species, utilize the arginine dihydrolase pathway, resulting in the production of ATP, ammonia, ornithine and carbon dioxide [Razin *et al.,* 1998; Prescott *et al.,* 2002; Dubvig and Voelker, 1996]. At least some mycoplasmas appear to have a functional pentose-phosphate pathway, but none seem to have a complete tricarboxylic acid cycle or any quinines or cytochromes. Their electron transport chain system is flavin terminated [Prescott *et al.,* 2002; Dubvig and Voelker, 1996]. Therefore, substrate-level phosphorylation is most probably the major route of mycoplasmal ATP production, resulting in low yields and fairly large quantities of metabolic end products, sometimes completely depleting the host of the specific substrate metabolized [Razin *et al.,* 1998; Dubvig and Voelker, 1996].

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### 2.4.9 *In vitro* culture

Despite their inefficient energy yielding pathways and fastidious nutritional requirements, mycoplasmas are able to grow in the absence of host cells [Razin *et al.,* 1998]. Although mycoplasmas are notoriously difficult to cultivate, complex media, typically based on beef heart infusion, yeast extract, serum with a range of supplements, as well as peptone, are used to overcome the assimilative difficulties of these organisms. Mycoplasmal cultivation using complex undefined media has, however, hindered the preparation of mycoplasmal antigens free of serum components, the molecular description of mycoplasmal metabolic pathways, and the genetic analysis of mycoplasmas. Ongoing efforts, with the aim of reaching a defined growth medium have seen partial success, with defined media supporting the growth of some species having been described.

### 2.4.10 Membrane characteristics

Mycoplasmas lack intracytoplasmic membranes and thus possess only one type of membrane, namely the plasma membrane [Razin *et al.,* 1998; Razin, 1978]. Consisting of mostly lipids and proteins, the gross chemical composition of mycoplasmal plasma membranes resembles that of most prokaryotes.

#### 2.4.10.1 Membrane Lipids

Nearly all mycoplasmal lipids are found in the cell membrane and, similar to other biological membranes, include phospholipids, glycolipids and neutral lipids [Razin *et al.,* 1998; Razin, 1978]. The acidic phospholipids, phosphatidylglycerol and to a lesser degree diphosphatidylglycerol, can be found nearly everywhere in the mycoplasmal membrane, whereas a large part of the membrane in many, but not all mycoplasmas, may also consist of glycolipids. The fatty acid residues of membrane phospholipids, glycolipids and cholesterol make up the main portion of the hydrophobic core of the membrane. However, as previously stated, mycoplasmas are incapable of synthesizing fatty acids and as a result are dependent on their host for their supply.

The majority of mycoplasmas require cholesterol for growth, a trait which is unique among the prokaryotes [Razin *et al.,* 1998; Razin, 1978]. The cell membranes of these sterol-requiring *Mollicutes* generally contain much greater levels of cholesterol than *Mollicutes* which do not require cholesterol for growth. These cholesterol levels are similar to those found in eukaryotic cell membranes.

Glycosyl diglycerides containing from one to five sugar residues is another common constituent of many mycoplasmal cell membranes [Razin, 1978]. Immunogenic lipopolysaccharides with chemical compositions unrelated to those found in Gram negative bacteria have also been found in many mycoplasmal species.

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2.4.10.2 Membrane Proteins

More than two thirds of the mass of mycoplasmal membranes is accounted for by proteins, the rest consisting of membrane lipids [Razin *et al.,* 1998]. Thirty five of the 200 membrane proteins known to occur in *M. gallisepticum* are covalently modified with acyl chains. Acylation of proteins with long chain fatty acids is an effective means of securing surface-exposed proteins with functions outside of the cell to the plasma membrane. Compared to the eubacteria, mycoplasmas have a remarkably high percentage of genes encoding putative lipoproteins, most of which are acidic (pI < 5 to 7) and immunogenic. In the absence of a cell wall, surface proteins embedded or anchored (lipoproteins) in the cell membrane play an essential role in the interactions between the mycoplasmas and their hosts [Beneina, 2002]. Thus, lipoproteins are some of the leading antigens in mycoplasmas, and most of the mycoplasmal cell surface antigens known to undergo size and/or antigenic variation, are lipoproteins.

The dependence of mycoplasmas on the exogenous supply of several nutrients would predict that they require several transport systems [Razin *et al.,* 1998]. Mycoplasmas, however, do not have a higher proportion of genes dedicated to transport, than do *H. influenza*, *E. coli* and *B. subtilis*. The occurrence of only one permeability barrier in the wall-less mycoplasmas, compared to at least two barriers found in Gram positive and Gram negative bacteria, may be a potential reason for the small number of transport systems observed in mycoplasmas. The significant gene saving witnessed in this category may also be aided by the low substrate specificity of some mycoplasmal transport systems, for example those involved in the transport of amino acids.

2.4.10.3 Membrane Transport Systems

To date, three types of transport systems have been identified in mycoplasmas [Razin *et al.,* 1998]. The first involves membrane proteins acting as specific carriers to carry out facilitated diffusion of substrates across the membrane. Another group is the highly efficient phosphoenolpyruvate-dependent sugar phophotransferase transport system (PTS). It is, however, the ATP-binding cassette (ABC) transporter family, which is also a transporter of oligopeptides, which is a major focus of this thesis and which will be discussed in greater detail.

## 2.5 ATP-binding cassette transporters

ABC transporters are unidirectional transporters which are known for their ability to couple the energy released during the hydrolysis of ATP to the transport of a large range of molecules, often against a concentration gradient, across cell membranes [Moutran *et al.,* 2008; Nepomuceno *et al.,* 2007]. ABC transporters regulate the movement of vital nutrients into and out of cells and are copiously present in all kingdoms of life [Nepomuceno *et al.,* 2007; Doeven *et al.,* 2004].

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ABC transporters may take up as much as 5% of bacterial genomes and may function either as importers or exporters [Nepomuceno *et al.,* 2007]. Importers typically amass amino acids and sugars, whereas exporters generally remove a number of compounds from the cytoplasm [Davidson and Maloney, 2007]. ABC transporters are usually composed of four functional domains: two highly conserved peripheral domains which attach to and hydrolyze ATP, as well as two transmembrane domains which form a permeation pathway across the membrane. ABC importers are substrate binding protein-dependant ABC transporters and as a result require the assistance of an additional domain. This domain binds and delivers substrate to the membrane complex. Exporters, on the other hand, whose substrates enter the translocation pathway directly from the cytoplasm or lipid bilayer, do not require the aid of additional binding proteins.

Oligopeptide permease (Opp) is a multicomponent ABC transporter which is widespread amongst several species of bacteria and archaea and can be seen in Figure 2.4 [Moutran *et al.,* 2008; Nepomuceno *et al.,* 2007]. It is a typical ABC transporter and is thus composed of the four domains which are characteristic to ABC transporters [Nepomuceno *et al.,* 2007]. OppB and OppC are the two pore-forming transmembrane domains, while OppD and OppF are the two peripheral membrane-associated ATPase domains. Since oligopeptide permease is an importer, it includes an additional ligand binding domain which is referred to as OppA. The function of OppA is to recognize, bind and guide peptide substrates to the oligopeptide permease complex. OppA is therefore thought to confer affinity and specificity to the oligopeptide permease transport system.

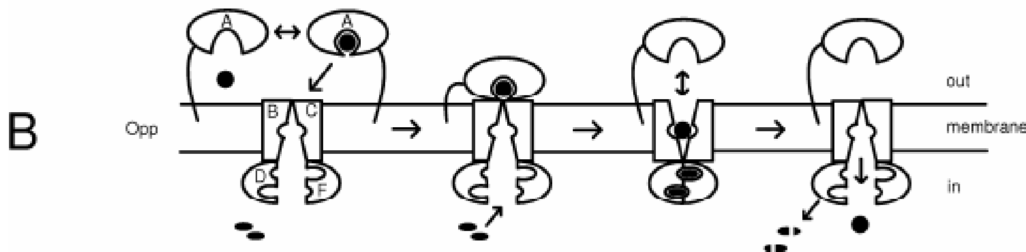


**Figure 2.4** An illustration of the mechanism with which oligopeptide permeases accomplish peptide transfer. The respective subunits of the oligopeptide permease complex are indicated by the corresponding letters [32].

In 1993, Heinrich *et al*. [1993] identified a 100 kDa protein in *M. hominis* thought to be involved in the adherence of the mycoplasma to its host cells, a crucial step in the pathogenicity of mycoplasmas. In reference to its size, the protein was termed the P100 protein. Subsequently, in 1999, the same authors verified the identity of the P100 protein as the OppA protein, a substrate-binding subunit of the oligopeptide permease complex of *M. hominis* [Heinrich *et al.,* 1999].

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### 2.5.1 Structure of OppA

OppA proteins in Gram negative bacteria are free-floating periplasmic proteins [Nepomuceno *et al.,* 2007]. Contrastingly, the OppA proteins of Gram positive bacteria are lipoproteins with an N-terminal cystein residue which is covalently bound, via a thioether linkage, to a diacylglycerol in the plasma membrane. Since mycoplasmas lack cell walls and are the phylogenetic relatives of Gram positive bacteria, their OppA proteins by implication need to be attached to the cell membrane in a manner similar to that seen in Gram positive bacteria.

An N-terminal leader peptide sequence, followed by a conserved cysteine residue which is the site where the lipoprotein attaches to the plasma membrane of Gram positive bacteria, can normally be found at the beginning of the amino acid sequence of OppA [Heinrich *et al.,* 1999, Heinrich *et al.,* 1993]. Tam and Saier [cited by Peltoniemi *et al.,* 2002] have proposed the amino acid sequence $A(X)_7D(X)_4T(X)_3R(X)_3K$ as a signature for oligopeptide permeases, while amino acid sequence alignments by Heinrich *et al*. [Heinrich *et al.,* 1999] have found the consenses sequence F/Y-I/LRK to be present in most peptide binding proteins.

While OppA is generally assumed to be monomeric, many early reports have indicated that these substrate binding proteins may actually be dimers [31. Davidson and Maloney, 2007]. Doeven *et al.* [2004] have, however, found that unanchored OppA is monomeric under a number of conditions and that no higher oligomeric aggregates are formed by membrane-bound OppA. The same authors also indicated that monomeric OppA is able to bind to a peptide, and in this liganded state, to OppBCDF. A single OppA molecule may thus, according to these authors, be adequate for peptide translocation.

The structure of the OppA protein in mycoplasmas has not been determined yet, but much can be deduced from OppA proteins whose structures have been determined [Figure 2.5]. The OppA protein consists of three structural domains [Moutran *et al.,* 2007]. Domains I and III are coupled by a flexible hinge and form a cleft in which ligand binds. This enables oligopeptides to be totally engulfed by the protein in a mechanism likened to that of the “Venus flytrap”. α-Helices surrounding mixed stretches of β-sheets, typify the bilobate ellipsoidal α/β configuration of the OppA protein in *Xanthomonas citri*. Domain I is composed of three non-contiguous sequences, consisting of residues 1 to 44, 154 to 260 and 491 to 521, which make up a 7-stranded mixed β-sheet with four α-helices on its sides. The rest of the non-contiguous segments (1-44 to 154-260) of this ensemble are held in a disulphide bond between the Cys 25 and Cys 165 residues. Nine α-helices flanking a 7-stranded central β-sheet composed of amino acids 261 to 490, constitute domain III. One side of the four anti-parallel β-sheet strands which make up Domain II, is circled by seven α-helices to create a very hydrophobic core, while the other side of the structure is exposed to the surface of the molecule. Since only a portion of periplasmic receptor proteins contain domain II, OppA orthologs are larger in comparison to other ABC-transporter nutrient-binding proteins.

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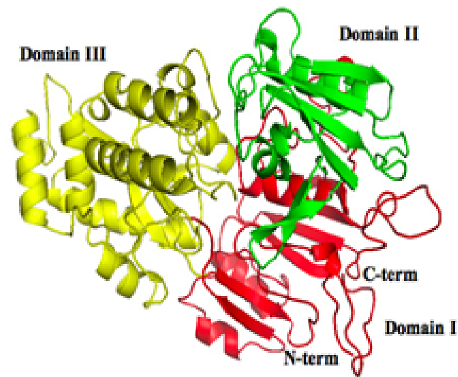


**Figure 2.5** The structure of the OppA protein of *X. citri* [Moutran *et al.,* 2007].

### 2.5.2 Function

Besides its role in peptide translocation, the oligopeptide permease system regulates several cellular processes which affect the virulence and physiology of a number of species [Nepomuceno *et al.,* 2007]. Oligopeptide permease is accountable for recycling as much as 50% of cell wall muropeptides in *Salmonella typhimurium* and *E. coli*. Moreover, a functional oligopeptide uptake system containing at least one active OppA ortholog has to be present in order for conjugation and transformation, which are quorem-sensing responses mediated by peptides in Gram positive bacteria such as *Streptococcus pneumonia* and *Bacillus subtilis*, to occur. OppA is also thought to be involved in sporulation and in the modulation of gene expression in a number of Gram positive bacteria. In addition, many studies have connected the presence of OppA orthologs to the expression of several virulence-associated traits, for instance the expression of adhesins. The sensitivity of *S. typhimurium* and *E. coli* to aminoglycoside antibiotics and toxic peptides is thought to be augmented by the expression of OppA in these bacteria, and recently there have been suggestions of a link between the expression of the OppA protein and the formation of biofilm in *Vibrio fluvialis*. The ecto-ATPase activity of the OppA protein in *M. hominis* is, furthermore, thought to be a virulence associated trait utilized by mycoplasmas following colonization, as discussed in greater detail later in this thesis [Hopfe and Heinrich, 2008].

## 2.6 The importance of mycoplasmas in disease

It may be surprising that, despite their simplicity, several mycoplasmal species are able to cause far-reaching effects in both animal and plant hosts [Bradbury, 2005]. Many mycoplasmal species are well-recognized respiratory pathogens, especially under conditions where animals such as pigs, calves, poultry and even laboratory rodents are intensively housed, and considerable economic loss can be caused by these pathogens, especially if the animals are stressed [Beneina, 2002; Bradbury, 2005]. Interestingly, there have even been cases in which outbreaks of *Mycoplasma pneumonia* have occurred amongst military recruits and university students, which are probably the nearest human equivalents to intensively housed and stressed animals [Bradbury, 2005].

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Whilst more than 20 species of *Mycoplasma* are able to infect chickens and turkeys, only four species are considered to be major avian pathogens [Dufour-Gesbert *et al.,* 2006]. The disease potential of these veterinary important mycoplasmas, which include *M. gallisepticum*, *M. synoviae*, *M. iowae* and *M. meleagridis* are most clearly defined and well established amongst the avian infecting mycoplasmas [Beneina, 2002; Bradbury, 2005].

**2.7 Pathogenicity of mycoplasmas**

Bacterial infection involves an intricate set of interactions between the host and the invading organism, as well as the environment in which the host finds itself [Bradbury, 2005]. Through evolution, the host has developed several strategies with which to defend itself against invading pathogens, whilst invading pathogens have developed numerous tactics with which to overcome the defenses of the host.

In order to be successful, a pathogen must find a way to enter its host, reach its target tissue and perhaps attach itself to the target tissue [Bradbury, 2005]. A successful pathogen should be able to evade the host defenses, enter the target tissue (or cells depending on the organism), multiply as well as cause some damage to the host. It should then be able to escape from the infected host to infect new hosts. Since there is no information currently available on the pathogenic nature of *Ms01*, much of the following report is based upon what is currently known about other mycoplasmas, particularly *M. hominis*, a close phylogenetic relative of *Ms01*, as well as other significant avian mycoplasmas.

**2.7.1 Transmission and entry into the host**

Mycoplasmas are able to enter the host by means of inhalation [Bradbury, 2005]. As such, the spread of poultry mycoplasmosis has unwittingly been encouraged by man as a result of birds being kept at high stocking densities in very large populations. Although mycoplasmas are not able to thrive in the environment outside the host, they may survive for a number of days on materials which include cotton clothing, hair, crates, utensils, equipment and feathers [Bigland, 1969; Kleven, 2008]. *M. gallisepticum* is thus regularly introduced to its host by humans, rodents and free-flying birds (who in themselves are not infected with *M. gallisepticum*) and as a result, commercial poultry producers continue to struggle with the horizontal transfer of *M. gallisepticum* [Kleven, 2008]. Vertical transmission of *M. gallisepticum* may also occur through the fertile egg to the offspring (poults and chicks), and airborne transmission of *M. gallisepticum* usually occurs sporadically, but does not normally exceed a distance of two kilometers. *M. synoviae* infections are also transmitted both laterally and vertically [Kleven, 2008; Dufour-Gesbert *et al.,* 2006]. In addition, the spread of *M. iowae* and *M. meleagridis* occurs through the venereal route [Bradbury, 2005]. This route of spread is often aided through the use of artificial insemination during which female flocks can be infected when semen from turkey stags are pooled, since contaminated semen from a single infected stag may potentially be distributed to an entire female flock.

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### 2.7.2 Target tissues

Mycoplasmas are usually organ and tissue specific [Razin *et al.,* 1998]. These organisms, which may be commensal, parasitic or saprophytic, primarily inhabit the mucous surfaces of the respiratory tract, alimentary canal, joints, urogenital tracts, eyes and mammary glands of human and animal hosts [Razin *et al.,* 1998; Prescott *et al.,* 2002; Beneina, 2002].

In avians, the epithelial surfaces are the main target tissues of mycoplasmas, those of the respiratory tract in particular [Bradbury, 2005]. However, some mycoplasmas also target the urogenital tract epithelial surface and there is speculation that, in poultry, cross infection may occur between a colonized air sac and the adjacent ovaries of a female. This may result in a developing embryo becoming infected and enable transmission to the egg, even though, as stated previously, transmission to the egg may also occur as a result of contaminated semen. Mycoplasmas also occasionally target the joints, a tendency typically displayed by *M. synoviae*. This, however, usually follows infection at another site, such as the respiratory tract. In human beings, *M. hominis* initially infects the urogenital tract, subsequently spreading to the respiratory tract as well as the joints [Razin *et al.,* 1998].

### 2.7.3 Motility

Motility has been demonstrated in various pathogenic mycoplasmas, although this trait may be surprising in an organism with such a tiny genome [Bradbury, 2005]. The movement, which has been compared to that of a flock of sheep grazing a field, is called ‘gliding motility’. The speed of motility seems to differ between species when examined *in vitro*. The composition of the medium, the age of the culture as well as the incubation temperature may affect the gliding ability of the organism, while particular antibodies may inhibit the motility of the organism completely. Being motile helps mycoplasmas reach their target tissues and may also be of aid in overcoming specific physical defenses in the host, for example the mucin layer in the respiratory tract as well as ciliary activity, making it a virulence-associated trait [Bradbury, 2005; Hatchel *et al.,* 2006]. Even though the mechanism of gliding motility has not yet been unraveled, studies have linked motility in mycoplasmas such as *M. pneumoniae* to a structure referred to as the tip organelle (discussed in greater detail in the next section), and have identified mutations in the adhesion protein P30 to be associated with a reduction of speed in such mycoplasmas. Other studies suggest the presence of a contractile cytoskeletal protein which aids motility and that the tip organelle is not directly involved in mycoplasmal motility [Dubvig and Voelker, 1996; Trachtenberg, 1998; Hatchel and Balish, 2008].

### 2.7.3 Host cell interactions

Infection and colonization has been shown to, in several animal mycoplasmas, depend upon adhesion of the organism to the host tissues [Rottem, 2003]. The ability of these mycoplasmas to adhere to host tissues is the main virulence factor, and mutants which are adherence-deficient are avirulent. Since the adhesion of

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mycoplasmas to the epithelial cells is an essential step in tissue colonization, it is considered to be the first step in disease pathogenesis [Rottem, 2003; Bradbury, 2005].

*M. gallisepticum* as well as some other mycoplasmas related to *M. pneumonia*, colonize eukaryotic cell surfaces and mucus membranes with the aid of the tip organelle, a tapered cell extension at one of their poles. The tip organelle functions as the leading end in gliding motility as well as an attachment organelle [Rottem, 2003]. Structural and functional cooperation between adhesins, interactive proteins and adherence accessory proteins localized in the tip organelle, mobilize and concentrate adhesins at the tip, enabling colonization of eukaryotic cell surfaces and mucus membranes, perhaps by means of the host sulfated glycolipids and sialoglycoconjugates [Baseman and Tully, 1997]. The 169 kilodalton (kDa) P1 and 30 kDa P30 proteins present in *M. pneumonia* have been identified to be critical in the cytoadherence process [Razin *et al.,* 1998, Rottem, 2003]. Most mycoplasmas, including *M. hominis*, however, do not have a well-defined tip organelle and little is known about the cytoadherence of such species. Studies have, nevertheless, identified several structures, including the P50 and the P100 protein in *M. hominis*, which are associated with cytoadherence of the organism to its host [Heinrich *et al.,* 1999; Kitzerow *et al.,* 1999].

Since mycoplasmas have no rigid cell wall, intimate contact can occur between the plasma membrane of the mycoplasma cell and that of the host cell [Rottem, 2003]. Under the right conditions such contact may lead to cell fusion. Mycoplasmas, such as *M. fermentans*, which are capable of cell fusion, are referred to as fusogenic mycoplasmas. The fusogenicity of mycoplasmas depends on the unesterified cholesterol content of the cell membrane and only mycoplasmas requiring cholesterol for growth are capable of fusogenic activity.

Although most mycoplasmas were thought to be extracellular pathogens for many years, mounting evidence suggests that mycoplasmas have evolved ways (not including fusion) of entering host cells which are not phagocytic [Razin *et al.,* 1998; Heinrich, 1999]. The mechanisms of host cell entry, however, still remain unclear. Some mycoplasmas, including *M. genitalium* and *M. penetrans*, appear to enter host cells with the aid of their tip organelles, but several other mycoplasmas, such as *M. hominis* and *M. fermentans*, which do not have tip organelles, are also capable of entering their host cells [Razin *et al.,* 1998; Bigland, 1969]. Adherence to host cells is, however, thought to be the first step towards host cell invasion, and reiterates the key role of adherence related structures on mycoplasmal cells in the pathogenicity of these organisms. Notably, the outcome of experimental *M. gallisepticum* infections in chickens were found to be influenced by the invasiveness of the infecting strain, a more invasive strain being found in the liver, kidney, heart and brain of infected birds, suggesting haematogenous spread [Bradbury, 2005].

#### 2.7.4 Host evasion

The survival of a pathogen within its host greatly depends on its ability to dodge the host's immune system and the ability of some mycoplasmas to enter their host cells affords them protection from the host's antibody response as well as from several drug therapies, accounting somewhat for the difficulty

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experienced in eradicating mycoplasmas from infected hosts and to the establishment of latent or chronic infection states [Razin *et al.,* 1998; Rottem, 2003; Bradbury, 2005].

Molecular mimicry and phenotypic plasticity are, however, better documented mechanisms employed by most mycoplasmas to circumvent the host's immune system [Rottem, 2003; Minion, 2002; Kleven, 1998]. Molecular mimicry describes antigenic epitopes which are shared by different mycoplasmas and host cells, and thus recognized by the host's immune system as 'self' instead of 'foreign' and/or leads to the induction of autoantibodies [Rottem, 2003]. Phenotypic plasticity refers to the capacity of a single genotype to alter its antigenic composition in order to generate more than one alternative form of morphology, physiological state and/or behavior in response to environmental conditions [Kleven, 1998; Rottem, 2003]. This may be accomplished as a response to environmental signals or as random changes of expression of single or multiple genes. The adhesin-related operons in mycoplasmas serve as an example. While such operons contain only a single full length copy of adhesin structural genes, several truncated and sequence related copies of adhesin genes occur throughout the genome, with specific areas of adhesin genes existing as single genomic copies, in contrast to the multiple copies of other areas which are homologous, but not identical [Baseman and Tully 1997]. Mycoplasmas thus seem to have a reservoir of partial adhesin-related gene regions with which to regulate the functional and structural characteristics of adhesins through recombination events. It is likely that mechanisms of phase and antigenic variation, which describes "the reversible high frequency gain or loss of cell surface components", occur, whereby adhesins display altered affinities and specificities, depending on the organization of variable and constant adhesin gene sequences [Baseman and Tully 1997; Rottem, 2003]. The host's antibody response mainly targets surface components on pathogens and the ability to rapidly change the antigenic repertoire of its components enables the pathogen to avoid detection by the host's immune system. Since mycoplasmas lack a cell wall, pilli, and flagella; membrane proteins are the major targets of the immune response [Rottem, 2003].

The immunomodulatory capabilities of mycoplasmas and other bacteria are increasingly recognized, not only as a method of host evasion, but also as a major mechanism of virulence [Rottem, 2003]. The production of systemic as well as local anti-mycoplasmal antibodies of various classes and subclasses, stimulation of cell-mediated immunity, opsonization and phagocytosis of organisms are amongst the specific protective defense mechanisms utilized by the host to combat infecting mycoplasmas [Razin *et al.,* 1998]. However, studies have shown that specific immune reactions drawn from the host by invading mycoplasmas aid in the development of lesions and exacerbate mycoplasma induced disease. Mycoplasmas exercise a wide array of nonspecific immunomodulatory effects on the cells of the immune system and may induce either suppression or polyclonal stimulation of T and B lymphocytes; increase cytotoxicity of T-cells, macrophages and natural killer cells; induce cytokines; activate the complement cascade; and enhance the expression of cell receptors. The ability of mycoplasmas to modulate their host's immune responsiveness has aided in their pathogenicity, enabling them to suppress or evade their host defense mechanisms and cause chronic, persistent infections.

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If all else fails, molecules located in the plasma membrane or outer surface of mycoplasmas, such as capsular polysaccharides, lipoproteins and biofilms composed of fibrillar polycarbohydrates surrounding cells, are thought to protect these organisms from the bactericidal effects of complement and other defenses, in addition to triggering inflammatory processes in the infected host [Pilo *et al.,* 2007].

### 2.7.5 Damage to the host

No typical principal virulence genes have been found in the genomes of the ten *Mycoplasma* species which have been sequenced completely, in contrast to other pathogenic bacteria, where virulence is mostly determined by toxins, cytolysins and invasions [Pilo *et al.,* 2007]. Instead, mycoplasmas appear to use intrinsic metabolic and catabolic pathway functions, or proper constituents of the mycoplasmal outer surface, to ensure their survival and cause disease in the infected host.

Competition for biosynthetic precursors, such as fatty acids and vitamins, is thought to upset host cell integrity and alter its function when mycoplasmas attach to their host [Rottem, 2003]. Nonfermenting *Mycoplasma* species such as *M. hominis* and *M. arthritidis* serve as examples [Dubvig and Voelker, 1996]. Such mycoplasmas rapidly deplete the host's arginine reserves, affecting host protein synthesis, host cell division, and growth; and additionally inducing chromosome breakage, reduction in chromosome number, the appearance of new and/or additional chromosome varieties, and multiple translocations [Rottem, 2003]. The depletion of L-arginine has also been shown in many studies to stimulate several immunosuppressive effects in immune cell systems *in vitro* [Razin *et al.,* 1998]. In addition, mycoplasmas can interfere with membrane receptors and alter transport mechanisms of the host cell, for example interference with $K^+$ channels of ciliated bronchial epithelial cells by *M. hyopneumoniae*, when they attach to the surface of the host [Rottem, 2003].

Despite the fact that they are not associated with toxins, mycoplasmas are known to excrete cytolytic enzymes, cytotoxic metabolites, and peroxide and superoxide radicals which damage the host cell membrane [Razin *et al.,* 1998; Rottem, 2003]. Potent membrane-bound phospholipases present in many mycoplasmas may also hydrolyse host cell phospholipids, activating particular signaling cascades or the release of cytolytic lysophospholipids which are able to disrupt the integrity of the host cell membrane.

Additionally, apart from upsetting the normal functions of the host cells, mycoplasmas deliver potent hydrolytic enzymes when they enter or fuse to host cells [Rottem, 2003]. Examples include endonucleases of *M. penetrans*, which degrade host cell DNA, and phosphoprotein phosphatase which may interfere with host cell signal transduction. Fusion of the mycoplasma membrane (components) to the host cell membrane may also alter receptor recognition sites, and influence the stimulation and expression of cytokines, upsetting cross-talk between host cells.

Recent studies by Hopfe and Heinrich [Hopfe and Heinrich, 2008] indicate that the ecto-ATPase OppA protein in *M. hominis* induces the release of ATP from HeLa cells and that OppA subsequently hydrolyzes

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this ATP. The degradation of extra-cellular ATP to adenosine inhibits the growth of numerous cell types and may even induce apoptosis. This correlates with previous findings by Zhang and Lo [cited by Hopfe and Heinrich, 2008] that *M. hominis* and *M. salivarium* accelerates apoptosis and inhibits proliferation of 32 D cells. Hopfe and Heinrich [2008] hypothesize that *M. hominis* is an extra-cellular colonizing pathogen and suggest that this pathogen enhances necrosis and apoptotic cell death in HeLa cells, while OppA promotes apoptosis only, in contrast to invasive mycoplasmas such as *M. fermentans* and *M. penetrans*, which have been shown to prevent apoptosis and stimulate cell proliferation.

**2.7.6 Epidemiology**

No information is currently available on the epidemiology of *Ms01*, but most avian species are infected by a unique species of mycoplasma [Bigland, 1969; Kleven, 2008]. Examples of minor mycoplasmal pathogens include *M. columbinum*, and *M. columbinasale* which infect pigeons, *M. anatis* which infects ducks and *M. imitans*, which is pathogenic to several avian species and closely related to *M. gallisepticum* [Kleven, 2008].

*M. gallisepticum* is the most significant mycoplasmal pathogen infecting chickens and turkeys [Kleven, 2008]. Although *M. gallisepticum* may also infect other avian species including ostriches, the gallinaceous species are known to be most at risk [Cline *et al.,* 1997; Kleven, 2008]. *M. gallisepticum* occurs worldwide. However, as a result of the intense control programs utilized by developed countries, key poultry breeds, as well as turkey and broiler production in Western Europe, North America and several other developed countries, are now free of this pathogen [Bradbury, 2005; Kleven, 2008; Kleven, 1998]. However, even in developed countries, *M. gallisepticum* is still a regular occurrence in big multi-age commercial egg production flocks [Kleven, 2008].

*M. synoviae* is another species of *Mycoplasma* which infects both chickens and turkeys [Kleven, 2008]. *M. synoviae* infections occur in layers, turkeys and commercial broilers all around the world. Breeding flocks are, however, generally free of infection [Dufour-Gesbert *et al*., 2006]. *M. meleagridis* infects turkeys only, whilst *M. iowae* is a newly discovered pathogen infecting young turkeys and turkey embryos [Kleven, 2008].

**2.7.7 Clinical Signs**

The virulence and clinical manifestations of poultry *Mycoplasma* species vary widely, perhaps as a result of the high intraspecies variability between strains, as well as the ability of mycoplasmas to interact with other disease-causing organisms and environmental factors [Pretorius, 2009; Kleven, 2008]. However, when a bird becomes infected with a pathogenic mycoplasma species, the infection lingers for the lifetime of the host, despite severe antibiotic medication and a strong immune response [Kleven, 2008]. The course of avian mycoplasmosis is often prolonged and symptoms of the disease typically develop after 6 to 21 days in experimentally infected poultry. However, the incubation period is variable in naturally infected birds

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and may result in birds remaining asymptomatic for days or months, until they become stressed [Avian mycoplasmosis, 2007].

*M. gallisepticum* infections result in what is commonly referred to as 'chronic respiratory disease' in chickens and 'infectious sinusitis' in turkeys [Bigland, 1969; Kleven, 2008]. Infectious sinusitis in turkeys is characterized by severe upper and lower respiratory disease, whereas in chickens, depending on the infecting strain as well as other factors, infections with *M. gallisepticum* may vary from asymptomatic to severe, with more severe respiratory symptoms being seen where birds are concurrently infected with *E. coli*, infectious bronchitis virus (IBV), pneumovirus infection, Newcastle disease virus (NDV), *Hemophilus paragallinarum*, avian influenza and other pathogens [Kleven, 2008; Avian mycoplasmosis , 2007; Ley, 2008]. Infections by *M. gallisepticum* are characterized by a build up of inflammatory exudates in the trachea, an exfoliation of ciliated epithelial cells and a severe inflammatory response in the pulmonary air-sacs [Collier *et al.,* 2006; Bajwa *et al*., 1996]. Respiratory symptoms may include tracheal rales, sneezing, coughing, dyspnea and nasal discharges. Lesions include airsacculitis, tracheitis, sinusitis and conjunctivitis [Kleven, 2008]. More severe disease symptoms are often seen in turkeys and often include swelling of the paranasal (infraorbital) sinus [Avian mycoplasmosis, 2007; Ley, 2008]. Another common occurrence in turkeys is conjunctivitis accompanied by a frothy exudate, a condition which occurs only occasionally in chickens. When the birds are concurrently infected with *E. coli*, lesions frequently include perihepatitis and pericarditis [Kleven, 2008]. *M. gallisepticum* infections result in decreased feed conversion, poor growth rate, decreased egg production in adults and hatchability losses [Bradbury, 2005; Kleven, 2008; Abdulmoumen and Roy, 1995]. Mortality may be considerable where birds are concurrently infected with *E. coli*. *M. gallisepticum* infections contribute to the downgrading or condemnation of the meat at poultry processing plants [Bradbury, 2005; Collier *et al.,* 2006].

Infections by *M. synoviae* are characterized by a comparatively mild respiratory infection and/or airsacculitis and infectious synovitis [Kleven, 2008; Dufour-Gesbert *et al.,* 2006; Lockaby *et al.,* 1998]. Synovitis frequently occurs as a swelling of the joints accompanied by poor growth and lameness [Kleven, 2008]. In this condition, a thick gelatinous fluid frequently fills the synovial membranes of the feet, mandibles, hock and breast, in response to inflammation of the joint synovial membrane [Bigland, 1969]. There are, however, considerable differences in the virulence of different strains of *M. synoviae* and the virulence of the infecting strain may determine how severe the resulting disease will be [Kleven, 2008; Lockaby *et al.,* 1998]. A small number of strains seem to be rather virulent, whereas several strains of *M. synoviae* seem to be non-pathogenic and result in little or no disease.

Ostrich mycoplasma infections are primarily associated with the respiratory tract, resulting in inflammation of the air sacs, trachea and nose, as well as in severe lung lesions [Botes *et al.,* 2005; Pretorius, 2009; Air sac infection, 2006]. Swollen sinuses, nasal exudates, rattle sounds in the throat, foamy eyes and excessive swallowing or shaking of the head are clinical signs associated with the ostrich specific mycoplasmas. Direct and indirect consequences of ostrich mycoplasma infections include erosion disease; increased

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treatment costs; downgrading of carcasses; and increased susceptibility to secondary infections, for example *E. coli*.

**2.7.8 The effect of concurrent infections**

In 1969, Bigland described *M. gallisepticum* as "an organism of moderate pathogenicity which often acts as an opportunist, waiting for other conditions to lower the defenses of the bird then acting synergistically to bring on the typical syndrome of chronic respiratory disease" [Bigland, 1969]. This statement resulted from early studies involving *M. gallisepticum*, where scientists became aware that infection of healthy birds did not always result in chronic respiratory disease. It came to their attention that additional factors, which include stress causing conditions such as bad ventilation, overcrowding, feed and water starvation, and reactions to vaccinations against NDV, fowl pox, IBV and infectious laryngotracheitis were often required, in conjunction with *M. gallisepticum* infection, to bring about chronic respiratory disease. Concurrent infections involving mycoplasmas, viruses and bacteria are now well recognized and additional agents which have since been found to be involved include reovirus, *Heamophilus gallinarium*, adenovirus and molds [Bradury, 1984].

It is now known that concurrent infection with even the mild or vaccine strains of IBV or NDV may promote the multiplication of mycoplasmas and amplify the severity of respiratory disease [Bradury, 1984]. The resulting infection may, however, be influenced by the breed, resistance and age of the birds, as well as the sequence of infection, the route and dose of infection, and the tropism and virulence of the infecting organisms. Amplification of pathogenicity, interference between pathogens or simply co-existance may be causative agents of the ensuing disease. It is thought that viral damage to the mucous epithelium may improve the habitat for the mycoplasma infection and a study by Hopkins and Yoder [cited by Bradury, 1984], where a tissue reaction to IBV appears to predispose *M. synoviae* to air sac lesion production, supports this theory. Humidity and environmental temperature may, however, influence the extent to which the lesions are formed.

Bacterial disease may also be involved and pathogenic strains of *E. coli* are frequently at the forefront of such infections [Bigland, 1969; Bradury, 1984]. Concurrent (mycoplasma) infections with NDV or IBV are known to enhance the vulnerability of the host to *E. coli* bacteraemia, resulting in perihepatitis, airsacculitis and pericarditis. It also appears as if previous infection with *M. gallisepticum* or *M. gallisepticum* and NDV or IBV may aid in the bacterial invasion of the air sacs [Bajwa *et al.,* 1996, Bradury, 1984]. Concurrent *E. coli* infections are characterized by aggravated clinical symptoms as well as increased morbidity and mortality, and are often associated with adverse management conditions, especially in broiler houses with a lot of dust in the air [Bradury, 2005; Bigland, 1969; Kleven, 2008; Bajwa *et al.,* 1996]. In addition to *E. coli*, bacteria such as *Pseudomonas aeruginosa*, *Avibacterium paragallinarum*, *Bordetella avium* and *Pasteurella* species have been implicated in mycoplasma-associated clinical conditions in South African feedlot ostriches [Botes *et al.,* 2005].

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### 2.7.9 Factors contributing to avian mycoplasma infections

Stress, adverse weather conditions, lack of biosecurity and poor hygiene are factors which add to the incidence and severity of mycoplasma infections [Pretorius, 2009; Kleven, 1998]. In 1984, Yoder *et al.* [cited by Mohammed *et al.,* 1987] suggested that mycoplasmal disease is augmented by an intricate interaction that takes place between temperature and humidity. This is evident during winter months in the Western Cape, when windy and wet weather amplifies the severity of ostrich mycoplasma infections due to the increased susceptibility of ostriches to secondary infections [Pretorius, 2009; Kleven, 1998]. A higher incidence of ostrich mycoplasma infections is, however, observed during autumn and spring, when fluctuations in temperature occur. Moldy feed, dirty water troughs, change in feed, high population densities, transport of birds, as well as other unhygienic and stress-causing conditions are also thought to contribute to ostrich mycoplasma infections. An increased risk of spread occurs with poor biosecurity programs, for example when birds from various sources are mixed. A lack of ventilation to decrease the amount of dust and ammonia, increased human contacts, the presence of wild birds, the absence of frequent stirring and replacement of manure to reduce moisture content, and the absence of bird-proofing are factors that have been linked to the spread and severity of other avian mycoplasmas [Mohammed *et al.,* 1987; Kleven, 1998].

### 2.7.10 Diagnosis

Confirmation of mycoplasma infections requires laboratory diagnosis, since several different poultry pathogens display similar clinical signs [Kleven, 2008]. Mycoplasmas should, however, be considered in the preliminary diagnosis if there is a drop in egg production or lesions and respiratory symptoms occur. The fact that mycoplasmal infections are frequently associated with additional pathogens should also be kept in mind.

Serological procedures are usually the means by which mycoplasmosis is diagnosed [Kleven, 2008]. The easiest and most commonly utilized test is the serum plate agglutination (SPA) test. An enzyme-linked immunosorbent assay (ELISA) may also be performed and either ELISA or SPA tests of 10-30 serum samples should always be included in early diagnostic efforts. Positive SPA or ELISA results are usually confirmed using the very precise and dependable hemagglutination inhibition (HI) tests. However, as a result of the problems associated with acquiring suitable antigen, HI tests are not accessible in several regions of the world. Nonetheless, the best diagnostic technique for a mycoplasma infection is isolation and identification of the organism [Avakian and Kleven, 1990; Kleven, 2008]. The majority of laboratories around the world, however, lack this capability, and culture techniques are frequently substituted by the polymerase chain reaction (PCR), a technique which is commonly used in many laboratories.

### 2.7.11 Prevention and Control

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The method of control which is generally favored against *M. gallisepticum* is the use of all-in, all-out management techniques, the exclusive use of *M. gallisepticum*-free breeder sources for replacements, and the implementation of strict biosecurity measures to ensure that flocks are kept free of infection [Collier *et al.,* 2006; Kleven, 2008]. Similarly, there is as yet no means with which ostrich mycoplasma infections can be prevented, except for maintaining good biosecurity practices [Olivier, personal communication]. However, ostriches infected with mycoplasmas frequently do not show any symptoms, but may act as carriers of mycoplasmal disease, making disease control difficult. In addition, the ability of these pathogens to conceal themselves from the host's immune system makes mycoplasmas difficult to eradicate.

2.7.11.1 Antibiotics

There is no known course of medication which is able to eradicate *M. gallisepticum* and *M. synoviae* from a flock [Kleven, 2008]. However, antibiotics such as chloramphenicol and related products, tetracyclines, fluoroquinolones, ethromycin, tiamulin, tylosin and its derivatives; and other antibiotics whose actions are not based upon the inhibition of cell wall synthesis, have proven relatively effective, with the most effective results obtained when the medication is applied prophylactically. Similarly, advocin, tylosin, doxycline and oxytetracyclin are typically used to manage ostrich mycoplasma infections, but are not able to eradicate the mycoplasmas [Olivier, personal communication]. In addition, when antibiotics are used for extended periods of time, mycoplasmas tend to become resistant [Kleven, 2008]. The development of an effective vaccine thus appears to be the most promising avenue of control.

2.7.11.2 Vaccines

In literature it is stated that although mycoplasma "vaccines provide some protection against disease, almost universally, they fail to prevent colonization, and disease outbreaks still occur at unacceptable frequencies" [Minion, 2002]. Thus, while there are vaccines available which aid in the reduction of symptoms associated with avian mycoplasma infections, no conventional vaccine has yet been able to completely thwart infection during field challenge [Collier *et al.,* 2006; Kleven, 2008]. There are no vaccines currently available for use against *M. meleagridis* infection and inactivated, oil-emulsion vaccines, which are generally applied intramuscularly or subcutaneously, are thought to be the safest vaccines for use against *M. gallisepticum*. Oil emulsion vaccines are, however, associated with the formation of granulomas and abcesses when applied subcutaneously to ostriches [Pretorius, 2009].

**2.8 DNA Vaccines**

Since its initial description by Edward Jenner more than 200 years ago, vaccination has become one of the most versatile and effective medical tools with which infectious pathogens of humans and animals can be combated [Liu, 2003; Saurez and Shultz-Cherry, 2000; Babiuk, 2002]. One of the greatest triumphs of medical science was the successful elimination of an entire wild-type of disease (smallpox) from the planet, an accomplishment largely attributed to the ability of vaccines to, instead of treating the disease directly,

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(as other therapeutics, for example antibiotics do), teach the body to protect itself against the wild-type pathogen. It is a principle which is eloquently expressed in an old Chinese saying: 'Give a man a fish and you feed him for a day. Teach a man to fish and you feed him for a lifetime'.

Vaccines are usually composed of attenuated, but still infectious microorganisms (live attenuated vaccines) or of whole inactivated microorganisms or purified components thereof (inactivated vaccines) [Gregersen, 2001]. Since live attenuated vaccines imitate natural infection, they are able to stimulate long-lasting immune responses after only a single application, whereas inactivated vaccines typically require booster injections and immunopotentiating adjuvants in order to stimulate sufficient humoral protection.

Despite the various successes accomplished by their use, vaccines against many pathogens throughout the world, for example tuberculosis, malaria and human immunodifficiency virus (HIV), remain unavailable or ineffective [Guranathan *et al*., 2000]. While efficient antibody responses are generated by all currently licensed vaccines, one of the obstacles to successful vaccinations against the abovementioned organisms is that protection is only afforded by a cellular immune response, a response which is only efficiently achieved with the use of live attenuated vaccinations. A major disadvantage of live attenuated vaccines is, however, the existence of a narrow and delicate balance between (over) attenuation and (lack of) efficacy, and between replication competence and lack of pathogenicity [Gregersen, 2001]. Thus, while some live attenuated vaccines may be available, widespread use is barred by practical constraints such as manufacturing and safety concerns [Guranathan *et al.,* 2000]. The observation that a recently developed group of vaccines, referred to as DNA vaccines, are able to induce both humoral and cellular immune responses, without the concern of "reversion back to virulence", has therefore resulted in a lot of excitement within the vaccine development community [Oshop *et al.,* 2002].

**2.8.1 History**

The science behind the use of DNA vaccines, also referred to as third generation vaccines, was initiated more than fifty years ago when both Stasney *et al*. [cited by Abdulhaqq and Weiner, 2008] and Ito [cited by Abdulhaqq and Weiner, 2008] demonstrated in separate studies that when mice were injected with mouse-derived tumor DNA, it not only resulted in the induction of tumors, but also precipitated seroconversion [Gregersen, 2001; Abdulhaqq and Weiner, 2008]. An increase in investigations into the *in vivo* expression of injected plasmid and linear DNA occurred in the 1980s and activity was demonstrated in several animal models. Such studies included a demonstration that growth hormone and insulin genes could result in the manufacture of these hormones in rats and that hepatitis could be induced in chimpanzees through the use of Hepatitis B Virus DNA. Support for the possibility of immune response induction using this *in vivo* delivery platform, came from studies conducted by Seeger *et al*. [cited by Abdulhaqq and Weiner, 2008], who established that Ground Squirrel Hepatitis Virus (GSHV) surface antigen was produced in experimental animals injected intrahepatically with GSHV genomic DNA [Shedlock and Weiner, 2000; Abdulhaqq and Weiner, 2008].

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**Table 2.1** The differences between inactivated, live attenuated and DNA vaccines, as tabulated by Gregersen [2001].

| | Inactivated vaccines | DNA vaccines / Live vaccines |
|---|---|---|
| ANTIGEN/TARGETS | extracellular<br>e.g. pyrogens, allergens, toxins, circulating microorganisms, secreted, in blood, lymph | cytosolic/cellular<br>e.g. virus infected cells, tumor cells, tissue transplants, autoantigen |
| APC PROCESSING | in intracellular vesicles | in proteasomes |
| PRESENTATION | via MHC II | via MHC I |
| REGULATION | Th2 type | Th1 type |
| SPECIFIC CYTOKINES | IL-4 ,5, 10, 13 | IL-2, IFN-γ, TNF-ß |
| DOMINANT IgG | IgG 1 | IgG 2a |
| EFFECTOR MECHANISMS | Neutralization, Complement lysis, Antibody-dependent cell-mediated cytotoxicity | Cytotoxic T-cells (CTL) |

These early studies, while essential in showing that injected DNA could be expressed *in vivo*, frequently depended upon particular DNA preparations to aid transfection [Abdulhaqq and Weiner, 2008]. Examples of such preparations include calcium phosphate precipitation or liposome encapsulation. The first demonstration that transfection of murine muscle cells could occur when a purified DNA plasmid which encoded a reporter gene was injected into the muscle tissue of an animal subject, came from Wolf *et al.* [cited by Oshop *et al.,* 2002] in 1990 [Guranathan *et al.,* 2000; Stevenson and Rosenburg, 2001]. This study provided the basis for the concept that 'naked plasmid DNA' could be used to express proteins which are foreign to the host, as a means of immunization [Oshop *et al.,* 2002; Guranathan *et al.,* 2000]. This was confirmed by Tang *et al.* in 1992 [cited by Shedlock and Weiner, 2000], when they showed that Human Growth Hormone-specific antibodies were produced in mice after 'genetic immunization' with the Human Growth Hormone gene [Saurez and Shultz-Cherry, 2000; Shedlock and Weiner, 2000; Abdulhaqq and Weiner, 2008]. The immunoprotective capabilities of DNA vaccines were illustrated simultaneously in different studies by Fynan *et al.* [cited by Guranathan *et al.,* 2000] and Ulmer *et al.* [cited by Guranathan *et al.,* 2000] one year later, with the use of hemaglutinin and the influenza virus respectively, and in the same year Robinson *et al.* [cited by Guranathan *et al.,* 2000] demonstrated that DNA vaccines could be used to elicit a protective immune response against influenza in chickens.

These initial studies launched the field of DNA vaccines and were followed by several investigations in which different DNA constructs were used in an extensive array of animal disease models [Abdulhaqq and

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Weiner, 2008]. The ability of DNA vaccines to stimulate appropriate immune responses is now being clinically investigated [Stevenson and Rosenburg, 2001; Abdulhaqq and Weiner, 2008].

### 2.8.2 Composition

2.8.2.1 Identification and characterization of the antigenic gene

The identification of an appropriate candidate gene is usually the first step in the development of a DNA vaccine. Proteins associated with the virulence of pathogens as well as structures of a pathogen which interact with the host are usually recognized by the host immune response, and consequently responsible for immune response induction [Heinrich, 1993]. Genes which encode such structures are referred to as antigenic or immunogenic genes.

Several studies have identified ABC transporters as good targets for vaccine development against pathogenic bacteria [Tanabe *et al.,* 2006]. These studies have established the immunogenic character of the OppA protein [Nowalk *et al.,* 2006]. One study in particular, which focused on the production of a subunit vaccine against *Yersinia pestis*, assessed the potential of several ABC transporters in the induction of protective immunity in a mouse model of infection [Tanabe *et al.,* 2006]. This study found that a significant increase in survival rate occurred in mice immunized against the OppA protein, when compared to the controls as well as the other proteins investigated. Immunoglobulin G (IgG) titers measured suggested that antibody to OppA was responsible for the protection afforded.

2.8.2.2 Eukaryotic Expression Vectors

In addition to an immunogenic gene of a pathogen of choice, a DNA vaccine usually comprises a specially modified bacterial plasmid [Donelly *et al.,* 1997; Saurez and Shultz-Cherry, 2000; Guranathan *et al.,* 2000; Oshop *et al.,* 2002]. This plasmid, which is specifically designed for optimal expression in eukaryotic cells, is commonly known as a eukaryotic expression vector. A eukaryotic expression vector, such as can be seen in Figure 2.6, typically comprises *(a)* an origin of replication to allow for the production of high yields of plasmid in *E. coli*, *(b)* a strong eukaryotic promotor to drive the expression of the target gene in eukaryotic cells, *(c)* an antibiotic resistance gene to allow for antibiotic selective growth of *E. coli*, and *(d)* a polyadenylation or termination signal sequence to stabilize mRNA transcripts [Donelly *et al.,* 1997; Saurez and Shultz-Cherry, 2000; Guranathan *et al.,* 2000]. The immune response induced by a DNA vaccine relies to a large extent on the components of the expression vector employed [Guranathan *et al.,* 2000]. It is thus required that the regulatory elements of the plasmid be optimized according to the expressing cell type [Oshop *et al.,* 2002]. An important consideration during the optimization of DNA vaccine efficacy is therefore the choice of a suitable expression vector which allows for optimal expression of the antigenicgene in eukaryotic host cells.

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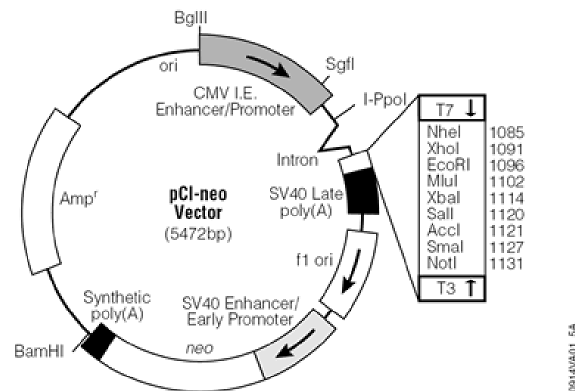


**Figure 2.6** An example of a eukaryotic expression vector (pCIneo, Promega) which may be utilized as part a typical DNA vaccination strategy.

The majority of the promotors utilized in eukaryotic expression vectors are derivatives of the immediate early human cytomegalovirus gene or are derived from other virus genes such as simian virus 40 [Guranathan *et al.,* 2000; Gregersen, 2001]. A number of studies conducted to determine the best promoter for use in plasmids employed during avian DNA vaccinations, have found the cytomegalovirus promoter to be the better choice between the group of promoters evaluated (including the Rous Sarcoma virus, simian virus 40, chicken beta actin and cytomegalovirus) [Oshop *et al.,* 2002]. Some promoters, for example the myocyte-specific desmin promoter, may be also used to limit antigen expression to a particular cell type, thus enabling a cell type of interest to be targeted directly. In addition, bicistronic vectors containing two genes whose transcription is regulated by a single promoter, and alternatively, vectors expressing multiple genes which are each regulated by an individual promotor and termination signal, may also be utilized [Gregersen, 2001].

The polyadenylation termination signals utilized in eukaryotic expression vectors are usually obtained from bovine growth hormone or specific viruses [Gregersen, 2001]. A study evaluating the effect of the termination signal on avian DNA vaccines found no significant difference between the bovine growth hormone signal and a synthetic poyadenylation signal [Oshop *et al.,* 2002]. Also, the single study conducted to investigate the best plasmid conformation for use (super coiled compared to linear) found that the linear plasmid had greater effectiveness. All other avian DNA vaccination studies were, however, conducted using super coiled plasmids and most produced high efficacy. Antibiotics such as neomycin, kanamycin or ampicillin are the most frequently utilized for plasmid selection [Gregersen, 2001]. Good DNA vaccination efficacy in avians may thus be achieved by using one of several common eukaryotic expression vectors containing the cytomegalovirus promoter and bovine growth hormone termination signal [Oshop *et al.,* 2002].

When the immunogenic target gene has been inserted into the eukaryotic expression vector, it is replicated (cloned) in bacteria, purified and delivered to the host using one of several routes.

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### 2.8.3 Administration

No particular formulations are required in order to make eukaryotic expression vectors appropriate for their intended utilization [Gregersen, 2001]. Most experimental DNA vaccines are applied merely as a physiological saline solution, making it possible for any laboratory to conduct investigations into the use of trial DNA vaccine products without the concern of having to prepare acceptable buffers, stabilizers or adjuvants.

DNA vaccines are usually administered in doses of around 100 µg to mice, between 0.25 and 750 µg (depending on the route of administration) to chickens, while doses of more than 1000 µg have been administered to calves and monkeys [Gregersen, 2001; Oshop *et al.,* 2002; Pretorius, 2009]. Suggestions have, however, been made that the amount of DNA required to induce an immune response is more dependent on the route of administration than on the size of the vaccinated animals. It is, however, imperative that vaccination occurs before field challenge.

The possible routes of vaccine delivery in the avian include intramuscular injection, intra-dermal injection, gene gun inoculation (uses plasmid-coated gold beads to force DNA into epidermal cells), orally, intranasally, ocularly, intravenously, topically, intratracheally, intraperitoneally, subcutaneously, intrabursally, epidermally, *in ovo* to the developing embryo and *in utero* [Oshop *et al.,* 2002]. Additional delivery routes which have been studied in other animal models include injections into thyroidal tissues and into lymph follicles [Gregersen, 2001]. Although vaccination by means of the gene gun has been shown to require less DNA to elicit a response in chickens, making it more efficient, this method is not economically feasible [Oshop *et al.,* 2002]. A study by Fynan *et al.* [cited by Oshop *et al.,* 2002], however, evaluated several of the above routes of plasmid delivery in the avian and found that none produced better efficacy than the intramuscular injection. Thus, due to its efficacy and simplicity, DNA vaccines are most frequently delivered to avian species by means of the intramuscular injection [Gregersen, 2001].

Several other routes through which DNA vaccines may be administered to various animal models are currently being investigated [Gregersen, 2001]. Such techniques include aerosol applications, formulation into biodegradable nanoparticles, liposome formulations, inoculations together with cholera toxin, formulation into bioadhesive polymers, and through the use of microbial carriers which cannot replicate, for example specific viruses or enteric bacteria such as *Salmonella typhimurium*, into which plasmids encoding antigen are inserted. Interestingly, Fan *et al.* [cited by Oshop *et al.,* 2002] also illustrated in 1999 that fairly satisfactory humoral and cellular immune responses were generated when purified expression plasmids encoding the hepatitis B surface antigen, applied to the skin in aqueous solution, were taken up by hair follicles. This finding suggests that DNA vaccines may be administered by any means. However, not all modes of administration illustrate the high degree of reliability required for the standardized administration of vaccines, which often occurs under variable circumstances. As yet, the only conclusion

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that can be drawn is that the character and the strength of the immune response generated are influenced by the species studied, the route of administration, the dose and the boosting scheme used.

### 2.8.4 Optimization of DNA vaccines

The identification of an immunogenic plasmid and antigen is rarely effective enough to immediately qualify as a vaccine [Gregersen, 2001]. Modifications of the plasmid framework, the antigen's configuration and/or the antigen's expression characteristics may improve immunity and protection. The antigen may prove more effective with or without its membrane anchor sequence, or when jointly expressed with a specific ligand, and even after optimization, single antigen may not sufficiently stimulate protective responses under practical circumstances, and may require antigen combinations, either in the same plasmid or by mixing different expression plasmids.

Particular nucleotide sequences may be used to influence the immune response induced by a potential DNA vaccine [Guranathan *et al.,* 2000]. Synthetic oligodeoxynucleotides (ODNs) with sequences modeled after those found in bacterial DNA have been found to stimulate the secretion of IFN-γ by natural killer cells. In addition, the unmethylated cytidine-phosphate-guanosine (CpG) dinucleotide motif, a specific motif which is 20 times more common in bacterial than in mammalian DNA, was also found to have a prominent role in enhancing the immune response, following DNA vaccinations. Unmethylated CpG motifs activate the innate immune response by binding to Toll-like receptor 9 (TLR9), triggering a signal transduction pathway which induces the secretion of proinflammatory cytokines, including IL-6, IL-12, IL-18, tumour necrosis factor (TNF)-ά, IFN- ά and IFN-γ, from macrophages and dendritic cells [Guranathan *et al.,* 2000; Kim *et al.,* 2001; Takeshita *et al.,* 2004]. IL-12 and IL-18 subsequently activate natural killer cells. Also, binding of CpG motifs to TRL9 up regulates major histocompatibility complex (MHC) type I, MHC-II, CD80 and CD86 molecule expression and stimulates maturation of dendritic cells. In addition, CpG/TRL9 interaction directly activates proliferation and antibody production by B-lymphocytes, and directly or indirectly activates T-lymphocytes, depending on their baseline activation state.

Genes encoding cytokines and co-stimulatory molecules have also been used successfully to enhance or steer the immune response generated in a particular direction, through the expression of the said genes with a particular antigen [Gregersen, 2001]. An interesting example is the expression of ubiquitin, which targets proteins to the proteasome, thus leading to the rapid degradation of the expressed antigen before its release into the extracellular compartment, and subsequent stimulation of B-lymphocytes. Such investigations found that the antibody response which would otherwise have been generated was diminished or lost, but that cytoxic T-lymphocyte responses were still obtained.

Although DNA vaccines are usually administered free of any enhancers or adjuvants when administered intramuscularly to avians, attempts have been made to enhance the immune response generated with the aid of adjuvants, such as cationic lipids, calcium phosphate, bupivacaine, sucrose, hypertonic solutions,

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polybrene, DEAE dextran and cardiotoxin [Oshop *et al.,* 2002]. Such studies found that the use of cationic lipids proved most successful.

**2.8.5 Immune response generated**

Despite the fact that the amount of antigen produced following DNA vaccination usually ranges from picograms to nanograms, a sustained and broad-based immune response is often generated [Guranathan *et al.,* 2000; Oshop *et al.,* 2002]. The type of antigen presenting cell transfected and the immune enhancing properties of the DNA itself (i.e. CpG motifs) are thought to play a role in the induction of the host immune response.

Although the precise mechanism by which DNA vaccines initiate an immune response has not been fully elucidated, several theories have been suggested as to how this might occur [Oshop *et al.,* 2002]. It has been suggested that myocytes are the cells which are predominantly transfected, even though dentritic cells and to a large extent monocytes, may also be transfected [Oshop *et al.,* 2002; Gregersen, 2001, Abdulhaqq and Weiner, 2008]. The myocytes take up the plasmid DNA, although not very efficiently, to then translate and transcribe the encoded genetic information into a protein [Gregersen, 2001].

It is thought that the antigenic proteins produced by the myocytes are presented to the immune system in their native form and that the process by which the antigen is presented is akin to what occurs during natural infection [Oshop *et al.,* 2002, Abdulhaqq and Weiner, 2008]. Gregersen states that the "newly synthesized protein is processed through the proteasome complex and small peptides of the same are bound to MHC-I molecules" [Gregersen, 2001]. The same author then goes on to explain that the peptide-MHC-I complex would be presented to $CD8^+$ T-lymphocytes resulting in differentiation and priming of peptide-specific $CD8^+$ cytotoxic T-lymphocytes. Any cell bearing the same peptide on its surface would then be targeted for destruction.

There is a general consensus that dentritic cells and other antigen presenting cells are vital to the activation of adaptive immunity and that these cells sooner or later obtain the antigen for presentation to the $CD4^+$ helper T-lymphocytes and $CD8^+$ cytotoxic T-lymphocytes at the tissue-draining lymph nodes [Guranathan *et al.,* 2000; Gregersen, 2001; Abdulhaqq and Weiner, 2008]. It is also thought that dendritic cells may directly prime the cytotoxic T-lymphocytes by expressing the antigen endogenously [Guranathan *et al.,* 2000; Abdulhaqq and Weiner, 2008]. Exogenous acquisition of antigen by the dendritic cells may occur in several ways, and these may include attainment of the antigen through a process referred to as "nibbling" from actively expressing myocytes, or through direct attainment through the phagocytosis of dying or dead cells [Guranathan *et al.,* 2000; Gregersen, 2001; Abdulhaqq and Weiner, 2008]. In addition, dendritic cells may present the attained antigen via the MHC-II pathway, thus stimulating the helper T-lymphocytes which control B-lymphocyte responses. B-lymphocytes will subsequently produce antibody which specifically recognizes the antigen of interest.

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In some studies, the skin has been implicated as an organ which plays a key role in the generation of an immune response subsequent to intradermal vaccination or gene gun inoculations [Guranathan *et al.,* 2000; Gregersen, 2001, Oshop *et al.,* 2002]. These studies have shown that very effective dendritic antigen presenting cells present in the epidermis, referred to as Langerhans cells, as well as dendritic cells present in the dermis of the skin are able to induce MHC-II immune responses resulting in strong helper T-lymphocyte responses and subsequent antibody production by B-lymphocytes [Figure 2.7].

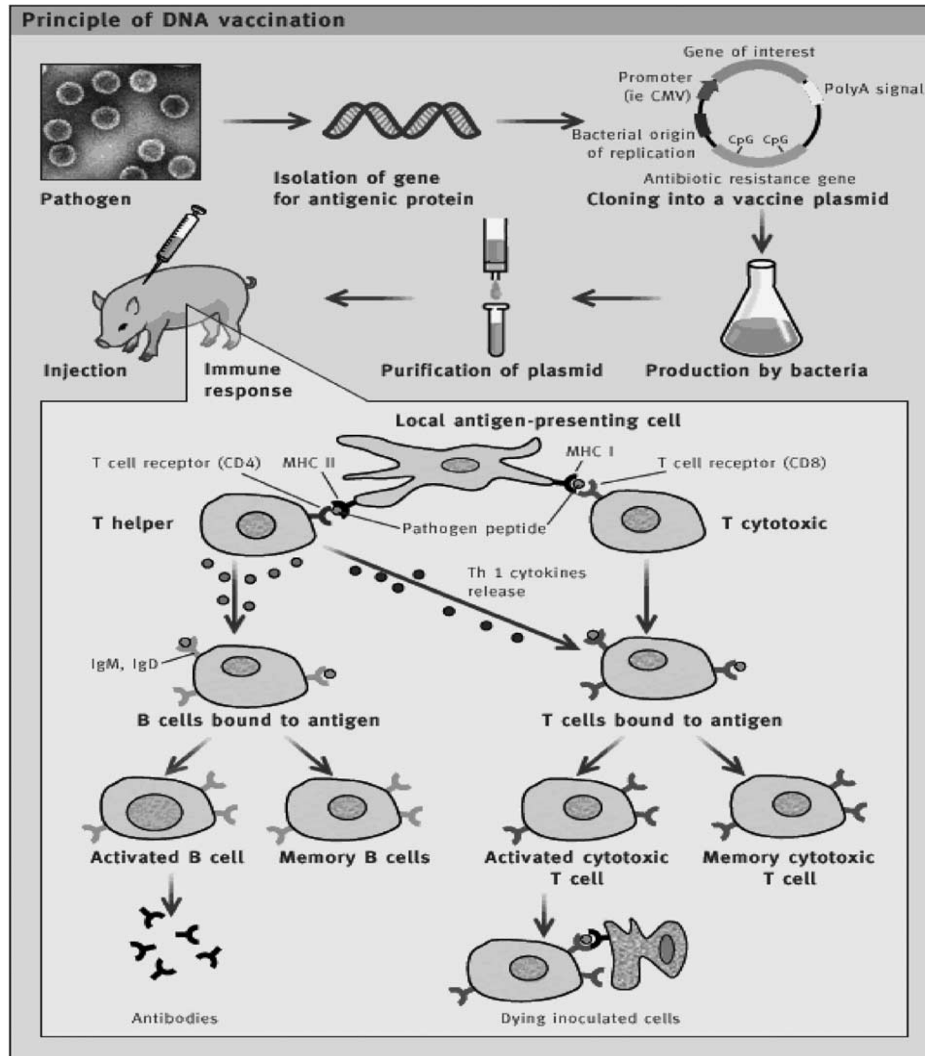


**Figure 2.7** A brief outline of the principal upon which DNA vaccination is based [Oshop *et al.,* 2002].

### 2.8.6 Advantages of DNA vaccines

DNA vaccines boast a number of important advantages when compared to the use of other currently available vaccines [Saurez and Shultz-Cherry, 2000; Oshop *et al.,* 2002]. One of the most important features is the inherent safety associated with the use of DNA vaccines when compared to inactivated viral

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vaccines and live attenuated vaccines, which are used at the constant risk of causing pathogenic infection *in vivo* [Abdulhaqq and Weiner, 2008]. Since DNA vaccines are able to induce both humoral as well as cell mediated immune responses, their use imitates that of live attenuated vaccines, but eliminates the safety concerns which are associated with the use of such vaccines [Saurez and Shultz-Cherry, 2000; Oshop *et al.,* 2002; Guranathan *et al.,* 2000]. A summary of the advantages associated with the use of DNA vaccines is provided in Table 2.2.

The design and production of DNA vaccines is reasonably straightforward and represents another important advantage [Abdulhaqq and Weiner, 2008]. In addition, DNA vaccines are decidedly more appropriate for mass production and circulation in both developed and developing countries, since they are fairly temperature insensitive and remarkably stable, thus eliminating requirement for a cold chain [Guranathan *et al.,* 2000; Abdulhaqq and Weiner, 2008]. The plasmids used for DNA vaccinations are also not immunogenic themselves, thus there is no risk of producing a heterologous immune response to the plasmid of choice, after a boost [Abdulhaqq and Weiner, 2008]. Lastly, several open reading frames from one or more pathogenic genes may be introduced into the expression vector to allow for a better or specifically directed immune response, resulting in nearly infinite possibilities in which genetic inserts and plasmids may be modified.

**Table 2.2** Advantages of using DNA vaccines, as tabulated by Shedlock and Weiner [2000].

| | |
|---|---|
| **Immunogenicity** | • Can induce both humoral and cellular immune responses<br>• Low effective dosages (micrograms) in animal models |
| **Safety** | • Unable to revert to virulence, unlike live vaccines<br>• Efficacy does not require the use of toxic treatments, unlike some killed vaccines |
| **Engineering** | • Plasmid vectors are simple to manipulate and can be tested rapidly<br>• Combination approaches are easily adapted |
| **Manufacture** | • Conceptually low cost and reproducible large-scale production and isolation<br>• Produced at high frequency in bacteria and easily isolated |
| **Stability** | • More temperature-stable than conventional vaccines<br>• Long shelf-life |
| **Mobility** | • Ease of storage and transport<br>• Likely not to require a cold chain |

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# Chapter 3 - Cloning, expression and purification of the *Ms01* OppA protein

## 3.1 Introduction

Ostrich mycoplasma infections cause significant losses to the South African ostrich industry each year. Mycoplasmosis is primarily a disease of the respiratory system, initially infecting the air sacs of the lungs and later spreading to the trachea and upper respiratory passages [Bajwa *et al.,* 1996]. Characterized initially by depression and decreased feed consumption, and later by sneezing, coughing and nasal discharges, the disease often results in decreased feed conversion, decreased viability, downgrading of carcasses and death of ostriches. Mycoplasmas are able to employ the minimal genetic information in their tiny genome to not only execute the essential functions for their replication, but also to damage the host cells in intimate proximity and obtain molecules, such as amino acids and nucleic acid precursors, for their own biosynthesis and survival. The lack of effective antibiotic and vaccine therapies with which to eradicate the mycoplasmas from South African ostriches has prompted a search for new alternatives. To this end, an immunogenic gene, specifically the *oppA* gene, was identified in *Ms01* and isolated, with the aim of developing a DNA vaccine with which to combat ostrich mycoplasma infections.

The oligopeptide transporter system has been identified in a variety of bacteria, and has been shown to be involved in a range of cellular processes, including nutrient accumulation and virulence [Tanabe *et al.,* 2006]. Such studies also identified the OppA lipoprotein as a good candidate for vaccine development. The *oppA* gene, which encodes the OppA lipoprotein, is referred to as the *P100* gene in *M. hominis* and was documented as an important adhesion-related protein present in its cell membrane [Heinrich *et al.,* 1993; Heinrich *et al.,* 1999]. Most mycoplasmas, including *M. hominis*, must adhere to host cells in order to successfully colonize the host and cause infection [Rottem, 2003]. Since lipoproteins are known to play a central role in the interactions between mycoplasmas and eukaryotic cells, these proteins are thought to trigger the pathogenic mechanisms of mycoplasmas [Pilo *et al.,* 2007]. Lipoproteins are also considered valuable targets for sensitive and specific sero-diagnosis, since these proteins are usually strongly antigenic and antibodies can be readily detected in the serum of infected hosts.

In 2009, Pretorius [2009] isolated the DNA of an ostrich-infecting mycoplasma, *Ms01*, after which a major part of the genome was sequenced using GS 120 sequencing. A total of 64 contiguous sequences were generated and from these sequences the *oppA* gene was identified, isolated by PCR from *Ms01* DNA, cloned and modified by site-directed mutagenesis [Pretorius, 2009]. The *Ms01 oppA* gene was subsequently subcloned into three different DNA vaccine vectors and purified. In this study, the three resulting DNA vaccines were amplified and then injected into ostriches as part of a vaccination trial. Serological analysis of the antibody responses generated would, however, require the use of a purified immunogenic protein for use in enzyme-linked immunosorbent assay of antibody levels. To this end, a strategy was followed in which the *oppA* gene was to be subcloned into a prokaryotic expression vector and expressed in *E. coli*.

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Expression of the protein was attempted after cloning of the *oppA* gene into the pET14b prokaryotic expression vector. Additionally, the *oppA* gene was cloned into the pGEX-4T-1 prokaryotic expression vector in an attempt to express the OppA protein as a fusion product with GST in order to enable the purification of this fusion product by means of affinity chromatography.

## 3.2 Materials and Methods

### 3.2.1 Cloning of *oppA* gene into pGEM-T easy vector

3.2.1.1 General Strategy

Primers were designed into which specific endonuclease restriction cut sites were incorporated on the 5' and 3' ends of the PCR amplification product, as can be seen in Figure 3.1. Following PCR amplification of the *Ms01 oppA* gene, adenine overhangs were created on the 3' ends of the *oppA* amplification product by means of polyA-tailing, after which the *oppA* gene was cloned into the pGEM®-T Easy cloning vector.

3.2.1.2 Primer design

Primers for the PCR amplification of the *oppA* gene were designed using Primer Designer (v1.01) based on the complete sequences of the *Ms01 oppA* gene that was previously sequenced by Pretorius (2009). *Nco*I and *Bam*HI endonuclease restriction sites are present in the cloning region of the pET14b and pGEX-4T-1 prokaryotic expression vectors, respectively, but not within the gene sequence of the *oppA* gene as modified by site-directed mutagenesis by Pretorius [8]. The *Nco*I and *Bam*HI endonuclease restriction sites were thus added to the 5' ends of the respective primers for later insertion of the modified *oppA* gene into the pET14b and pGEX-4T-1 vectors. The primers, which are shown in Table 3.1, were synthesized by Integrated DNA Technologies (IDT), Cape Town, South Africa.

3.2.1.3 Polymerase Chain Reaction

A pGEM®-T easy vector containing the modified (by prior site-directed mutagenesis) *Ms01 oppA* gene was provided by Benita Pretorius, Department of Biochemistry, Stellenbosch University. The *oppA* gene was amplified, using the P100-AmpF and P100-AmpR primers (Table 3.1), in 50 µl volumes containing 10 µl of 5x High Fidelity reaction buffer (Kapa Biosystems, Cape Town, South Africa), 1 µl of 1 U/µl HiFi proofreading *Taq* DNA polymerase (Kapa Biosystems), 1.5 µl of 10 mM deoxynucleotide triphosphates (dNTPs) (Kapa Biosystems), 0.5 µl of 25 mM $MgCl_2$ (Kapa Biosystems), 1.5 µl of each primer (20 µM), 250 ng of purified DNA and 29 µl of Milli-Q® deionized water. Amplifications were performed using a Labnet Thermal Cycler, with an initial denaturation step at 95°C for 2 minutes, followed by 25 cycles of denaturation at 98°C for 20 seconds, annealing at 50°C for 15 seconds and extension at 68°C for 90 seconds. A final extension step was performed at 68°C for 5 minutes.

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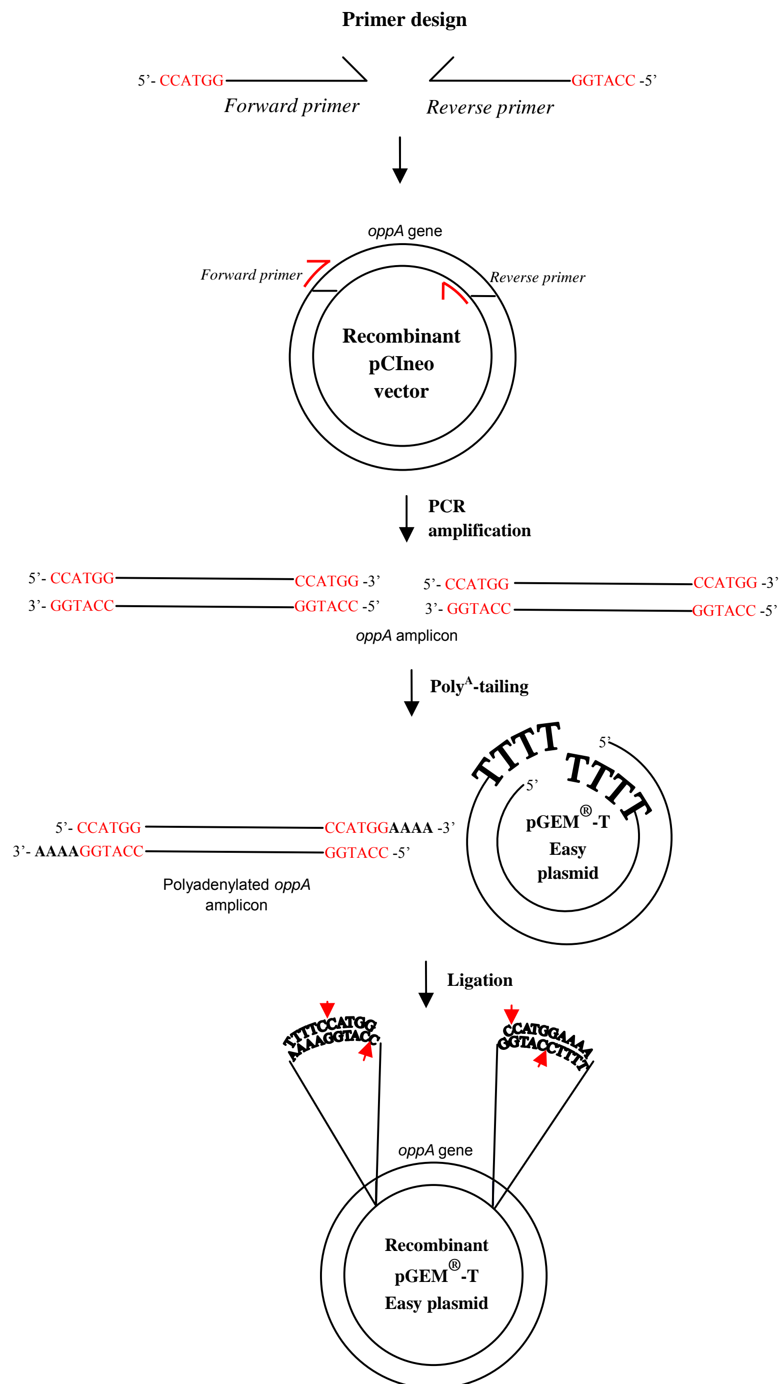


**Figure 3.1** A diagrammatic illustration of how the *Ms01 oppA* gene was cloned into the pGEM®-T Easy vector. Primers were designed with *NcoI* restriction endonuclease cut sites added to the 5' ends of the forward and reverse primer. These primers were used to amplify the *Ms01 oppA* gene present in the pCIneo eukaryotic expression vector, so as to obtain linear *Ms01 oppA* amplification product with *NcoI* restriction endonuclease cut sites on the 5' and 3' ends. Adenine overhangs were created on the 3' ends of the *oppA* amplification product by means of poly$^{A}$-tailing. The bonds formed between the poly-adenine overhangs of the *oppA* amplification product and the poly-thymine overhangs of the

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pGEM®-T Easy plasmid could then be utilized to facilitate cloning of the *oppA* gene into the pGEM®-T Easy plasmid. Restriction endonuclease cut sites are indicated with red arrows.

3.2.1.4 Agarose gel electrophoresis

In order to determine whether the PCR yielded the desired DNA product, the amplicon was analyzed on a 1% (w/v) agarose gel (Molecular Grade Agarose D1-LE, Whitehead Scientific, Cape Town, South Africa) in 1x TAE buffer (0.04 M Tris acetate, 0.002 M EDTA, pH 8), to which 0.175 µg/ml of ethidium bromide was added for visualization of the DNA product under ultraviolet light. Ten microliters of PCR product, mixed with 2 µl of loading buffer [50% (v/v) glycerol, 50 mM EDTA, 100 mM Tris-base, 0.1% (v/v) bromophenol blue] was loaded onto the gel and run at 100 V for 50 minutes. A DNA Ladder (Fermentas, Inqaba Biotech, Pretoria, South Africa) was used to determine the approximate size and concentration of the amplicon.

3.2.1.5 Purification of PCR product

The PCR product was purified and concentrated using a GSF™ PCR DNA and Gel Purification Kit (GE Healthcare, Cape Town, South Africa) according to the manufacturer's instructions. Agarose gel electrophoresis was subsequently performed as previously described (section 3.2.1.4), using 2 µl of purified PCR product, in order to confirm that enough PCR product was available for further use. The concentration of the purified PCR product was determined using a NanoDrop ND-2000 Spectrophotometer.

3.2.1.6 Polyadenylation of the *Ms01 oppA* gene

The amplification product generated during the PCR was used as template for polyadenine-tailing ($poly^A$ -tailing). A reaction containing 1 µl of 10x reaction buffer (Super-Therm, JMR Holdings, USA), 6 µl of purified PCR product, 1 µl of 2 mM dATP (Bioline), 0.8 µl of 25 mM $MgCl_2$ (Super-Therm), 1 µl of 5 U/µl *Taq* DNA polymerase (Super-Therm) and 0.2 µl of Milli-Q® water, was incubated at 70°C for 25 minutes. The PCR product was subsequently purified and concentrated using the DNA Clean and Concentrator™ -5 Kit (ZYMO Research, USA) as previously described.

3.2.1.7 Cloning of the *Ms01 oppA* gene into the pGEM®-T easy vector

The polyadenylated PCR product was cloned into the pGEM®-T Easy cloning vector (Promega, USA) using the pGEM® -T Easy Vector Kit, in a reaction mixture containing 5 µl of 2x Ligase buffer, 2 µl of polyadenylated PCR product, 50 ng of pGEM®-T Easy vector, 1 µl of 3 U/ µl T4 DNA Ligase, and Milli-Q® water for a final volume of 10 µl. The reaction mixture was incubated at 24°C for 1 hour. Ligation reaction controls consisted of (a) a background reaction mixture containing no insert and (b) a positive control reaction containing control insert provided with the kit.

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3.2.1.8 Transformation of *E. coli* JM109 cells with recombinant pGEM®-T Easy plasmids

Agar plates containing Luria-Bertani (LB) medium were prepared according to the blue/white screening protocol of the pGEM®-T Easy Vector System (Promega). Plates were sterilized under ultraviolet (UV) light for 20 minutes. Thirty milliliters of sterilized LB/agar medium (15 g agar/ml LB medium), to which 48 µl of 0.1 M IPTG (Bioline), 24 µl of 50 mg/ml X-Gal (Bioline) and 15 µl of 100 mg/ml ampicillin (Sigma) was added, was subsequently poured into plates for setting. Two microliters of ligation reaction was added to 50 µl of competent *E. coli* JM109 cells (1 x $10^7$ cfu/µg DNA) (Promega) and incubated on ice for 20 minutes. The cell suspension was then heat shocked in a water bath at 42°C for 45 seconds and again incubated on ice for 2 minutes. The addition of 950 µl of LB medium to the cell suspension followed, as well as 60 minutes of incubation at 37°C on an IKA® KS 260 Basic orbital shaker (150 rpm) to allow for growth of the transformed cells. The cells were plated onto the LB/agar plates and subsequently incubated at 37°C for 16 hours. The plates could then be inspected for the development of colonies.

3.2.1.9 Diagnostic PCR

Successfully transformed colonies were identified with the aid of blue/white screening (as described in the Promega pGEM® -T Easy Vector System protocol), and subjected to a diagnostic PCR in order to identify the colonies containing the recombinant pGEM® -T Easy vector-insert construct. The T7 promotor (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAGAA-3') primers were utilized in 10 µl reactions, containing 1 µl of 10x Reaction buffer (Super-Therm), 0.1 µl of 5 U/µl *Taq* DNA polymerase (Super-Therm), 0.4 µl of 5 mM dNTPs (Bioline), 0.5 µl of each primer (20 µM), 0.6 µl of 25 mM $MgCl_2$ (Super-Therm) and 6.9 µl of Milli-Q® deionized water. A sterilized toothpick was used to scrape up cells from a colony of interest, after which the tip of the toothpick was placed into the PCR mixture and flicked 5 times to release some of the cells from the toothpick. The DNA contained within the cells of the chosen colony thus served as template DNA for the PCR. Amplification was performed with 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Initial denaturation occurred at 94°C for 5 minutes and final extension at 72°C for 7 minutes. Resulting PCR products were visualized using agarose gel electrophoresis, as described previously. A negative control reaction during which a blue colony was utilized as template DNA, as well as a positive control reaction during which previously purified plasmid known to contain insert of 700 bp was utilized as DNA template, was also performed.

3.2.1.10 Isolation of the pGEM® -T Easy vector-insert construct

Five milliliters of LB medium containing 5 µl of 100 mg/ml ampicillin (Sigma) was inoculated, using a sterilized toothpick, with a colony expected to contain the recombinant pGEM®-T Easy vector-insert construct. The inoculated medium was incubated for 16 hours at 37°C, on an IKA® KS 260 Basic orbital shaker, at 200 RPM, to allow the cells to grow. Plasmix Miniprep Kit (Talent, Italy) was used to isolate the pGEM®-T Easy vector-insert construct from the cultured cells according to the manufacturer's instructions. The presence of the plasmid was confirmed using agarose gel electrophoresis, performed as previously

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described. The successfully isolated recombinant pGEM®-T Easy plasmid containing the *Ms01 oppA* gene was then utilized as template for the subsequent DNA sequencing reaction.

3.2.1.11 Sequencing of the pGEM®-T Easy plasmid insert

In order to confirm that the *oppA* gene had been successfully inserted into the pGEM-T Easy plasmid, sequencing of the *oppA* gene insert was performed by means of cycle sequencing, with the aid of eight primers, each of which generated overlapping sequences in order to sequence the complete 2829 bp insert as illustrated in Figure 3.2. The internal primers utilized (P2F, P3F, P4F, P1R, P2R and P3R) are shown in Table 3.1. The T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAGAA-3') primers were used as external primers. Sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and was composed of 1 µl of Terminator mix, 3 µl of Half-dye buffer (Bioline), 300 ng of the isolated recombinant pGEM®-T Easy plasmid (refer to previous paragraph) and 3 µl of 3.3 µM of the respective primer solutions. Each of the eight individual reactions (each containing a different primer) was performed in 35 cycles of 96°C for 10 seconds, 52°C for 30 seconds and 60°for 4 minutes, with a final extension of 60°C for 10 minutes, in a Labnet ThermoCycler. Sequencing reaction products were then analyzed, using an ABI PRISM® 373 DNA Sequencer, by the Core DNA Cycle Sequencing Facility at Stellenbosch University. The DNA sequences obtained were subsequently aligned using Chromas v1.45 (Technelysium Pty) and BioEdit (v 7.0.5.2, Hall) and inspected for mutations**.** Only when an *Ms01 oppA* sequence, free of all mutations except those induced by site-directed mutagenesis, was obtained, could the next step be continued with (Addendum A).

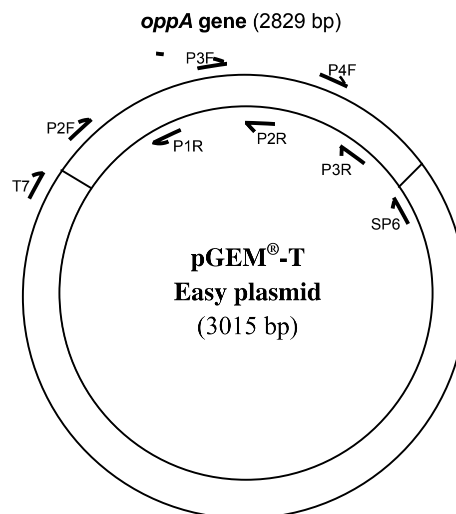


**Figure 3.2** A schematic illustration of the *oppA* gene (2829 bp) cloned into the pGEM®-T Easy plasmid (3015 bp) to indicate the positions of the primers in the recombinant vector for subsequent sequencing of the *oppA* gene.

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**Table 3.1** Primers utilized during PCR and cycle sequencing reactions. *NcoI* and *BamHI* enodonuclease recognition sites, for the cloning of the *Ms01 oppA* gene into the pET14b and pGEX-4T-1 prokaryotic expression vectors, respectivlely, are underlined and the cut sites indicated with arrows. The initiation and termination codons of the *Ms01 oppA* gene are indicated in bold.

| Primer name | Primer sequence (5'-3') | Restriction enzyme |
|---|---|---|
| **P100-pET-AmpF** | 5' – C↑CATGGTT**ATG**AAAAAAAGCGCAAGACT – 3' | *NcoI* |
| **P100-pET-AmpR** | 5' – C↑CATGG**TTA**TTTATTGATTCTCCAATCA – 3' | *NcoI* |
| **P100-pGEX-AmpF** | 5' – G↑GATCC**ATG**AAAAAAAGCGCAAGACT – 3' | *BamHI* |
| **P100-pGEX-AmpR** | 5' – G↑GATCC**TTA**TTTATTGATTCTCCAATCA – 3' | *BamHI* |
| **T7** | 5' – TAATACGACTCACTATAGGG – 3' | None |
| **T7 terminator** | 5' – GCTAGTTATTGCTCAGCGG – 3' | None |
| **SP6** | 5' – ATTTAGGTGACACTATAGAA – 3' | None |
| **pGEX-F** | 5' – GGGCTGGCAAGCCACGTTTGGTG – 3' | None |
| **pGEX-R** | 5' – CCGGGAGCTGCATGTGTCAGAGG – 3' | None |
| **P2F** | 5' – CTAAAGATTTCTATTATTCATGGCTAAGAACAAATCAAACAATTG – 3' | None |
| **P1R** | 5' – CTTCACCTTTTGAATTTACCCATTTTAAATTGTCTTTAAG – 3' | None |
| **P3F** | 5' – GCTAAATTAGGTGTTTACTGGTATGGTGTAACAGCAAATAC – 3' | None |
| **P2R** | 5' – CAATTGTTTGATTTGTTCTTAGCCATGAATAATAGAAATCTTTAG – 3' | None |
| **P4F** | 5' – CTTTATTACAAGCTGCAATTAACTGGAATACAGTAGCAGATGTAAGAAC – 3' | None |
| **P3R** | 5' – CTATTTGCTGTTACACCATACCAGTAAACACCTAATTTAGC – 3' | None |

### 3.2.2 Cloning of the *Ms01 oppA* gene into the pET14b and pGEX-4T-1 prokaryotic expression vectors

Restriction endonucleases recognizing the endonuclease cut sites flanking the *Ms01 oppA* gene in the recombinant pGEM® -T Easy vector were used to cleave the gene from the pGEM®-T Easy vector-insert construct. The resultant sticky-ended *oppA* gene could then be cloned into the pET14b and pGEX-4T-1 prokaryotic expression vectors as shown in Figure 3.3.

The pGEM®-T Easy vector-insert construct, the pET14b vector (Novagen, USA) and the pGEX-4T-1 vector (GE Healthcare) was each digested using *NcoI* (in case of pET14b) and *BamHI* (in case of pGEX-4T-1) restriction endonuclease in a reaction containing 2 µl of 10x reaction buffer (Promega), 0.5 µl of 10

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mg/ml

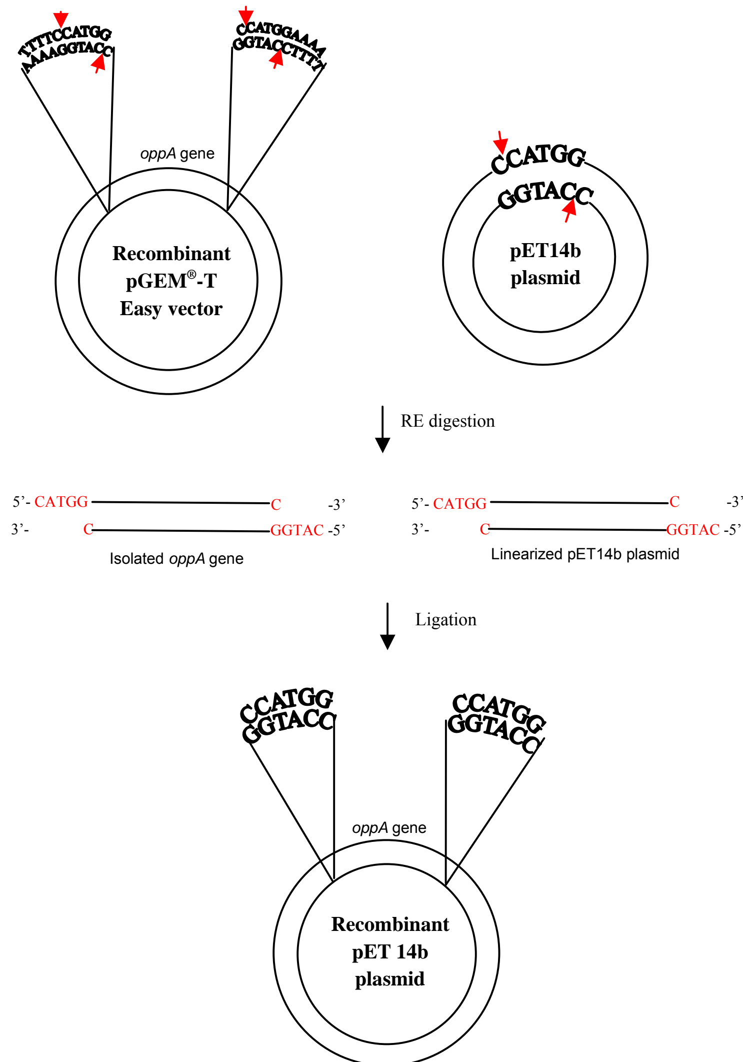


**Figure 3.3** A diagrammatic illustration of how the *Ms01 oppA* gene was cloned into the pET14b vector. *NcoI* restriction endonucleases were used to remove the *oppA* gene from the recombinant pGEM®-T Easy plasmid, as well as to digest and linearize the pET14b plasmid. The *NcoI* restriction endonuclease cut sites of the *oppA* gene and pET14b plasmid could then be bound together in a ligation reaction in order to produce the recombinant pET14b prokaryotic expression vector. Restriction endonuclease cut sites are indicated with red arrows.

acetylated BSA, 2 mg of vector DNA, 2 µl of 10 U/µl *NcoI* (Promega) and Milli-Q® deionized water to a final volume of 20 µl. The reactions were incubated at 37°C for 2 hours. Restriction endonucleases and other reactants were removed from the digested DNA products using the DNA Clean and Concentrator ™-5 Kit (ZYMO Research) according to the manufacturer's instructions. In order to prevent the digested ends of linearized pET14b and pGEX-4T-1 plasmids from re-attaching, a phosphate group was removed from the 5' and 3' ends of the vectors. This was executed in a reaction mix containing 1.5 µl of 1 U/µl Shrimp

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alkaline phoshatase (SAP) (Promega), 0.5 µl of 10x SAP buffer (Promega) and 1.5 mg of digested vector. The reaction was incubated for 15 minutes at 37°C and inactivated for 15 minutes at 65°C. The dephosphorylated vectors were then purified and concentrated using the DNA Clean and Concentrator ™-5 Kit (ZYMO Research) as previously described. The *oppA* gene was subsequently cloned into the pET14b and pGEX-4T-1 expression vectors in a reaction containing 1 µl of 10x Ligase buffer (Promega), 0.35 µl of 3 U/µl T4 DNA Ligase (Promega), 100 ng of linearised vector DNA and 181.7 ng of *oppA* insert DNA. The ligation reactions were incubated for 16 hours at 4°C. Various insert to vector ratios were experimented with, including 5:1, 3:1, 1:1, 1:3 and 1:5 ratios. The equation illustrated below was used to calculate the concentration of DNA required to obtain these ratios. Negative controls consisted of reactions to which no insert was added. The transformation reaction was then performed as previously described.

$$\frac{vector(ng) \times insert(kb)}{vector(kb)} \times \frac{3}{1}(ratio) = insert(ng)$$

3.2.2.1 Diagnostic PCR

In order to determine which colonies contained the recombinant pET14b and pGEX-4T-1 vector-insert constructs, a diagnostic PCR was carried out as previously described using the T7 promotor (5'-TAATACGACTCACTATAGGG-3') and T7 terminator (5-GCTAGTTATTGCTCAGCGG-3') primers. Results were visualized using agarose gel electrophoresis, also described previously.

3.2.2.2 Sequencing of the *oppA* gene in pET14b and pGEX-4T-1 vector-insert construct

Colonies containing the recombinant pET14b and pGEX-4T-1 constructs were cultured in liquid medium, isolated using the Plasmix Miniprep Plasmid Isolation Kit (Invisorb, Invitek, Germany), and sequenced, as previously described, using the T7 promotor (5'-TAATACGACTCACTATAGGG-3') and T7 terminator (5-GCTAGTTATTGCTCAGCGG-3') external primers and the P2F, P1R, P3F, P2R, P4F and P3R primers as internal primers**.** Once an *Ms01 oppA* gene sequence in the correct orientation with regard to the transcription promoter and free of any mutations (except those induced by site-directed mutagenesis) was obtained, expression of the *oppA* gene could be attempted.

**3.2.3 Expression of the recombinant *Ms01* OppA protein in *E. coli* BL21(DE3) pLysS cells using the pET14b and pGEX-4T-1 prokaryotic expression systems**

The recombinant pET14b and pGEX-4T-1 constructs (containing the *Ms01 oppA* gene) were cloned into competent BL21(DE3) pLysS cells (Novagen) using the transformation reaction described previously (PGEM®-T Easy transformation protocol). A diagnostic PCR was subsequently performed to confirm the success of the transformation reaction, and the results visualized using agarose gel electrophoresis as previously described. Two starter cultures, consisting of 5 ml of LB medium containing 250 µl of 20%

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(m/v) glucose (Saarchem), 5 µl of 34 mg/ml chloramphenicol (Sigma-Aldrich) and 2.5 µl of 100 mg/ml ampicillin (Sigma), were respectively inoculated, using a sterile toothpick, with a colony of BL21(DE3) pLysS cells (Novagen) containing the recombinant pET14b plasmid, and a colony of BL21(DE3) pLysS cells (Novagen) containing the recombinant pGEX-4T-1 plasmid. The starter cultures were then incubated at 37°C for 16 hours on an IKA® KS 260 Basic orbital shaker at 200 RPM. The starter cultures were subsequently diluted fifty times in 10 ml of tryptone broth (TB) medium containing 1 ml of phosphate buffer (Merck), 0.5 ml of 20% (m/v) glucose (Saarchem), 10 µl of 34 mg/ml chloramphenicol (Sigma-Aldrich) and 5 µl of 100 mg/ml ampicillin (Sigma) and allowed to continue growing at 37°C, under agitation, until an optical density ($OD_{600}$) of 0.6 was reached. Induction was attempted using varying amounts of IPTG, ranging from 0.1 mM to 1 mM, and incubated at temperatures ranging between 20°C and 37°C for up to 24 hours under agitation, with 500 µl samples being taken every hour. Controls consisted of BL21(DE3) pLysS cells containing (a) induced vector with no insert, (b) uninduced vector with the *oppA* gene insert, and in the case of expression with the pGEX system, (c) induced vector, not containing any insert. Following expression, samples were centrifuged (Beckman Model J2-21 Centrifuge, JA-20 rotor) at 10 000 x g for 5 minutes, resuspended in TEN50 buffer (20 mM Tris, 1 mM EDTA, 0.2 mM DTT, 0.1% Triton X-100, 50 mM NaCl and 10% glycerol, pH 8) and stored at -80°C.

3.2.3.1 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

In order to determine whether the OppA protein was successfully expressed, reducing SDS-PAGE was performed. Cell cultures were subjected to 3 cycles of freezing at -80°C for 10 minutes and thawing at 37°C for 15 minutes in order to lyse individual cells and release the intracellular product into the medium. This was followed by five cycles of 3 second sonication and 20 seconds cooling of cells on ice. Samples containing 20 µl of cell lysis product, 12 µl of bromophenol blue (0.1% m/v) and 20 ml of loading buffer (0.125 M Tris-Cl, 4% m/v sodium dodecyl sulfate (SDS), 20% v/v glycerol, 10% v/v 2-mercaptoethanol, pH 6.8) were subsequently boiled for 3 minutes and immediately placed on ice. The samples were then loaded onto a reducing SDS gel composed of 4.5% stacking and 12% resolving gel, and electrophoresed at 250 V, 20 mA, 100 W. Polyacrylamide gels were stained with staining buffer (0.125% Coomassie Briliant Blue R250, 50% v/v methanol, 10% v/v acetic acid) at room temperature on a rotary table (20 rpm) for 16 hours. This was followed by 16 hours of destaining with Destain I solution (50% v/v methanol, 10% v/v acetic acid) at room temperature on a rotary table (20 rpm) for and 16 hours of further destaining under the above conditions, using Destain II solution (5% v/v methanol, 7% v/v acetic acid).

3.2.3.2 Western Blot

Prior to the western blot all samples were subjected to 12% reducing SDS-PAGE, as described before. The separated protein was then transferred to a 0.45 µm nitrocellulose membrane (Schleicher and Schuel) by means of 16 hours of electrophoresis (120 mA 100 W, 250 V) in electrophoresis buffer (0.05 M Tris, 0.2 M glycine and 20% v/v methanol, pH 8.3). Following protein transfer, the nitrocellulose membrane was

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blocked with Casein buffer (154 mM NaCl, 0.5% w/v casein, 10 mM Tris HCl, 0.02% thiomersal, pH 7.6) for one hour at 37°C under agitation. Goat anti-GST antibody (GE Healthcare) was subsequently diluted 20 000 times with Casein-Tween buffer (154 mM NaCl, 0.5% w/v casein, 10 mM Tris HCl, 0.05% v/v Tween 20, 0.02% thiomersal, pH 7.6) and incubated with the nitrocellulose membrane for one hour at 37°C under agitation. The nitrocellulose membrane was then washed three times by means of incubation with PBS-Tween (1.4 mM NaCl, 1% v/v Tween 20, pH 7.2) for ten minutes at 37°C under agitation. Rabbit anti-goat IgG peroxidase conjugate (Sigma-Aldrich) diluted 5 000 times with Casein-Tween buffer (154 mM NaCl, 0.5% w/v casein, 10 mM Tris HCl, 0.05% v/v Tween 20, 0.02% thiomersal, pH 7.6) was subsequently added to and incubated with the nitrocellulose membrane for one hour at 37°C under agitation. This was followed by another washing step as previously described. Finally, substrate solution consisting of citrate buffer (0.1 M, pH 5), 0.05% w/v 2,2'-azino-bis(3ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 0.05% v/v $H_2O_2$ was prepared and added to the nitrocellulose membrane. The substrate reaction, visualized with the appearance of green protein bands on the nitrocellulose membrane, was terminated after 30 minutes of incubation at room temperature by washing the membrane with distilled water.

### 3.2.4 Protein Purification

3.2.4.1 Ammonium sulphate precipitation

An attempt was made to precipitate the recombinant OppA protein from the protein expression product of the recombinant pGEX-4T-1 vector containing the *oppA* gene. Therefore, 3 ml of phosphate buffered saline (PBS, pH 7.2) and subsequently 4.5 ml of saturated ammonium sulphate was added to 1.5 ml of lysed cell extract in a JA-20 centrifuge tube. Following incubation for 20 minutes at 4°C, the mixture was centrifuged (Beckman Model J2-21 Centrifuge, JA-20 rotor) at 4°C for 20 minutes at 27 216 × g. The supernatant was then decanted and the pellet redissolved in 3 ml of PBS. This was followed by the addition of a further 3 ml of saturated ammonium sulphate and incubation at 4°C for 20 minutes. Following centrifugation at 4°C for 20 minutes at 27 216 × g, the pellet was redissolved in 1.5 ml of PBS and dialyzed overnight against carbonate buffer (0.1 M, pH 8.3) at 4°C. The carbonate buffer used for dialysis was replaced with fresh carbonate buffer 8 hours after the beginning of dialysis. The presence and size of the isolated protein was subsequently determined by SDS-PAGE, and the protein concentration was determined by measuring the absorbance at 280 nm using a Nanodrop ND-2000 Spectrophotometer.

3.2.4.2 Glutathione affinity chromatography

An attempt was made to purify the OppA protein from the protein expression product of the recombinant pGEX-4T-1 vector containing the *oppA* gene by means of affinity purification. Affinity purification was initiated by the swelling, washing and equilibration of the Glutathione-Agarose resin (Sigma-Aldrich), according to the manufacturer's instructions. Following cell lysis, as described above, the soluble and insoluble fractions of the cell lysate were separated by centrifugation at 27 216 × g for 20 minutes. The soluble fraction of the cell lysate was subsequently pushed through a 0.45 μm Whatman® filter, as

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recommended by the Sigma-Aldrich and GE Healthcare GST column purification protocols, to reduce the viscosity of the cell lysate and prevent the clogging of the purification column. Five milliliters of cell lysate was loaded onto the column and incubated overnight at 4°C on a rotary table (10 RPM). All subsequent steps were also performed at 4°C. A total of 5 ml of elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl) was applied to the column, after which the column was closed and left to incubate for 30 minutes. Multiple fractions were subsequently collected in 1 ml volumes, followed by the washing and storing of the column as recommended in the Sigma-Aldrich Glutathione-Agarose column purification protocol. Collected fractions were stored at 4°C and their protein content subsequently analyzed by means of SDS-PAGE (as previously described).

## 3.3 Results

### 3.3.1 Primer design, PCR amplification and agarose gel electrophoresis of the *oppA* gene

The *oppA* gene was successfully amplified from the recombinant pCIneo vector by means of PCR, using the P100-AmpF and P100-AmpR primer pair, as shown by the presence of an amplicon of just under 3000 bp (*oppA* gene of 2829 bp + 2 × restriction endonuclease cut sites of 6 bp each = 2841 bp DNA product) shown in Figure 3.5. An amplification product was obtained at all three annealing temperatures tested (50°C, 55°C and 60°C). It was thus decided that an annealing temperature of 50°C was most appropriate for amplifications in which the P100-AmpF and P100-AmpR primer pair were utilized. The amplicon was subsequently successfully purified as can be seen in Figure 3.6.

### 3.3.2 Cloning the *Ms01 oppA* gene into the pGEM®-T easy vector

The *Ms01 oppA* gene was successfully cloned into the pGEM®-T Easy plasmid, with transformation into *E. coli* JM109 cells yielding several white colonies amongst much fewer blue colonies. A diagnostic PCR performed on white colonies, using the T7 and SP6 primer pair, confirmed the success of the transformation reaction, with several colonies displaying the presence of insert, about 3000 bp in size (*oppA* gene of 2829 bp + 2 × restriction endonuclease cut sites of 6 bp each + 128 plasmid DNA = 2969 bp DNA product), in the multiple cloning site of the pGEM®-T Easy plasmid as shown in Figure 3.7.

Isolation of the recombinant pGEM®-T Easy plasmid yielded the expected DNA product of just under 6000 bp (2829 bp *oppA* gene + 3015 bp pGEM®-T Easy plasmid + 2 × restriction endonuclease cut sites = 5856 bp recombinant pGEM®-T Easy plasmid) in size (separation on an agarose electrophoresis gel not shown). After analyzing 5 colonies containing the recombinant pGEM®-T Easy plasmid, a DNA sequence of the modified (by site-directed mutagenesis) *Ms01 oppA* gene, free of any mutations and flanked by the correct restriction endonuclease cut sites, was obtained.

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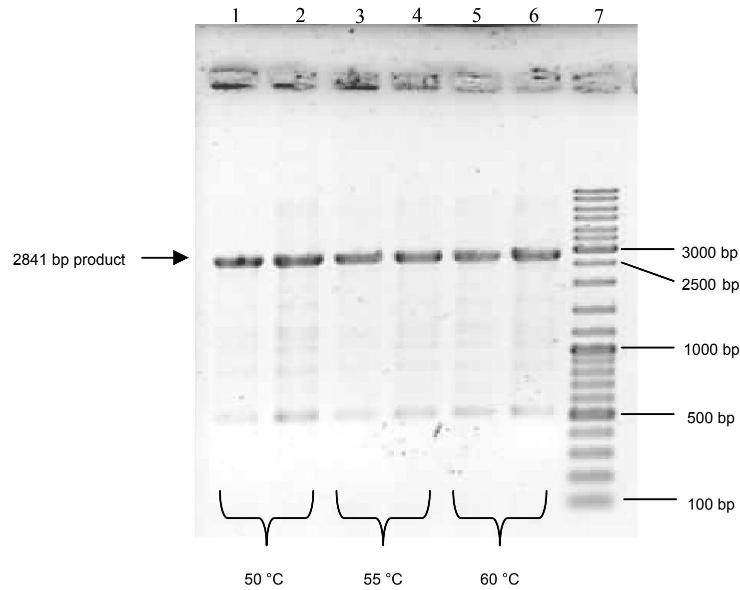


**Figure 3.5** PCR products obtained using the P100-AmpF and P100-AmpR primer pair. Amplification products were electrophoresed on a 1 % agarose gel, to which 0. 175 µg/ml ethidium bromide was added for visualization of the PCR product under UV light. Reactions were performed and analyzed in duplicate, with an annealing temperature gradient beginning at 50 °C and ending at 60 °C. Lane 7: 5 µl DNA ladder mix (Fermentans)

### 2.3.3 Cloning of the *Ms01 oppA* gene into the pET14b and pGEX -4T-1 vectors

Restriction enzyme digestion of the pET14b, pGEX -4T-1 and recombinant pGEM®-T Easy plasmids was performed successfully. Complete digestion of the restriction endonuclease recognition sites in the recombinant pGEM-T Easy vector was obtained after one hour of incubation with restriction endonuclease, as deduced from the single solid band running slightly above the band of the undigested control sample, as shown in Figure 3.8. The ligation efficiency of the ligation reaction was initially evaluated through agarose gel electrophoresis of the ligation reaction product. Agarose gel electrophoresis of the ligation reaction product yielded two bands, corresponding to the size of the *Ms01 oppA* gene (2829 bp) and the expected size of the recombinant pET14b / pGEX-4T-1 vector (2829 bp *oppA* gene + 1 × restriction endonuclease cut site + 4671 bp pET14b / 4969 bp pGEX-4T-1 vectors = 7506 bp recombinant pET14b / 7804 bp recombinant pGEX-4T-1 plasmid ), leading to the deduction that although the concentration of plasmid was limiting, ligation had occurred efficiently, and that the transformation reaction could be proceeded with.

Following numerous (more than 20) unsuccessful attempts and optimization, the *Ms01 oppA* gene was successfully cloned into the prokaryotic expression vectors (initially pET14b and later pGEX-4T-1), with several white colonies obtained on the LG/agar plates. The largest number of colonies were obtained on plates transformed using a 3:1 ligation reaction when the recombinant pET14b vector was utilized, and a 5:1 ligation reaction when the recombinant pGEX-4T-1 vector was utilized.

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**Figure 3.6** Purified PCR products obtained using the P100-AmpF and P100-AmpR primer pair. Purification was performed using a GSF™ PCR DNA and Gel Purification Kit. The purified amplification products were electrophoresed on a 1% agarose gel, to which 0. 175 µg/ml ethidium bromide was added for visualization of the DNA under UV light.

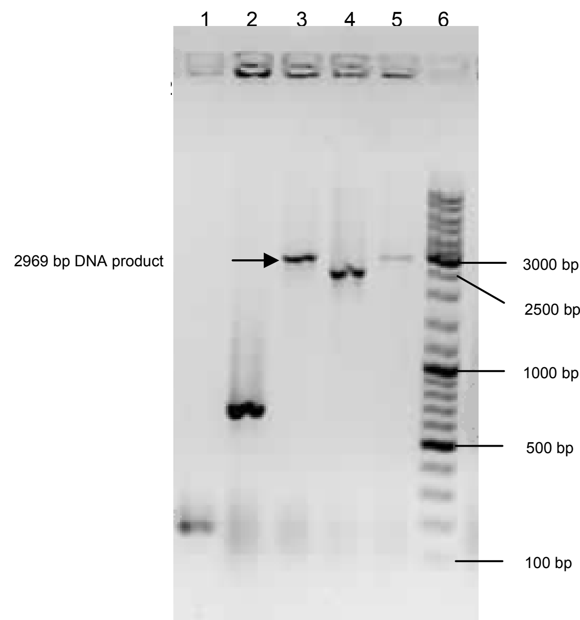


**Figure 3.7** PCR products obtained following diagnostic PCRs in which the T7 and SP6 primer pair were utilized. The PCR products were electrophoresed on a 1% agarose gel to which 0.175 µg/ml ethidium bromide was added for visualization of the PCR product under UV light. Lane 1: 10 µl PCR product where a blue colony was used as template DNA (negative control). Lane 2: 10 µl PCR product where a plasmid known to contain an insert with a size of 700 bp was used as template DNA (positive control). Lanes 3 - 5: 10 µl PCR product where white colonies were used as template DNA. Lane 3 displayed an insert of the expected size and was thus considered to be a positive result. Lane 6: 5 µl DNA ladder mix (Fermentas).

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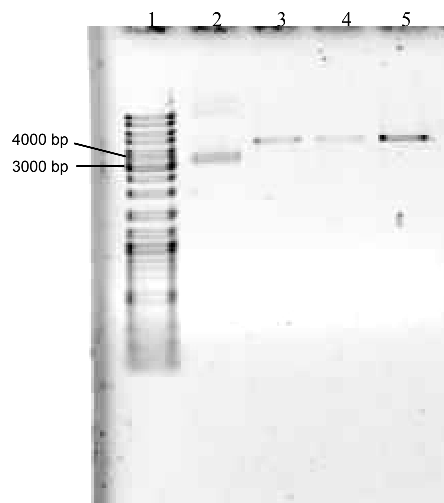


**Figure 3.8** DNA products observed following restriction enzyme digestion of the pET14b plasmid. The digested DNA product was electrophoresed on a 1% agarose gel to which 0.175 µg/ml ethidium bromide was added for visualization of the DNA under UV light. Lane 1: DNA Ladder mix (Fermentans). Lane 2: Undigested pET14b plasmid. Lane 3: pET14b plasmid incubated with *NcoI* restriction enzyme for 1 hour. Lane 4: pET14b plasmid incubated with *NcoI* restriction enzyme for 2 hours. Lane 5: pET14b plasmid incubated with *NcoI* restriction enzyme for 3 hours.

Transformation control (a), which was transformed with pET14b and pGEX-4T-1 expression vectors which did not contain any insert, yielded only blue colonies, whereas control (b), which was transformed using pET14b and pGEX-4T-1 vectors known to contain inserts, yielded mostly white colonies, as was expected.

Diagnostic PCRs performed on well over 3000 colonies, thought to contain the recombinant pET14b and pGEX-4T-1 vectors, eventually showed the presence of the *oppA* gene in some colonies, with DNA product of about 3000 bp (2829 bp *oppA* gene + 1 × restriction endonuclease site + 216 bp vector DNA (pET14b) / 150 bp vector DNA (pGEX-4T-1) = 3051 bp insert (pET14b)/ 2985 bp insert (pGEX-4T-1)) being obtained. Other colonies displayed band sizes of about 200 bp (216 bp vector DNA (pET14b) / 150 bp vector DNA (pGEX-4T-1)) in size, indicating that no insert was present in the vector (Figure 3.9).

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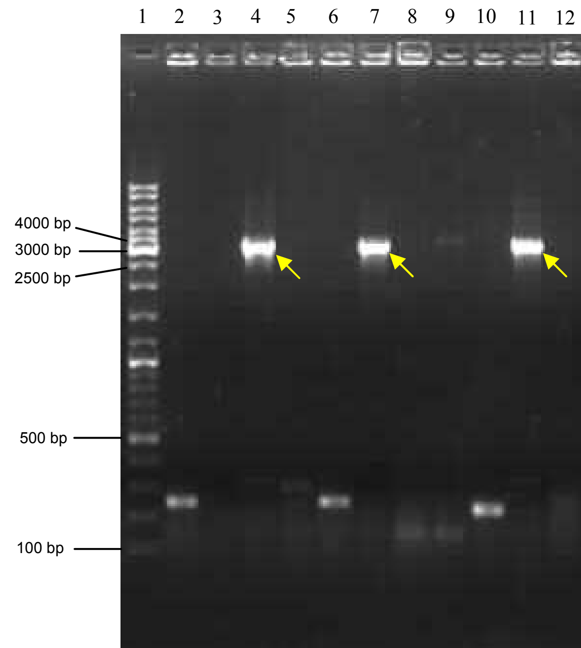


**Figure 3.9** PCR products obtained following diagnostic PCR in which the T7 and T7 terminator primer pair was used. The PCR product was electrophoresed on a 1% agarose gel to which 0.175 µg/ml ethidium bromide was added, for visualization of the DNA under UV light. Lane 1: 5 µl DNA ladder mix (Fermentas). Lane 2: 10 µl PCR product where a blue colony was used as template DNA (negative control). Lanes 3 - 12: 10 µl PCR product where white colonies were used as template DNA. Lanes 4, 7 and 11 displayed insert of the expected size (as indicated by the arrows) and were thus considered to be positive results.

The recombinant plasmids were isolated and DNA product corresponding to the expected size of the prokaryotic expression vectors containing the *Ms01* OppA protein (7506 bp recombinant pET14b / 7804 bp recombinant pGEX-4T-1 plasmid), could be seen on the respective agarose gels**.** Cycle sequencing yielded a DNA sequence in the correct orientation with regard to the transcription promoter, which could be aligned successfully with the modified *Ms01 oppA* DNA sequence previously sequenced [8].

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**3.3.4 Expression of the recombinant *Ms01 oppA* gene in *E. coli* BL21(DE3) pLysS cells using the pET14b prokaryotic expression system**

The transformation of the *E. coli* BL21(DE3) pLysS cells with the recombinant pET14b plasmid construct resulted in the formation of several colonies on the LB/agar plates. A diagnostic PCR and subsequent agarose gel electrophoresis revealed PCR products of around 3000 bp, which correspond to the size of the *Ms01 oppA* gene (2829 bp) (results not shown).

BL21(DE3) pLysS cells were grown for six hours to an $OD_{600}$ of 0.6. Cell samples taken hourly following induction of expression, appeared to contain a similar variation and quantity of protein products as the negative control when analyzed by means of reducing SDS-PAGE (Figure 3.10). A protein, around 130 kDa in size (inclusing the GST tag), was expected. Even after several attempts at optimization (outlined and discussed in section 2.4), no additional bands or difference in protein band size could be observed between the expression samples and the expression controls.

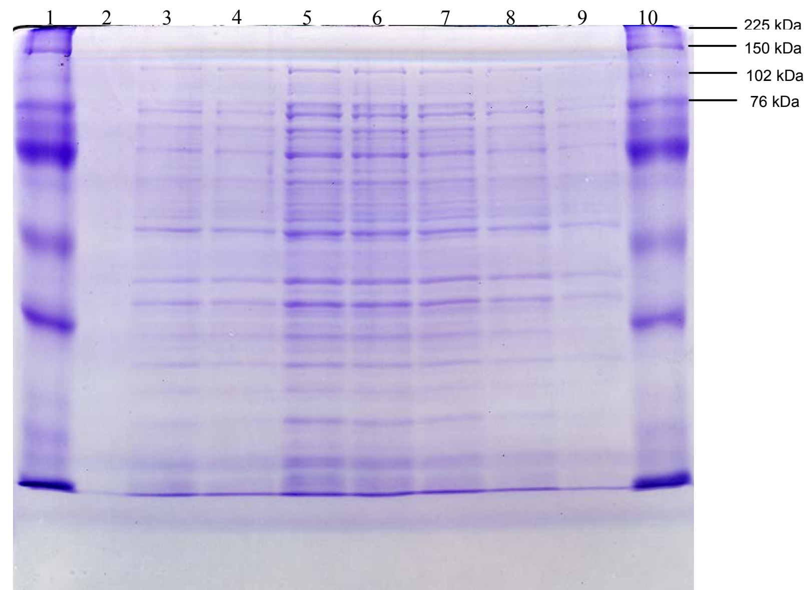


**Figure 3.10** Reducing SDS-PAGE analysis of the protein product of BL21(DE3) pLysS cells containing the recombinant pET14b prokaryotic expression vector, following induction of expression. Lane 2: Cell sample taken before induction. Lane 3: Cell sample taken two hours after induction. Lane 4: Induced sample of cells containing pET14b with no insert (negative control) taken two hours after induction. Lane 5: Cell sample taken four hours after induction. Lane 6: Negative control taken four hours after induction. Lane 7: Cell sample taken 3 hours after induction. Lane 8: Cell sample taken two hours after induction. Lane 9: Uninduced cells containing the recombinant pET14b prokaryotic expression vector grown for four hours after the induction of other cell samples. Lane 1 and 10: Rainbow™ Full Range Molecular Weight Marker (GE Healthcare).

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### 3.3.5 Expression of the recombinant *Ms01 oppA* gene in *E. coli* BL21(DE3) pLysS cells using the pGEX-4T-1 prokaryotic expression system

The recombinant *Ms01* OppA protein was successfully expressed in BL21(DE3) pLysS cells containing the recombinant pGEX-4T-1 plasmid, with a protein of about 150 kDa (100 kD OppA protein + 26 kDa protein = 126 kDa recombinantly expressed protein) becoming visible two hours after induction on a reducing SDS-PAGE gel. Expression for about eight hours after induction appeared to yield the highest concentration of recombinant OppA protein product, since longer expression resulted in a decrease in the protein product obtained, and was thus accepted to be the optimum expression time.

### 3.3.6 Purification of the recombinant OppA protein

3.3.6.1 Ammonium sulphate precipitation

Purification of the recombinant OppA protein expressed using the pGEX-4T-1 prokaryotic expression system was initially attempted by means of ammonium sulphate precipitation. The objective of this technique, which involves the addition of saturated ammonium sulphate to the cell lysate following expression, is to precipitate highly expressed soluble protein, isolate, and re-dissolve the protein. Although, this technique appeared to be relatively successful, results from the SDS-PAGE gel, shown in Figure 3.11, did not allow accurate protein size determination. Furthermore, the SDS-PAGE analysis suggested that another highly expressed protein had been isolated (lane 3), instead of the recombinant OppA protein. In addition, the isolated protein product appeared to contain smaller sized proteins, indicating that it was not completely pure. It was thus decided to purify the recombinant OppA protein by means of glutathione-agarose affinity purification.

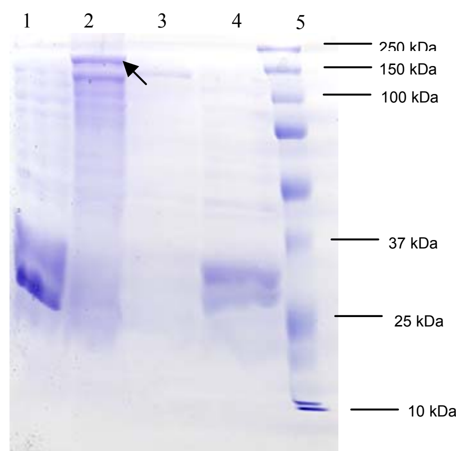


**Figure 3.11** Reducing SDS-PAGE analysis of protein products of BL21(DE3) pLysS cells containing the recombinant pGEX-4T-1 plasmid, after expression and ammonium sulphate precipitation of the recombinant OppA protein. Lane 1: Induced sample of cells containing pGEX-4T-1 plasmid with no insert. Lane 2: Cell sample taken eight hours after induction of expression. Lane 3: Purified protein. Lane 4: Purified GST protein. Lane 5: PrecisionPlus Protein™ All Blue Standard (Bio Rad). The arrow indicates the position of the *Ms01* OppA protein.

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3.3.6.2 Affinity purification

Glutathione affinity purification of the recombinant OppA protein proved successful, with a single protein of about 150 kDa (100 kD OppA protein + 26 kDa protein = 126 kDa recombinantly expressed protein) having been obtained on an acrylamide gel, seen in Figure 3.12. The presence of the recombinant protein was successfully confirmed by means of western blot using anti-GST antibodies as shown in Figure 3.13.

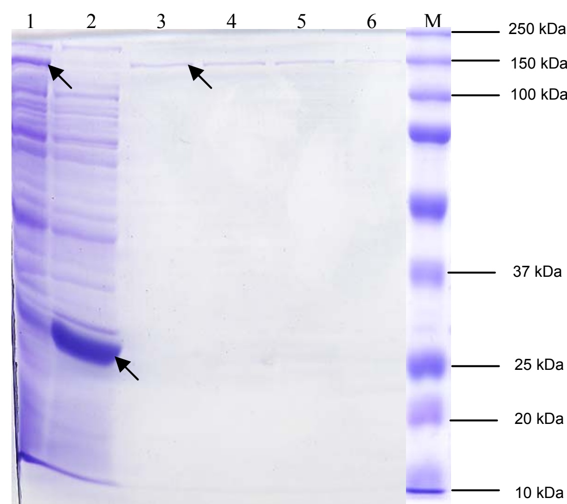


**Figure 3.12** Reducing SDS-PAGE analysis of the protein product of BL21(DE3) pLysS cells containing the recombinant pGEX-4T-1 plasmid, following expression and affinity purification of the recombinant OppA protein. Lane 1: Cell sample taken eight hours after induction of expression. Lane 2: Induced sample of cells containing pGEX-4T-1 plasmid with no insert (control sample) taken two hours after induction. Lanes 3-6: Purified recombinant *Ms01* OppA protein. Lane M: PrecisionPlus Protein$^{TM}$ All Blue Standard (Bio Rad). The arrow in lane 1 indicates the position of the expressed recombinant protein, while the arrow in lane 2 points to the expressed GST protein (positive control). The arrow in lane 3 indicates the position of the purified recombinant protein.

## 3.4 Discussion

From the results obtained in this study, it can be concluded that the cloning strategy employed for the insertion of the *oppA* gene into the selected expression vectors was successful. The primers designed to amplify the *Ms01 oppA* gene were successfully used to yield a PCR product with the expected band size of 3000 bp. The cloning of the purified *Ms01 oppA* gene into the pGEM-T easy cloning vector appeared to be successful, based on the diagnostic PCRs which showed a PCR product of the expected band size. The subsequent plasmid isolation, where a plasmid of the expected size was visualized on an agarose gel, and DNA sequencing reaction confirmed the presence of the *Ms01 oppA* gene flanked by the *NcoI* restriction endonuclease recognition sites in the cloning region of the plasmid.

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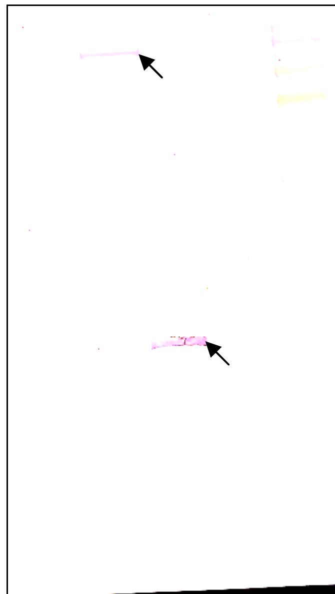

**Figure 3.13** Western blot analysis of the purified protein. The top-left arrow indicates the position of the recombinant OppA protein, while the bottom-right arrow points to GST control.

The *Nco*I restriction endonuclease was successfully used to cleave the gene from the cloning vector for subsequent subcloning, with a difference in band size being observed between the digested and undigested samples. The change in band size was thought to occur as a result of the linearization of the circular plasmids during digestion, resulting in slower migration of the digested linear DNA through the gel matrix, and causing the linear DNA to migrate more slowly in the gel than the circular DNA.

Cloning of the *Ms01 oppA* gene into the pET14b and pGEX-4T-1 prokaryotic expression vectors yielded positive results, with the diagnostic PCR that followed transformation revealing the presence of an insert the size of the *oppA* gene (around 3000 kb). Insertion of the *oppA* gene into the correct position in the expression vectors was confirmed by analysis of the size of the recombinant vectors (with the expected sizes having been attained) and subsequent DNA sequencing.

Despite numerous attempts at problem-solving and optimization, expression of the recombinant *Ms01* OppA protein could not be achieved using the pET14b system of expression. A subsequent literature study indicated that the mode of expression of recombinant proteins influences the production and physical properties of the polypeptide that is synthesized [Guan and Dixon, 1991]. The expression system utilized is also said to influence the level of expression obtained. Problems commonly associated with expression include protein insolubility, incorrect protein folding, protein aggregation (formation of inclusion bodies), plasmid instability and codon bias. All of the above factors were considered as possible causes of the problems experienced and investigated accordingly.

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Studies investigating the structure of the OppA protein have confirmed it to be a soluble protein, thus protein insolubility was unlikely as a possible reason for the problems with expression [Garmory and Titball, 2004; Balan *et al.,* 2008; Hopfe and Heinrich, 2008]. Proteins which are folded incorrectly are usually either broken down into their constituent amino acids by cytoplasmic proteases or precipitate into inclusion bodies [Baneyx, 1999]. Thus, incorrectly folded proteins may not be readily detected by means of SDS-PAGE, the detection technique used during this study. For this reason, incorrect protein folding could thus not be ruled out as a possible problem of expression. An attempt was thus made to express the protein at lower temperatures. Literature indicates this to be a measure of reducing inclusion body formation [Simionatto *et al.,* 2010]. Inclusion bodies are amorphous protein aggregates which are thought to occur during situations which impair protein folding *in vivo*, resulting in the aggregation of protein folding intermediates [Sorensen and Mortensen, 2005]. The exact mechanism of formation is unknown, but Simionatto *et al*. [2010] suggests that inclusion bodies are formed when the protein translation rate exceeds the cells capacity to fold newly synthesized proteins. Expression at a lower temperature would therefore reduce the rate of protein translation and lessen the formation of inclusion bodies. However, even with expression performed at temperatures as low as 20˚C, no bands of the expected size could be observed on the SDS-PAGE gels after staining. Expression was also attempted using a higher concentration of IPTG for induction, as recommended in the pET system manual, but no increase in expression was detected.

The plasmid stability of the recombinant pET14b vector was then investigated. Literature indicates that recombinant protein expression is always enhanced with higher plasmid stability and that plasmid stability may be influenced by various factors, including medium composition, type of insert and plasmid load [Xu *et al.,* 2005]. A study conducted by Xu *et al*. [2005] found that the ratio of plasmid-containing cells decreased significantly (from 67.2% to 0.053%) with increasing numbers of multiple (heterologous) joined genes in the pET28a plasmid. This indicates that increased gene size may greatly reduce plasmid retention when using the pET expression system. Since the *Ms01 oppA* gene is a relatively large gene (2829 bp), its size could be viewed to be of serious concern in this regard. A diagnostic PCR was thus conducted with samples taken before, during and after protein expression to determine whether the recombinant pET14b plasmid was retained by the *E. coli* BL21(DE3) pLysS cells during expression. Positive results were obtained in each case indicating that at least some plasmid was retained by the induced cells even after expression. The percentage of expressing cells actually containing plasmid was, however, not determined.

Xu *et al*. [2005] also found that the addition of glucose to the medium significantly increased retention of the pET28a recombinant plasmid by BL21(DE3) cells. Cells containing plasmid typically grow slower than cells which do not contain any plasmid, particularly when a recombinant strain is fully induced. This is the result of an increased metabolic burden on the cells due to the synthesis of the recombinant protein. This burden is likely to be increased with the size of the protein synthesized and probably accounts for the decrease in protein stability witnessed by Xu *et al*. [2005]. Nonetheless, even with the addition of glucose to the medium, no protein bands of the expected size were found on the SDS-PAGE gel after staining.

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It has also been reported that plasmid stability can be improved by decreasing the strength of the promoter [Xu *et al.,* 2005]. The pET14b expression system is the most powerful prokaryotic expression system to date and comprises a T7 promoter and T7 RNA polymerase that are so active that, when fully induced, most of the cell's resources are converted to recombinant gene expression, such that the recombinant protein synthesized may comprise more than 50% of the cells protein within only a few hours [Novagen pET System manual]. This strong promoter may therefore have negatively affected plasmid stability and protein production, as has been shown in previous studies [Xu *et al.,* 2005].

Examination of the nucleotide sequence of the *Ms01* OppA protein raised the additional concern that the occurrence of high numbers of rare codons was likely to be hampering expression of the recombinant protein. All cells, especially *E. coli* in this case, utilize a particular subset of 61 amino acid codons for the manufacturing of most mRNA molecules [Kane, 1995; Sorensen and Mortensen, 2005]. Rare codons are codons which are utilized by *E. coli* at a frequency of less than 1%. Rare codons usually occur in genes which are expressed at low levels, whereas major codons are typically found in highly expressed genes. While genes which contain a distribution of codons similar to that which is normally encountered by *E. coli* will usually be expressed without much difficulty, genes containing rare codons are likely to undergo translational problems resulting in a reduction in either the quantity or quality of the protein synthesized. The AGG and AGA codons occur at the lowest frequency in *E. coli* (0.14% and 0.21%, respectively) and were the first codons demonstrated to have a detrimental effect on protein expression [Kane, 1995]. Studies investigating the effect of the said codons on gene expression found that the expression additionally depended on the number and position of the codons within the mRNA. Thus, as the number of rare codons increased, the expression decreased, and the effects on the protein decreased as the codons approached the carboxyl-terminus.

About 15% of the codons encountered in the *Ms01* OppA are rare codons, with some occurring in clusters and others occurring singly [Addendum B]. The first rare codon to appear in the *Ms01* OppA transcript occurs as a single AGA codon at position number six on the mRNA transcript. The other AGA codons occur at positions 83, 166, 223, 249, 254, 455, 485, 489, 550, 566, 637, 639, 667, 673, 680, 711, 739, 786, 820, 821, 851 and 939. A tandem double repeat AGA codon also occurs at position 821. The AGA codon occurs in the *Ms01* OppA transcript at a frequency that is ten times more than that which is normally encountered in *E. coli* mRNA transcripts. In addition, several other rare codons are placed throughout the length of gene, with several of these codons occurring in clusters, as is shown in Addendum B. Table 3.2 gives an indication of the types and frequencies of all rare codons encountered in the *Ms01* OppA protein.

Two strategies may be employed to overcome codon bias during expression. The first is site-directed mutagenesis of the target sequence in order to generate codons which reflect the tRNA pool of *E. coli* [Sorensen and Mortensen, 2005]. This is, however, a time consuming process which may result in unstable mRNA transcripts. The second strategy is the co-transformation of *E. coli* with a plasmid possessing a gene which codes for the tRNA of the problematic codons. *E. coli* can be manipulated to match the codon usage

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frequency in heterologous genes by increasing the copy number of the limiting tRNA species. The Rosetta-gami B(DE3) pLysS cell line is an example of such a strain of *E. coli*, and as such possesses a plasmid to remedy problems experienced with codon bias. However, although such a cell line was initially considered as expression host, following the failed expression attempts using the BL21(DE3) pLysS cell line, a decision was taken against it, due to its failure to influence problematic expression resulting from codon bias during previous studies in this laboratory [Rothman, 2007]. Instead a fusion partner was considered for use.

**Table 3.2** A summary of the type and number of rare codons present in the *Ms01* OppA mRNA transcript compared to the frequency at which these codons are typically observed in the mRNA transcripts of *E. coli*. The *Ms01* OppA mRNA transcript comprises 929 codons.

| **Rare codon** | **Number of codons encountered in the *Ms01* OppA mRNA transcript** | **Frequency of codons in a typical *E. coli* mRNA transcript** | **Frequency of codons in *Ms01* OppA transcript compared to that in *E. coli*** |
|---|---|---|---|
| AGA | 23 / 943 codons | 2.1 / 1000 codons | 10 times more |
| UGU | 1 / 943 codons | 4.7 / 1000 codons | |
| CCU | 11 / 943 codons | 6.6 / 1000 codons | 1.5 times more |
| UCA | 44 / 943 codons | 6.8 / 1000 codons | 6 times more |
| AGU | 14 / 943 codons | 7.2 / 1000 codons | 2 times more |
| UCG | 1 / 943 codons | 8 / 1000 codons | |
| AUA | 10 / 943 codons | 4.1 / 1000 codons | 2 times more |
| ACA | 40 / 943 codons | 6.5 / 1000 codons | 6 times more |
| CCA | 23 / 943 codons | 8.2 / 1000 codons | 3 times more |
| GGA | 13 / 943 codons | 7 / 1000 codons | 2 times more |
| CUA | 6 / 943 codons | 3.2 / 1000 codons | 2 times more |

Although most fusion partners are employed for specific purification strategies, they may also be beneficial *in vivo*, where their use in protein expression has proven advantageous for a number of reasons. The most important reason, with regards to this study, is that amino-terminal fusion partners often facilitate the synthesis of otherwise poorly expressed proteins (specifically with regard to translation) [Baneyx, 1999; Sorensen and Mortensen, 2005]. This is thought to occur as a result of mRNA stabilization as well as the

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occurrence of a higher efficiency of the translation of passenger proteins containing rare codons. Fusion partners have also been shown to protect passenger proteins from intra-cellular proteolysis and to enhance the solubility of the passenger proteins (lessen or eliminate inclusion body formation) [LaVallie and McCoy, 1994; Sorensen and Mortensen, 2005]. While several hypotheses have been presented to explain the successes observed with the use of fusion partners during expression, none have yet been substantiated. Some authors suggest that fusion proteins act as "intramolecular chaperones", while others believe that fusion partners favour on-pathway isomerization reactions, thus promoting the acquisition of the correct protein structure [LaVallie and McCoy, 1994; Baneyx, 1999]. In addition, superior fusion partners also permit controlled transcription [LaVallie and McCoy, 1994]. This has been shown to minimize plasmid loss and hold off accumulation of mutations in passenger protein products. Thus, the use of a fusion partner during expression would enable a range of problems encountered during expression to be addressed simultaneously.

The glutathione S-transferase (GST) gene fusion system is a multipurpose system for the expression, purification and detection of fusion proteins consisting of a *Schistosoma japonicum* GST moiety at the amino terminus and the target protein at the carboxyl terminus [GST gene fusion system handbook, GE Healthcare]. The pGEX vector is controlled by a $P_{tac}$ promoter, a weaker promoter than the T7 promoter [Xu *et al.,* 2005]. The pGEX system is one of the expression systems which have proven most successful for the synthesis of soluble, correctly folded proteins in the bacterial cytoplasm [LaVallie and McCoy, 1994]. Several studies involving the expression of mycoplasma genes have been able to successfully express these proteins with the utilization of the pGEX expression system [Rosati *et al.,* 2000; Robino *et al.,* 2007]. In addition, a previous study conducted in this laboratory accomplished successful expression of a gene containing rare codons after employing the pGEX expression system, when the pET expression system had failed [Rothman, 2007].

Once the *Ms01 oppA* gene was successfully cloned into the pGEX-4T-1 vector, expression was attempted again. The initial observation made was that the pGEX-transformed cells grew much slower than was observed during utilization of the pET expression system, taking about 6 hours to reach an $OD_{600}$ of 0.6 compared to the 2.5 hours required by the pET-transformed cells. Expression utilizing the pGEX system proved successful, yielding a protein band of about 150 kDa. Although the expected size of the *Ms01* OppA protein was around 100 kDa, the addition of the 26 kDa GST protein was expected to increase the size of the fusion protein product to about 130 kDa. The fact that the product obtained was slightly larger than expected was not a serious concern and could be attributed to an initial net positive charge of the recombinant protein (resulting from a protein composition of more positively charged than negatively charged amino acids), which probably decreased its overall negative charge during SDS-PAGE, resulting in slower migration of the protein through the gel matrix. This theory was, however, disproved when further investigation revealed the recombinant protein has an initial net negative charge. Western Blot analysis

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with anti-GST antibodies, nevertheless, indicated that a GST moiety was present on the protein product obtained and served as a confirmation of successful expression.

Although the reason for the success of the pGEX expression system compared to the pET expression system remains largely unknown, it may have been through the accumulation of various factors. These include gene size, incorrect protein folding, plasmid instability and codon bias. The weaker promoter of the pGEX system possibly aided in correct protein folding and to maintain plasmid stability, in combination with the GST fusion partner which may also have contributed to correct protein folding, as explained earlier in this chapter. The highly expressed GST fusion partner may also have enabled expression of the recombinant protein, despite codon bias and large gene size, as a result of mRNA stabilization and a higher efficiency of the pGEX gene fusion system in translating the rare codons in the *Ms01 oppA* gene. Also, while no protein was detected on SDS-PAGE gels when the pET expression system was employed, it does not exclude the possibility that expression did occur. The expression product was not tagged and thus could not be analysed using the Western Blot. Expression levels may thus simply have been too low to detect when the pET system was utilized.

The subsequent purification of the *Ms01* OppA protein was initially attempted by means of ammonium sulphate precipitation. Using this technique, large soluble proteins are precipitated out of solution and isolated, by adding saturated ammonium sulphate solution to the expressed cell extract. Although relatively pure protein could be isolated using this technique, the SDS-PAGE gels did not display even protein separation and were thus unreliable for use in determining the exact size of the protein isolated. This was likely the result of the high salt content of the protein samples analysed (even after dialysis) influencing the overall charge, and thus migration of the proteins through the gel matrix. Even so, close analysis of the isolated protein product suggested that the protein product was not sufficiently pure and that the isolated protein might in fact be another large, highly expressed protein present in the cell extract of the expressed cells. Thus, for the sake of accuracy and efficiency, it was decided that affinity purification would be the best method of purification. This decision proved to be right, as indicated by later SDS-PAGE analysis. Following successful affinity purification, a decision was made not to remove the affinity tag from the fusion protein, since the GST protein is much smaller than the *Ms01* OppA protein and would not likely interfere with antibody detection of the *Ms01* OppA protein during the ELISA. In addition, the ostriches were unlikely to have been in contact with the GST protein of *Schistosoma japonicum* so they were highly unlikely to react sero-positively to this protein. Interestingly, however, when left for a period of time, the recombinant protein samples degraded in a manner such that the GST moiety became detached from the OppA protein, while the OppA protein remained intact. This was determined by means of SDS-PAGE analysis, where a protein of about 100 kDa was observed, and confirmed by western blot analysis of the same sample, where only a protein of about 25 kDa could be detected, instead of the 150 kDa protein previously detected in this sample. The 100 kDa protein was thought to consist largely of the OppA protein, whereas the 25 kDa protein is probably the GST moiety. A probable explanation for the degradation of the

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recombinant OppA protein is the co-purification of an *E. coli* protease(s) together with the expressed protein. While there are no specific references to proteases, the co-purification of host proteins with GST-fusion proteins have been previously documented [GST gene fusion system handbook, GE Healthcare]. Since the BL21(DE3) pLysS cell line which was utilized for protein expression is a Lon and OmpT protease deficient cell line, the addition of protease inhibitors to the cell extract was deemed unnecessary [Sorensen and Mortensen, 2005; GST gene fusion system handbook, GE Healthcare]. It is therefore possible that a tiny amount (as judged by the very slow rate of protein degradation and the fact that it was not detected during the SDS-PAGE) of another active host protease may have co-purified with the recombinant OppA protein. The use of a protease deficient expression cell lines and/or the addition of protease inhibitors to the cell extract after expression are the generally employed measures with which unwanted degradation of the expressed protein is prevented.

The successfully expressed and purified *Ms01* OppA protein could now be utilized as the antigenic protein in the ELISA to enable analysis of the serum samples which were collected during the DNA vaccine trial.

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# 4. An evaluation of the antibody responses elicited in ostriches by the pCIneo, VR1012 and VR1020 DNA vaccines

## 4.1 Introduction

The stringent nutritional requirements of mycoplasmas have made these organisms notoriously difficult to cultivate [Razin *et al.,* 1998]. The current utilization of complex undefined media to this end, have hindered the preparation of mycoplasmal antigen which is free of any contaminating serum components, making traditional vaccine development strategies, in which the organism is cultivated and then inactivated, difficult. Although poultry vaccines against *M. gallisepticum* and *M. synoviae* were thought, until recently, to provide some protection against the *Ms01*, *Ms02* and *Ms03* ostrich-infecting mycoplasmas, a study by Pretorius [2009] found that these vaccines did not prevent ostrich-specific mycoplasma infections. This effectively means that no vaccine is currently available for use against mycoplasma infections in ostriches. In addition, despite the use of currently available antimicrobial agents, none are able to prevent or eliminate mycoplasmal disease from flocks, and outbreaks still occur at unacceptable frequencies in South African ostriches. As a result, the South African ostrich industry has launched a search for a more effective therapy with which to combat ostrich-specific mycoplasma infections.

DNA vaccines represent a novel way in which protective immunity may be achieved in a host, and have been successfully utilized against various microorganisms in a number of different hosts [Saurez and Shultz-Cherry, 2000]. DNA vaccines stimulate both a humoral and cellular immune response by utilizing the genes encoding (immunogenic) proteins of pathogens, as an alternative to an attenuated pathogen, a live replicating vector or the protein itself [Donelly, 1997]. DNA vaccines consist of a plasmid containing a strong viral promoter; an immunogenic gene; possibly immunostimulatory sequences and a polyadenylation or transcription termination sequence, which is dissolved in saline and injected into the host. The plasmid does not integrate into the host chromosomal DNA and its lack of a eukaryotic origin of replication prevents it from replicating within the host. The host cells take up the DNA plasmid and subsequently manufacture the encoded protein, resulting in the generation of an immune response. However, the DNA plasmid that is taken up by the cell is broken down again and therefore only leads to a short-term production of the encoded vaccine protein, which means that a booster vaccination is required to establish long-term immune memory.

Following DNA vaccine development and preparation, a technique was required to determine whether or not the DNA vaccines, as administered to the ostriches in this study, elicited an antibody response. For this purpose an indirect ELISA was developed. In its simplest form such an ELISA requires an immunogenic protein which will be recognized by a primary antibody (which should be present in the serum of animals exposed to the antigenic protein), an enzyme (such as horse radish peroxidase (HRP)) labeled secondary

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antibody which will recognize the primary antibody and an enzyme substrate which reports upon the success of the reaction (Figure 4.1).

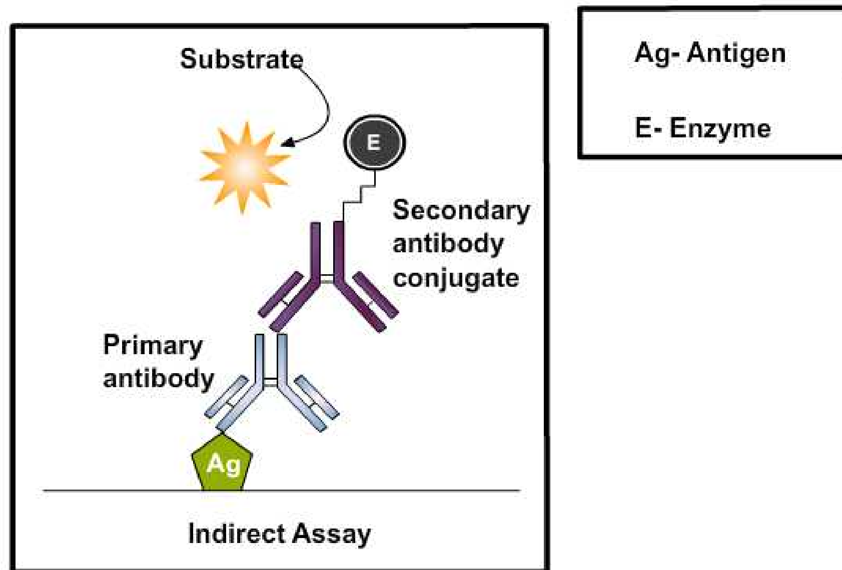


**Figure 4.1** A diagrammatic representation of an indirect ELISA [ELISA technical guide and protocols, Thermo Scientific]. Immobilization of the antigen may be achieved either by direct adsorbtion or by means of an antibody adsorbed to a microtiter plate. Following the blocking of non-specific binding to the plate, a primary antibody is used to probe for the antigen. Subsequently a secondary antibody labelled with a signal generating enzyme is added to detect the binding of the primary antibody. An appropriate enzyme substrate is then added (in the case of enzymatic detection), and the strength of the signal is proportional to the amount of antibody present in the sample. The maintenance of only high affinity binding events is ensured by washing the plate between steps.

Since its initial description by Engval and Perlmann [cited by Jordan, 2002] more than 30 years ago, the ELISA has had an enormous impact in both basic scientific applications as well as in clinical diagnostics [Busolo *et al.,* 1980; Jordan, 2002]. An ELISA is a simple and sensitive technique with which molecules recognized by an antibody are specifically and quantitatively detected [Jordan, 2002]. This technique, which is based upon the detection of specific antibodies against "solid phase" adsorbed antigens using enzyme-conjugated immunoglobulins, is one of several serological tests which have been developed for the detection of avian mycoplasma infections [Busolo *et al.,* 1980; Abdulmoumen and Roy, 1995]. The ELISA offers more sensitivity than HI tests, in which false negatives are common, and more specificity than the serum plate agglutination test, in which false positives are common.

As part of this study, an ELISA was developed and optimized for the detection of antibodies against the *Ms01* OppA protein and the *Ms01* whole organism. By comparison with the ELISA format illustrated above, this ELISA was modified to include an additional step in order to increase its sensitivity. Instead of only using an enzyme labelled secondary antibody, the secondary antibody was labelled with biotin, after

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which an additional step was included which involved the addition of HRP enzyme labelled streptavidin, followed by substrate addition (Figure 4.2). The inclusion of this step increased the sensitivity of the ELISA and decreased background binding, thus resulting in an increased signal to noise ratio.

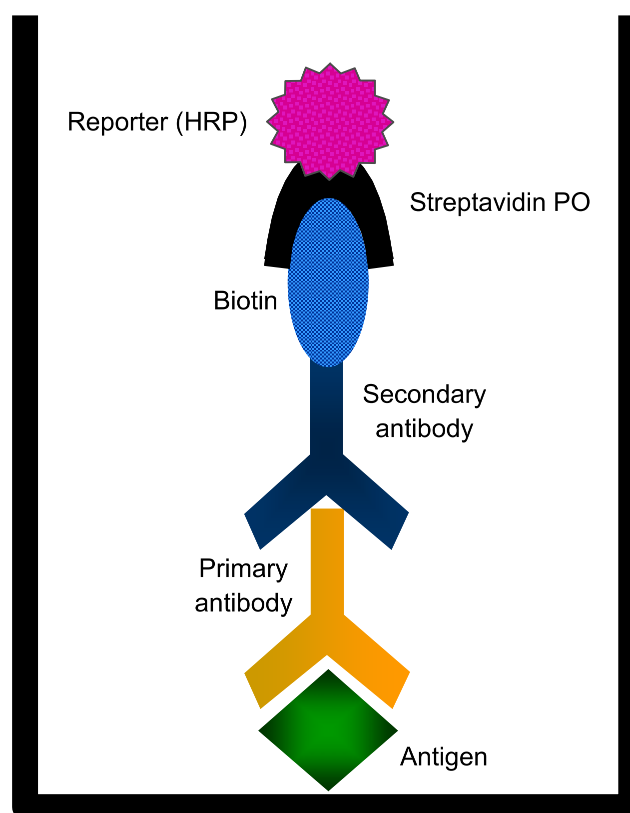


**Figure 4.2** A diagrammatic illustration of the modified indirect ELISA. Following immobilization of the antigen on the microtiter plate by direct adsorbtion and the blocking of non-specific binding to the plate, a primary antibody is used to probe for the antigen. A biotinylated secondary antibody is subsequently added to detect the binding of the primary antibody. A signal generating enzyme (horseradish peroxidase (HRP) in this case) conjugated to streptavidin PO is then added. Streptavidin PO binds to biotin with high affinity. The inclusion of this step decreases background binding and increases the sensitivity of the ELISA, resulting in an increased signal to noise ratio. Following the addition of the appropriate substrate, the strength of the signal detected is proportional to the amount of antibody present in the sample. The maintenance of only high affinity binding events is ensured by washing the plate between steps.

A DNA vaccine trial was conducted in which the ability of recently developed DNA vaccines, the *Ms01 oppA* gene cloned into pCIneo, VR1012 and VR1020 respectively, to elicit antibody responses against the ostrich-specific mycoplasma, *Ms01*, was evaluated. The antibody responses of six month old ostriches against the above vaccines were analyzed following initial and booster vaccinations.

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## 4.2 Materials and Methods

### 4.2.1 Vaccine preparation

The three DNA vaccines utilized comprised the eukaryotic expression vectors, pCIneo, VR1012 and VR1020, into which the *Ms01 oppA* gene was cloned. The above recombinant eukaryotic expression vectors were obtained ready for use and vaccine preparation thus only required increasing the amount of vector available. For this purpose, single colonies of *E. coli* JM109 cells containing the respective recombinant eukaryotic expression vectors were used to inoculate aliquots of 5 ml of LB medium containing 2.5 µl of 100 mg/ml ampicillin respectively, and incubated for eight hours at 37°C. Each of these starter cultures were then transferred to 400 ml of LB medium containing 200 µl of 100 mg/ml ampicillin respectively, and incubated for a further 16 hours at 37°C. Isolation and purification of the respective recombinant eukaryotic expression vectors followed, using a Nucleobond® Endotoxin-free Plasmid DNA Purification Kit (Macherey-Nagel, Germany) as per the manufacturer's instructions, with MilliQ® analytical quality water as eluting agent. The concentration of the isolated plasmids was then determined using a Nanodrop ND-2000. The purified recombinant eukaryotic expression vectors were then ready to be inoculated into ostriches as DNA vaccines.

### 4.2.2 Vaccination trial

Pre-immunization blood samples were collected from all ostriches on the first day (day 0) of the vaccination trial. Three groups consisting of ten birds each were immunized with 100 µg of pCIneo, VR1012 or VR1020 DNA vaccine in 1 ml of PBS by intra-muscular injection in the large upper thigh muscle, using a 25 G ×11/4" needle (Nipro) and 2 cc syringe (Promex), respectively. The control group (10 birds) was not immunized. Blood samples were collected in 5 ml Vacuette® Z Serum Sep Clot activator tubes (Greiner-bio one) using Vacuette® 18 G ×1" multiple drawing blood collection needles (Greiner-bio one) from the main vein under the wing of the ostriches on a weekly basis, on days 7, 14, 21, 28 and 35. Following blood sample collection on day thirty-five, the vaccinated ostrich groups received an additional 100 µg of the respective DNA vaccine in 1 ml intramuscularly. Once again, the control group received no vaccine. Blood samples were subsequently collected on days 42, 49, 58 and 64 as calculated from the first day of vaccination. The blood samples collected were incubated for 30 minutes at 37°C and subsequently at 4°C overnight to facilitate the clotting process. Samples were then centrifuged at 5 000 x g for one minute to separate the clotted cell components of the blood from the serum. The serum obtained was removed using a 1 ml Gilson pipette and subsequently stored at -20°C for later use.

### 4.2.3 Antigen preparation

The concentration of the *Ms01* OppA protein, prepared as described in the previous chapter, was determined using the Bradford protein determination assay. The Bradford reagent was prepared as described in the original publication [Bradford, 1976]. Bovine serum albumin (BSA) was utilized in the

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preparation of a standard curve, ranging in concentration from 0 to 175 µg/ml. Five microliters of each standard solution was transferred, in triplicate, to a Greiner Bio-one 96-well microplate, after which 250 µl of Bradford reagent was added. Once the protein concentration was determined, the OppA antigenic protein was diluted, using carbonate buffer (50 mM $NaHCO_3$, pH 9.6), to 1 µg/ml and used to coat Nunc© 96-well microtiter plates.

**4.2.4 Secondary antibody purification and biotinylation**

Serum samples collected, on day 97, from a rabbit immunized with ostrich antibodies were provided by Dr Annelise Botes, Department of Biochemistry, Stellenbosch University. The presence and immunogenicity of rabbit anti-ostrich immunoglobin (Ig) in the collected serum was established in a previous investigation by an Honours student in this laboratory, Ms Amanda van Tonder [89]. Rabbit anti-ostrich antibodies were isolated from serum by means of ammonium sulphate precipitation, as described in Chapter 3. Biotinylation of the isolated rabbit anti-ostrich Ig was initiated with a 1:10 dilution of the isolated rabbit anti-ostrich Ig in carbonate buffer. Following protein concentration determination by measuring the absorbance at 280 nm on a Nanodrop ND-2000, the Ig was diluted with carbonate buffer to a final concentration of 5 mg/ml. Two mg of biotinomidocaproate N-hydroxysuccinimide ester (Sigma) was dissolved in 1 ml of N,N-dimethylformamide (DMF), and 250 µl added per 1 ml of Ig fraction of the rabbit anti-ostrich Ig, with gentle stirring for 2 hours at room temperature. The biotinylated Ig was subsequently dialysed against PBS at 4°C for 8 hours followed by a change of PBS and further dialysis overnight. Glycerol was then added in a ratio of 1:1 to the biotinylated rabbit anti-ostrich Ig, which was subsequently stored at -20°C.

**4.2.5 Enzyme-linked immunosorbent assay (ELISA)**

4.2.5.1 Optimization

Nunc© 96-well microtitre plates (Nunc, Denmark) were coated with 100 µl of *Ms01* OppA protein ranging from 2.5 µg/ml to 0.125 µg/ml in concentration, or with $10^9$ *Ms01* cells/ml diluted 100, 1 000 and 10 000 times, and incubated at 4°C for 16 hours. Plates were then blocked with 200 µl of casein buffer (154 mM NaCl, 0.5% w/v casein, 10 mM Tris HCl) for 2 hours at 37°C. Ostrich serum was diluted between 40 to 5620 times in casein buffer containing Tween 20 (0.05% v/v Tween 20), after which 100 µl of diluted serum was added to each well, followed by incubation at 37°C for one hour. At least two birds from each group of ostriches were utilized to determine the optimal serum dilution for that group. Plates were then washed three times with PBS-Tween 20 (1.4 mM NaCl, 1% v/v Tween 20, pH 7.2). Biotinylated rabbit anti-ostrich antibody (5 mg/ml) was subsequently diluted 100 times in casein buffer containing Tween 20 and 100 µl of the diluted antibody incubated for one hour at 37°C in each well. Plates were then washed as described. This was followed by the addition of 100 µl of HRP–Streptavidin conjugate, diluted 8000 times in casein buffer containing Tween 20, and subsequently incubated at 37°C for one hour. Plates were again washed as previously described. Substrate solution consisting of citrate buffer (0.1 M, pH 5), and substrate,

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0.05% w/v 2,2'-azino-bis(3ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 0.05% (v/v) $H_2O_2$ was subsequently prepared, of which 100 µl was added to each well, and readings taken on a Labsystems Multiscan Original spectrophotometer at $A_{405}$ at five minute intervals for 30 minutes. Results were presented graphically, with the antigen dilution/serum dilution plotted on the x axis and the absorbance values obtained plotted on the y axis.

4.2.5.2 Detection of humoral antibodies to the Ms01 OppA protein in ostrich serum

The ELISA was performed as described above, with Nunc© 96-well micrititre plates coated with 100 µl of 1 µg/ml *Ms01* OppA protein and $10^6$ *Ms01* cells, respectively. Ostrich serum diluted 160 times in casein buffer containing Tween 20 was utilized as primary antibody. As coating controls, wells were coated with the respective antigen, but not incubated with serum. As negative serum controls, wells were not coated with antigen. Results were presented graphically, with the absorbance values (Y axis) obtained plotted against time (X axis).

4.2.5.3 Comparative analyses

The results obtained from the ELISA using the *Ms01* OppA protein as coating antigen were compared to those obtained from the ELISA using the *Ms01* whole organism as coating antigen. In addition, comparisons were drawn between the antibody responses observed in ostriches of similar weight in the same group as well as in different groups. An unexpected avian influenza outbreak during the trial also necessitated a careful analysis of the antibody responses obtained during the trial.

## 4.3 Results

### 4.3.1 Optimization studies

Before a new ELISA can be attempted, the optimum concentration of antigen and antibody for use in the ELISA, must be determined. To determine the optimal concentration of antigen for use in the ELISA, the absorbance values obtained at different coating concentrations of antigen were plotted against a serum dilution series with serum samples diluted between 1/40 and 1/5 120 times (Figures 4.3 and 4.4). The antigen coating concentration was optimized utilizing serum samples from one of the ostriches in the VR1012 vaccinated group.

Based on the results obtained using the OppA protein as coating antigen, a concentration of 1 µg/ml *Ms01* OppA protein was chosen as the optimal coating concentration for subsequent ELISAs. Based on the results obtained using the *Ms01* whole cells as coating antigen, a concentration of $10^6$ *Ms01* whole cells/ml was chosen as the optimal coating concentration for the subsequent ELISAs. Furthermore, although the day 0 serum should not give any absorbance readings in both of these ELISAs, antibodies were consistently detected in the serum of the prebleeds of the ostriches included in this trial and this will be placed into context in the discussion that follows this section.

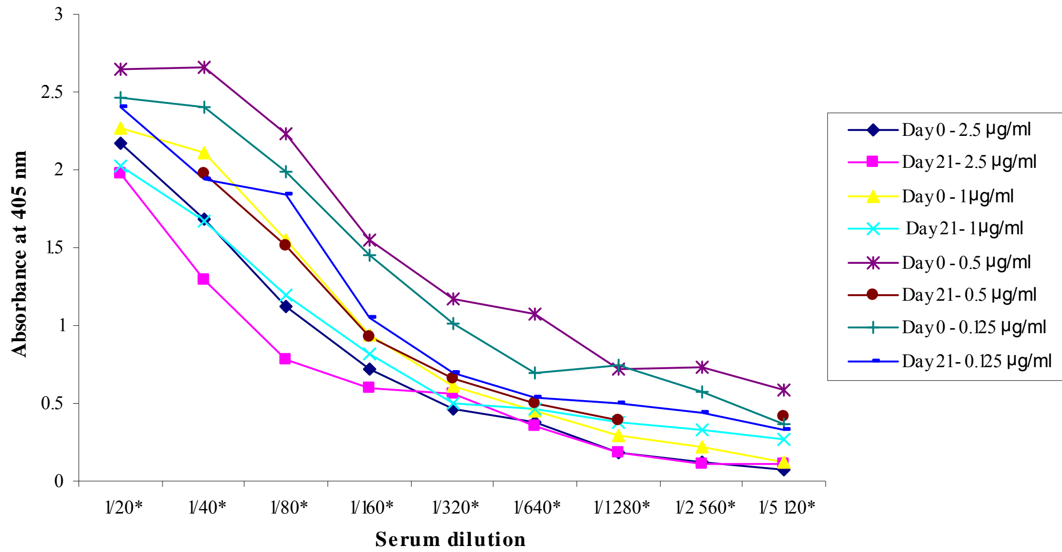


**Figure 4.3** Optimization of coating concentrations using the *Ms01* OppA protein as antigen. ELISA plates were coated with OppA protein concentrations ranging between 2.5 µg/ml to 0.125 µg/ml. Serum samples taken on day 0 and day 21, from a VR1012 vaccinated ostrich (ostrich 209), were used for the serum dilution series.

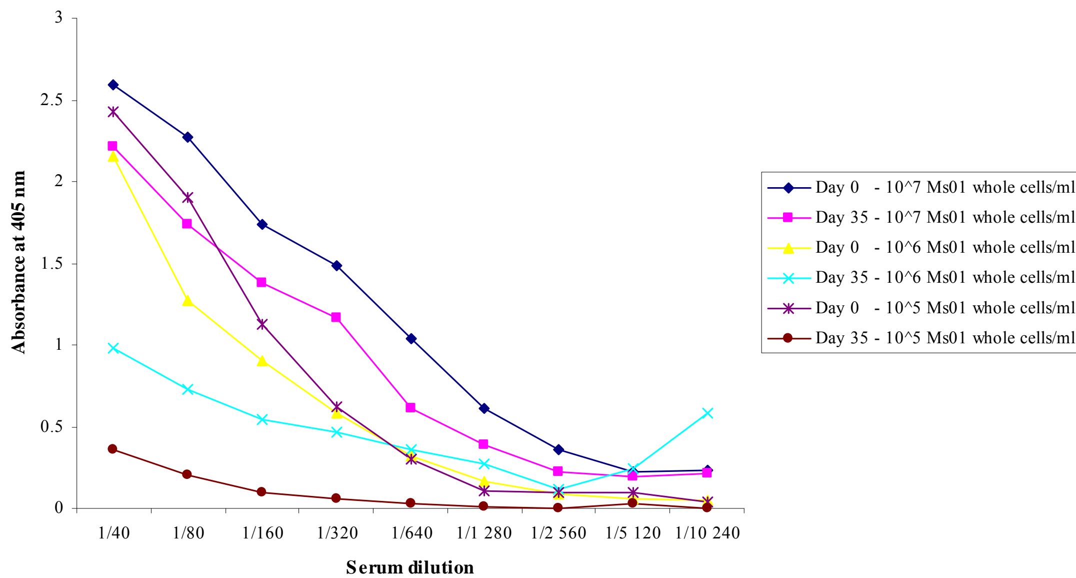


**Figure 4.4** Optimization coating concentrations using *Ms01* whole cells as coating antigen. Serum samples taken from a VR1012 vaccinated ostrich (ostrich 209) on day 0 and day 35 were used for the serum dilution series.

A serum dilution series was subsequently used in the ELISAs to determine if a single serum dilution could be used to assess the relative levels of antibodies in sera. Serum samples taken from an ostrich in each of the groups throughout the trial, were serially diluted between 1/40 and 1/5120 times. The $A_{405}$ values

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obtained when the ELISA was performed on plates coated with 1 µg/ml *Ms01* OppA protein (Figures 4.5, 4.7 and 4.9), and $10^6$ *Ms01* whole cells/ml (Figures 4.6, 4.8, and 4.10) were plotted against time.

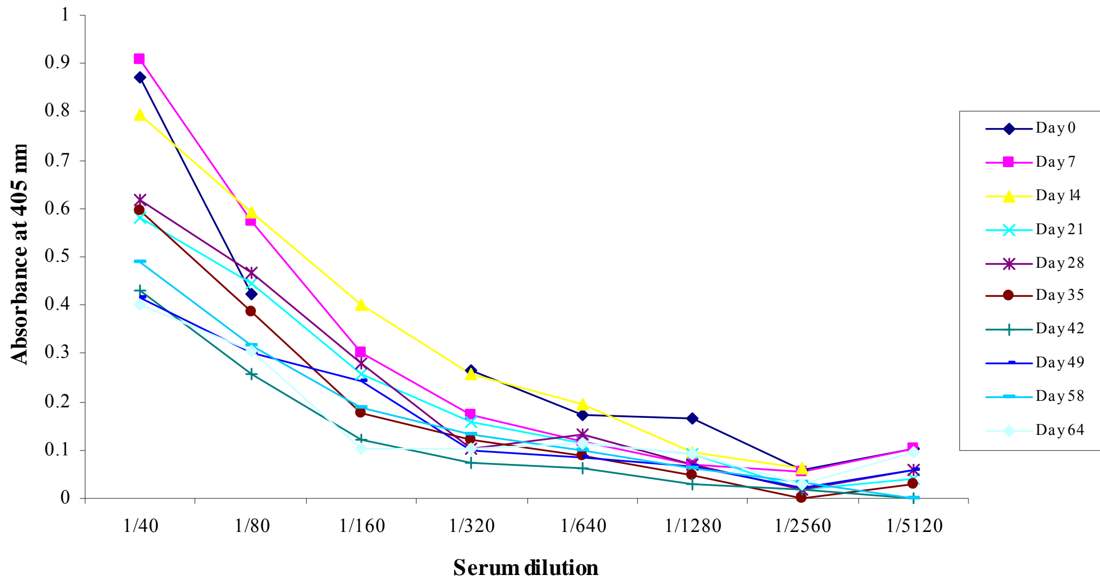


**Figure 4.5** Serum dilution analysis of serum samples collected from a pCIneo DNA vaccinated ostrich (ostrich 416). The ostrich was vaccinated on day 0 and day 35 with 100 µg of pCIneo DNA vaccine, administered intramuscularly. The ELISA plate was coated with *Ms01* OppA protein at a concentration of 1 µg/ml.

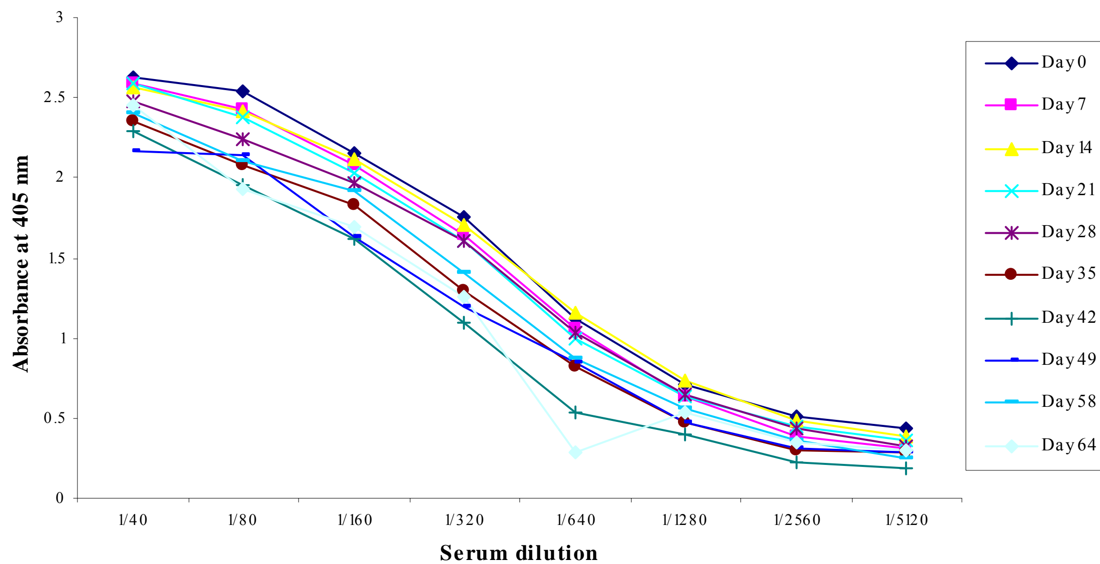


**Figure 4.6** Serum dilution analysis of serum samples collected from a pCIneo DNA vaccinated ostrich (ostrich 416). The ostrich was vaccinated on day 0 and day 35 with 100 µg of pCIneo DNA vaccine, administered intramuscularly. The ELISA plate was coated with $10^6$ *Ms01* whole cells/ml.

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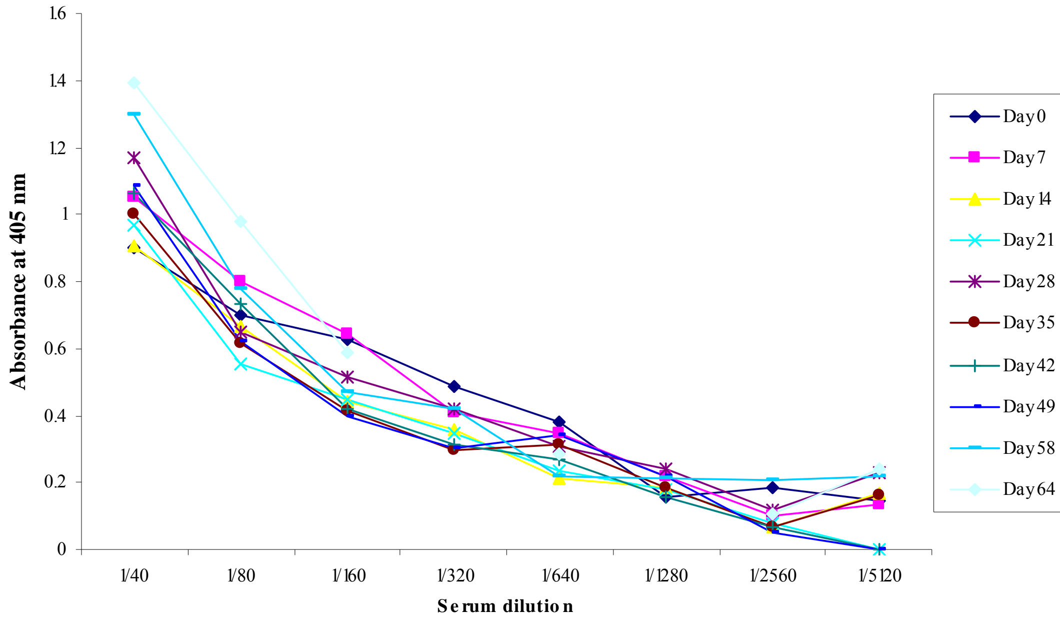


**Figure 4.7** Serum dilution analysis of serum samples collected from a VR1012 DNA vaccinated ostrich (ostrich 454). The ostrich was vaccinated on day 0 and day 35 with 100 µg of VR1012 DNA vaccine, administered intramuscularly. The ELISA plate was coated with *Ms01* OppA protein at a concentration of 1 µg/ml.

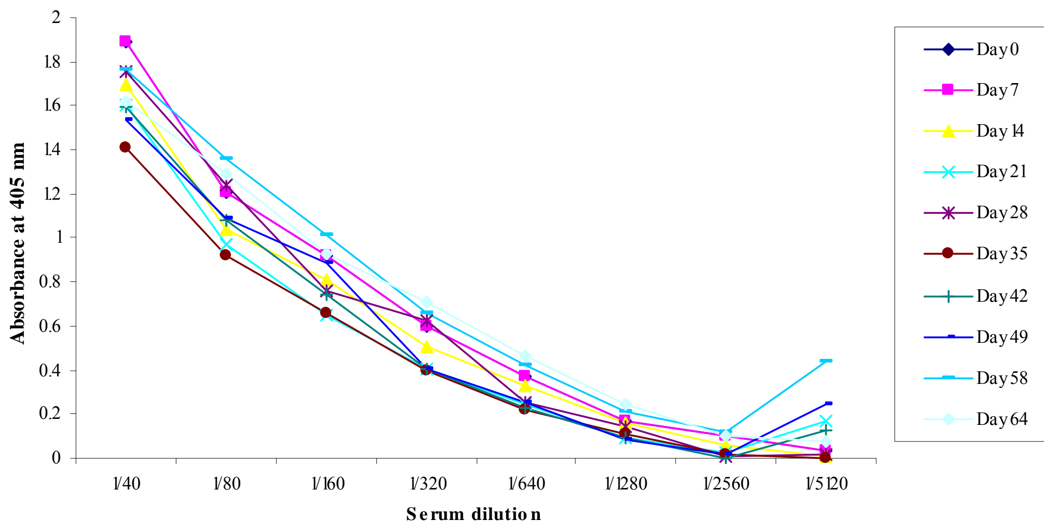


**Figure 4.8** Serum dilution analysis of serum samples collected from a VR1012 DNA vaccinated ostrich (ostrich 454). The ostrich was vaccinated on day 0 and day 35 with 100 µg of VR1012 DNA vaccine, administered intramuscularly. The ELISA plate was coated with $10^6$ *Ms01* whole cells/ml.

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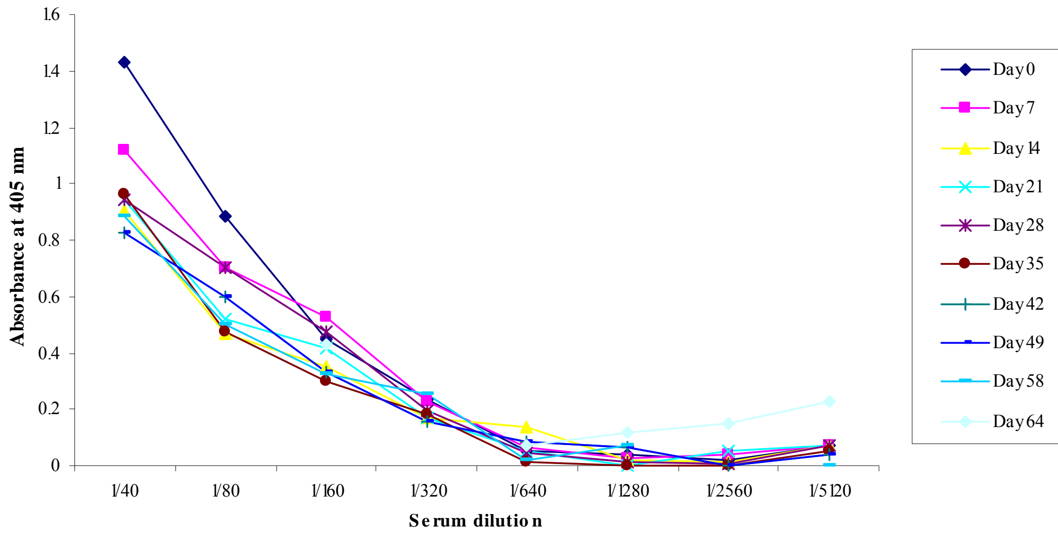


**Figure 4.9** Serum dilution analysis of serum samples collected from a VR1020 DNA vaccinated ostrich (ostrich 404). The ostrich was vaccinated on day 0 and day 35 with 100 µg of VR1020 DNA vaccine, administered intramuscularly. The ELISA plate was coated with a *Ms01* OppA protein concentration of 1 µg/ml.

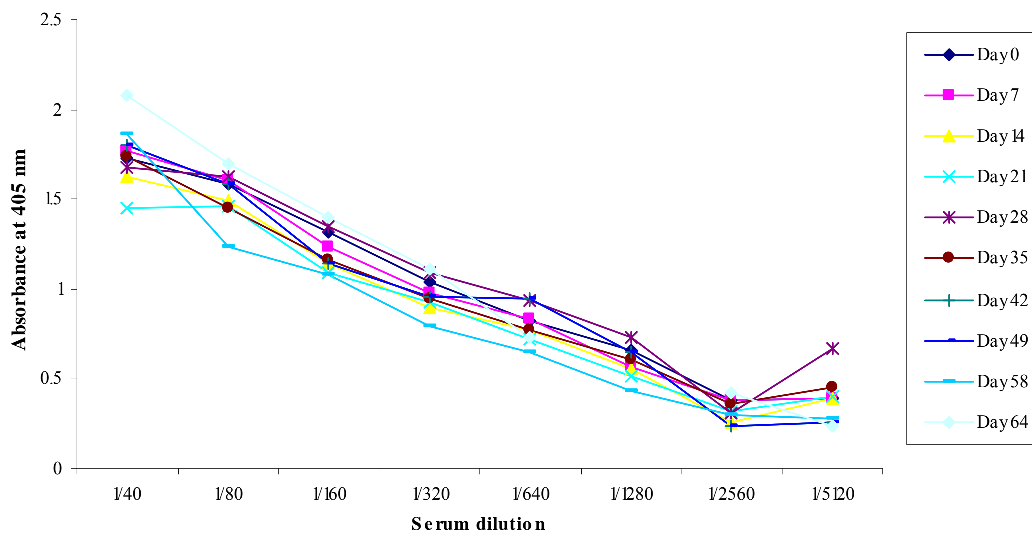


**Figure 4.10** Serum dilution analysis of serum samples collected from a VR1020 DNA vaccinated (ostrich 404). The ostrich was vaccinated on day 0 and day 35 with 100 µg of VR1020 DNA vaccine, administered intramuscularly. The ELISA plate was coated with $10^6$ *Ms01* whole cells/ml.

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A decrease in the $A_{405}$ values obtained could consistently be observed with each step in the dilution series. Thus, the more diluted the serum, the lower the $A_{405}$ values obtained. A serum dilution of 1/160 was determined to be optimal for use with all four groups of ostriches. In all the serum samples of the ostriches tested, high absorbance values were observed on day 0, indicating that anti-*Ms01* antibodies were already present in the sera of the birds before the vaccination trial was started.

### 4.3.2 Serum analysis of the pCIneo, VR1012 and VR10120 DNA vaccine trials

4.3.2.1 Antibody responses of ostriches measured by the ELISA using the *Ms01* OppA protein as coating antigen

The antibody responses elicited by ostriches vaccinated with the pCIneo, VR1012 and VR1020 DNA vaccines were presented as the individual titre values ($A_{405}$) obtained for each ostrich in each group. The individual immune responses of the ostriches in each of the four groups, analyzed using the ELISA in which the *Ms01* OppA protein was used as coating antigen, are shown in Figures 4.11, 4.12, 4.13 and 4.14 respectively.

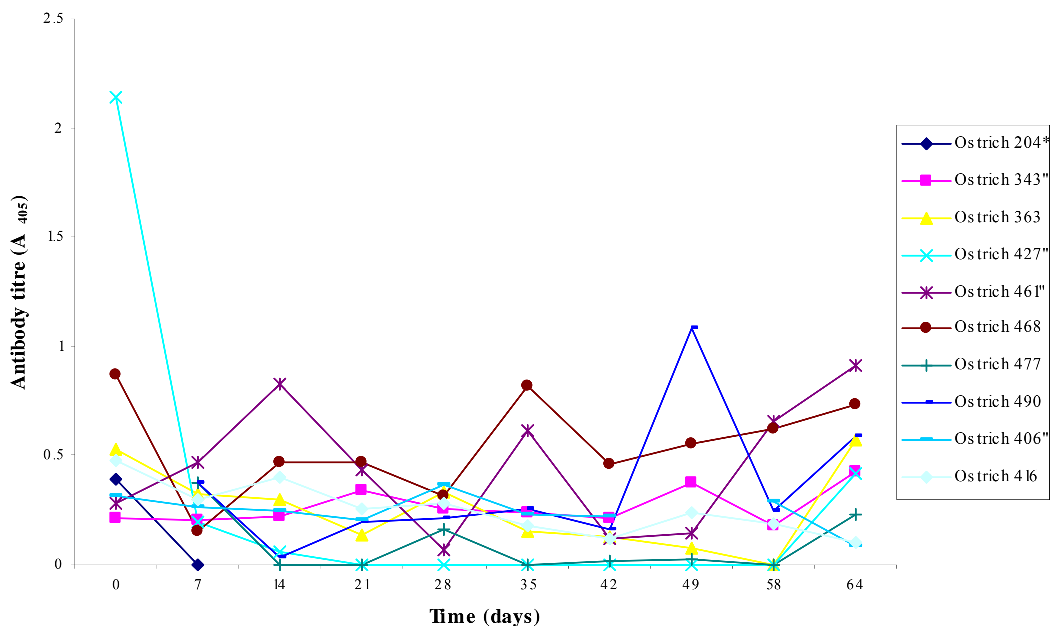


**Figure 4.11** The antibody response elicited in ostriches vaccinated with the pCIneo DNA vaccine, as determined by an ELISA utilizing 1 µg/ml *Ms01* OppA protein as coating antigen. 100 µg of pCIneo DNA vaccine was administered to ostriches on day 0 and day 35 through intramuscular injection. Ostriches which died during the trial are indicated with an asterisk (*). Ostriches diagnosed with avian influenza during the trial are marked with a quotation mark (“).

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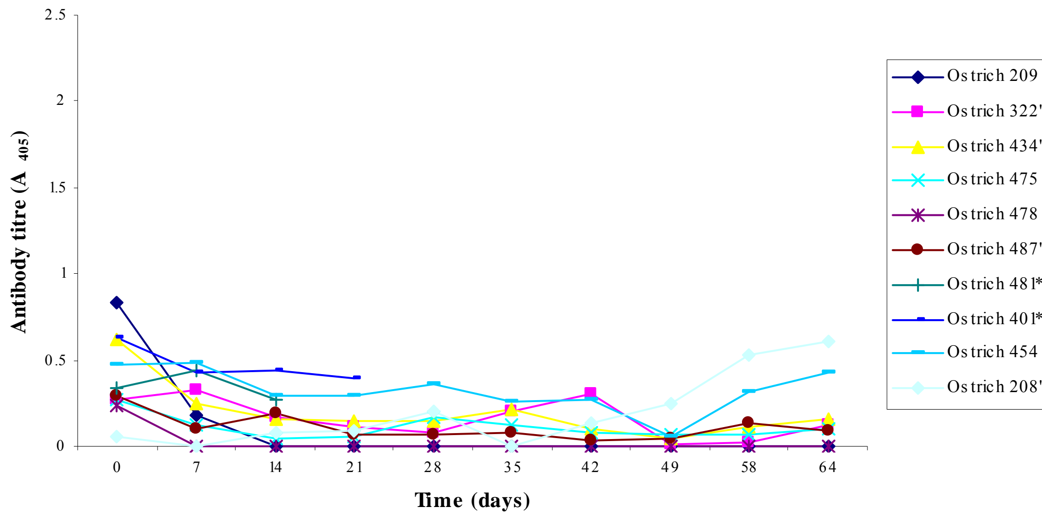


**Figure 4.12** The average antibody response elicited in ostriches vaccinated with the VR1012 DNA vaccine, as determined by an ELISA utilizing 1 µg/ml *Ms01* OppA protein as coating antigen. 100 µg of VR1012 DNA vaccine was administered to ostriches on day 0 and day 35, through intramuscular injection. Ostriches which died during the trial are indicated with an asterisk (*). Ostriches diagnosed with avian influenza during the trial are marked with a quotation mark (“).

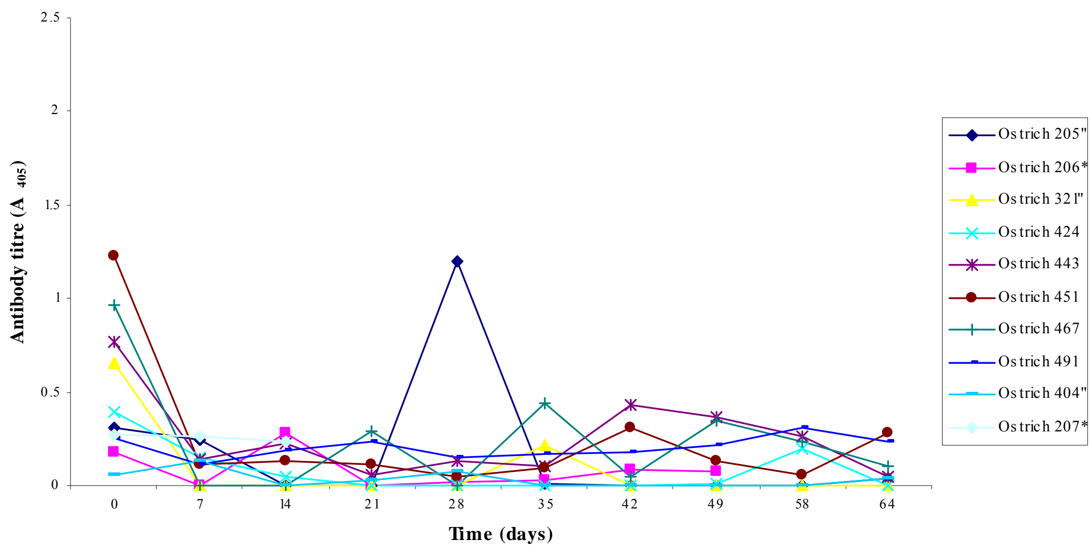


**Figure 4.13** The average antibody response elicited in ostriches vaccinated with the VR1020 DNA vaccine, as determined by an ELISA utilizing 1 µg/ml *Ms01* OppA protein as coating antigen. 100 µg of VR1020 DNA vaccine was administered to ostriches on day 0 and day 35, through intramuscular injection. Ostriches which died during the trial are indicated with an asterisk (*). Ostriches diagnosed with avian influenza during the trial are marked with a quotation mark (“).

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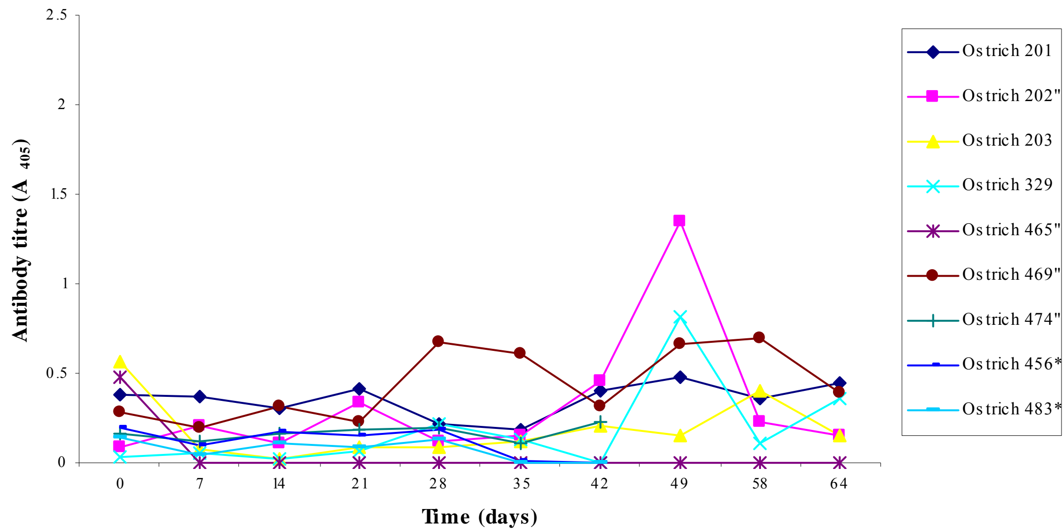


**Figure 4.14** The anti-*Ms01 OppA* antibody titres observed in the individual ostriches of the control group, as determined by an ELISA utilizing 1 µg/ml *Ms01* OppA protein as coating antigen. Ostriches in the control group did not receive any vaccine. Ostriches which died during the trial are indicated with an asterisk (*).Ostriches diagnosed with avian influenza during the trial are marked with a quotation mark (").

4.3.2.2 Antibody responses measured by the ELISA using *Ms01* whole cells as coating antigen

ELISAs using *Ms01* whole cells as coating antigen were performed on each group of ostriches in an attempt to corroborate and compare results with those obtained using the ELISA in which the *Ms01* OppA protein was used as coating antigen. The antibody responses elicited by ostriches vaccinated with the pCIneo, VR1012 and VR1020 DNA vaccines were represented as the individual titre values ($A_{405}$) obtained for each ostrich in each group. The individual immune responses of the ostriches in each of the three experimental and one control groups, analyzed using the ELISA in which *Ms01* whole cells were used as coating antigen, are shown in Figures 4.15, 4.16, 4.17 and 4.18, respectively.

If the antibody titre values, as measured with the OppA ELISA and the *Ms01* whole cell ELISA, of each individual ostrich within all the groups are compared, then firstly, it is apparent that considerable variation occurs over time. Although average values of antibody titres at each time point were calculated, the error margins were very large, and at no point could significant differences between titre values be observed. As a result, the titre values of each bird in each group were shown, and not averages and error bars as the error bars would have been very large. Secondly, there do not appear to be any general trends in any of the groups over time, i.e. an increase over time as a consequence of immunization was not observed. This applies to the ELISA results obtained using the *Ms01* OppA protein as coating antigen as well as the ELISA results obtained using *Ms01* whole cell as coating antigen.

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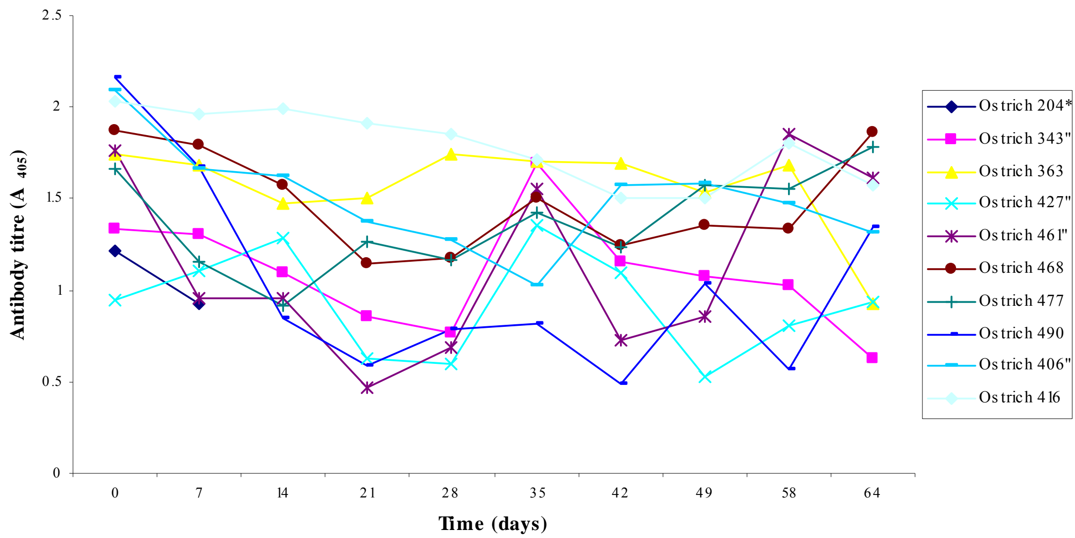


**Figure 4.15** The antibody response elicited in ostriches vaccinated with the pCIneo DNA vaccine, as determined by an ELISA utilizing $10^6$ *Ms01* whole cells/ml as coating antigen. 100 µg of pCIneo DNA vaccine was administered to ostriches on day 0 and day 35 through intramuscular injection. Ostriches which died are indicated with an asterisk (*). Ostriches diagnosed with avian influenza during the trial are marked with a quotation mark (").

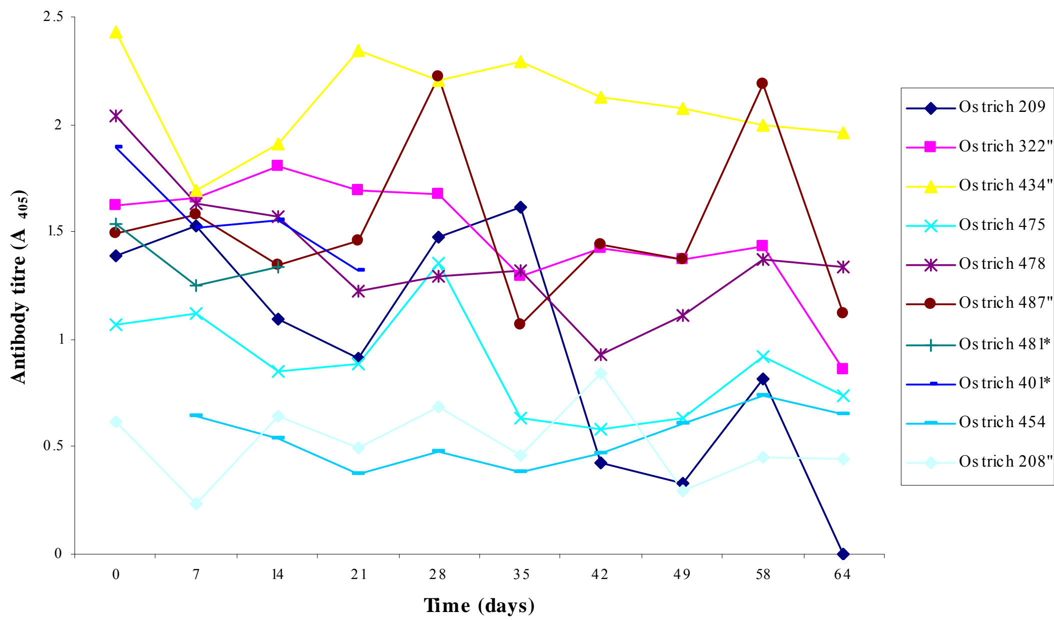


**Figure 4.16** The antibody response elicited in ostriches vaccinated with the VR1012 DNA vaccine, as determined by an ELISA utilizing $10^6$ *Ms01* whole cells/ml as coating antigen. 100 µg of VR1012 DNA vaccine was administered to test ostriches through intramuscular injection on day 0 and day 35. Ostriches which died are indicated with an astrix (*). Ostriches diagnosed with avian influenza during the trial are marked with a quotation mark (").

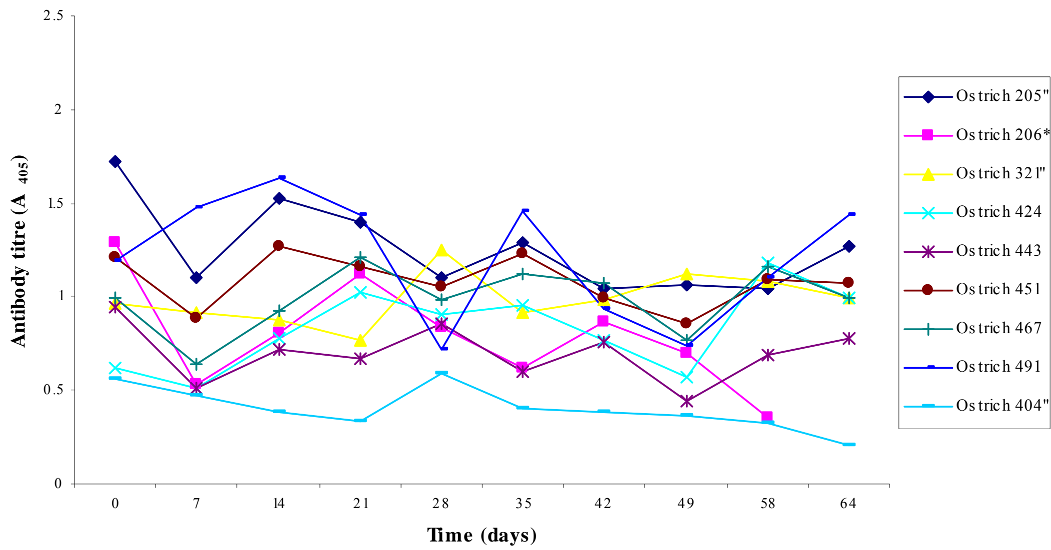


**Figure 4.17** The antibody response elicited in ostriches vaccinated with the VR1020 DNA vaccine, as determined by an ELISA utilizing $10^6$ *Ms01* whole cells/ml as coating antigen. 100 µg of VR1020 DNA vaccine was administered to test ostriches through intramuscular injection on day 0 and day 35. Ostriches which died are indicated with an asterisk (*). Ostriches diagnosed with avian influenza during the trial are marked with a quotation mark (“).

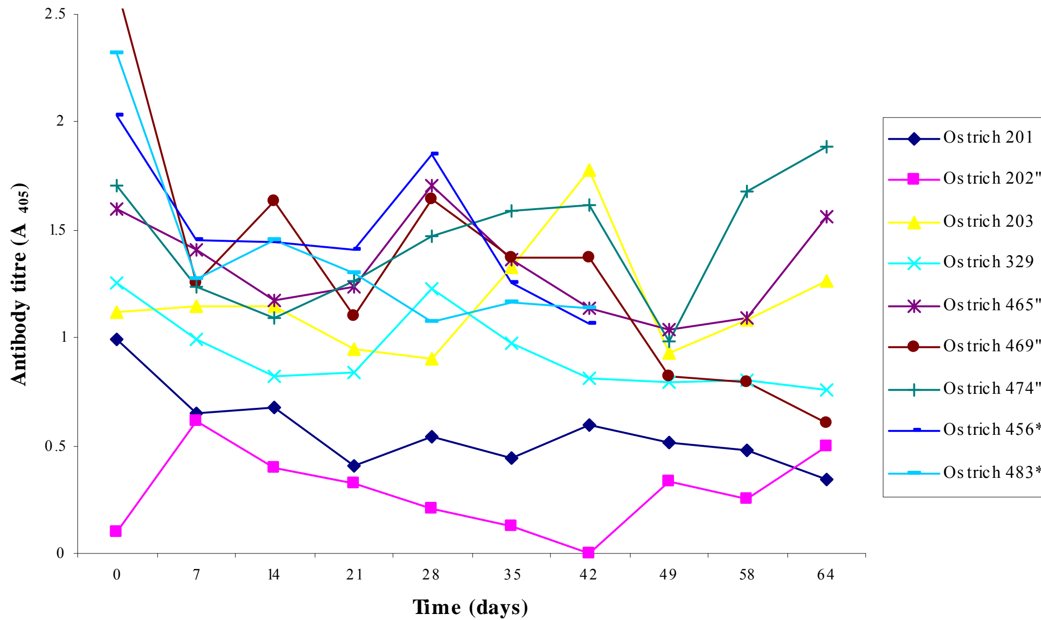


**Figure 4.18** The anti-*Ms01 OppA* antibody titres observed in the individual ostriches of the control group, as determined by an ELISA utilizing $10^6$ *Ms01* whole cells/ml as coating antigen. Ostriches in the control group did not receive any vaccine. Ostriches which died during the trial are indicated with an asterisk (*). Ostriches diagnosed with avian influenza during the trial are marked with a quotation mark (“).

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Unexpectedly high antibody titers were observed in a number of birds at the start of the trial, as determined by both ELISAs. However, high titre values of some birds were determined using the one ELISA but not in the other, for example bird 202 on day 35, where a high outlier titre value of bird 202, as determined with the ELISA using *Ms01* OppA protein as coating antigen, did not show a high value using the ELISA in which *Ms01* whole cells were used as coating antigen. The antibody responses of individual birds fluctuated, with no observed increase in antibody titres over time. In addition, high outliers frequently occurred in all groups of ostriches in the trial. As a result of this apparent variation in titre values using the two ELISAs, an attempt was made to try to establish general trends in which the results obtained with the two ELISAs did correlate and which gave explicable results. Firstly, the titre values of all birds that died during the trial were investigated, and secondly, the effects of an outbreak of avian influenza were investigated.

4.3.2.3 The possible role of mycoplasma infections on the vaccination trial

Although the ostriches in the trial were not properly inspected for symptoms of mycoplasma infections before the immunization trial was started nor at any given time point during the trial, symptoms of mycoplasma infection were noted in at least one ostrich prior to its death, and included foaming eyes and mucus exudates (ostrich 207). As mycoplasma infections prior to the start of the vaccination trial could have had a significant effect on the results of the trial, a careful investigation was made of the titres of the birds that died during the trial.

One bird (ostrich 204) that was vaccinated with the pCIneo vaccine, showed a decrease in its antibody titre on day 7, as determined by both the *Ms01* OppA protein ELISA and the *Ms01* whole cell ELISA, prior to its death. Two ostriches (ostrich 401 and 481) that were vaccinated with the VR1012 DNA vaccine died. Ostrich 401 died following a slight decrease in its antibody titre on day 21, as determined by both the *Ms01* OppA protein ELISA and the *Ms01* whole cell ELISA, while ostrich 481 died following a decrease in its antibody titre on day 14, as determined by the *Ms01* OppA protein ELISA, and an increase in antibody titre, as determined by the *Ms01* whole cell ELISA. Two birds (ostrich 206 and 207) vaccinated with the VR1020 DNA vaccine died. Ostrich 207 died following a decrease in antibody titre on day 14, while ostrich 206 died following a slight decrease in antibody titre on day 49, as determined by both the *Ms01* OppA protein ELISA and the *Ms01* whole cell ELISA. Three ostriches (ostrich 483, 456 and 421) in the control group died during the DNA vaccination trial. A decrease in the antibody titre of ostrich 483 was observed on day 35, as determined by both the *Ms01* OppA protein ELISA and the *Ms01* whole cell ELISA, prior to the death of the ostrich. Ostrich 456 died following a slight increase in antibody titre on day 28, as determined by the *Ms01* OppA protein ELISA, and a decrease in antibody titre, as determined by the *Ms01* whole cell ELISA. Although not statistically analyzed, the general trend that was observed was that birds which died showed a drop in the antibody titre, as determined by both the ELISA using *Ms01* cells as coating antigen as well as the ELISA using the *Ms01* OppA protein as coating antigen.

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4.3.2.4 An avian influenza outbreak during the vaccination trial

A state veterinarian inspected the birds that were included in the vaccination trial, as well as breeding birds that were housed in adjacent pens at regularly intervals. Six weeks after the initiation of the trial, the veterinarian suspected that some of the birds had been infected with avian influenza and placed the whole flock under quarantine. As blood samples had been collected for the vaccination trial, these blood samples were used for the detection of antibodies against avian influenza as an indication of infection. Pathologists use the appearance of antibodies as an indication of whether ostriches have been infected with avian influenza, because the virus can only be detected for a very short period after infection, whilst antibody levels in the serum of infected birds remain there for longer periods. The technical term of "seroconversion" is used if the diagnosis is based on antibody levels and this is the standard practice for the detection of avian influenza in the outbreak that is currently infecting ostriches in South Africa.

Four pCIneo vaccinated birds (ostrich 343, 406, 427 and 461) tested sero-positive for avian influenza on day 64 of the trial, after having tested negative in all the weeks prior to week 7 of the trial. Similarly, four VR1012 vaccinated ostriches (ostrich 208, 322, 434 and 487) tested sero-positive for avian influenza during the trial, with ostrich 208 and 322 testing positive for the first time on day 35, while ostrich 434 and 487 tested positive for the first time on day 64. Three VR1020 vaccinated ostriches (ostrich 205, 321 and 404) tested sero-positive for avian influenza during the vaccination trial. Ostrich 404 first tested positive for the virus on day 35 (having tested negative in all prior weeks of the trial), whereas ostrich 205 and 321 tested positive for the virus for the first time on day 64. Four ostriches (ostrich 202, 465, 469 and 474) in the control group tested sero-positive for avian influenza. Ostrich 202 tested positive for the virus for the first time on day 42, while ostrich 465, 469 and 474 first tested positive for avian influenza on day 64 of the trial.

Thus, to summarize, on day 35 of the DNA vaccination trial two ostriches in the VR1012 vaccinated group and one ostrich in the VR1020 vaccinated group tested sero-positive for avian influenza, after having tested negative on all previous days of the trial. More birds became infected as the trial progressed, to the point where 15 birds tested positive at the end of the trial on day 64 (one bird which tested positive on day 35 and 42, tested negative on day 63, but was included in the final count). None of the birds which tested positive for avian influenza died during the trial and it should be emphasized that all eight birds which did die during the trial, tested negative for avian influenza. The antibody response curves of birds which tested positive for avian influenza were equally as erratic as those observed for birds which tested negative to the virus (as determined by both the *Ms01* OppA protein ELISA and the *Ms01* whole cell ELISA). Also, no consistent similarities, for example a decrease in the antibody titre, could be detected in the *Ms01* antibody responses of birds which tested positive for avian influenza. For this reason, the titre values of the birds which tested positive for avian influenza were not removed from the graphs of the titre values shown before.

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**Table 4.1** A summary of the avian influenza outbreak which occurred during the DNA vaccine trial.

| | VR1012 vaccinated | VR1020 vaccinated | pCIneo vaccinated | Control group | Totals |
|---|---|---|---|---|---|
| **Number of birds Taking part in the trial** | 10 | 10 | 10 | 10 | 40 |
| **Number of birds which died during the trial** | 2 | 2 | 1 | 3 | 8 |
| **Number of birds which became infected with avian influenza during the trial** | 4 | 3 | 4 | 4 | 15 |
| **Number of birds which died of avian influenza during the trial** | 0 | 0 | 0 | 0 | 0 |

#### 4.3.2.5 Ostrich weight comparisons

Due to the high variation in the weights of ostriches in each of the respective vaccination groups, an analysis was conducted to compare the antibody responses obtained for specific ostriches in each group, with the weight of the individual ostriches, as measured on day 35 of the trial (Addendum C). No correlations between the weights of ostriches and the antibody response measured could be detected in any of the vaccinated groups, with ostriches as heavy as 95 kg displaying similar responses to ostriches weighing 53 kg, and ostriches of similar weights sometimes displaying very different antibody responses.

#### 4.3.2.6 Comparison between *Ms01* OppA protein ELISA results and *Ms01* whole cell antigen ELISA results

Since no increase in the antibody titres of vaccinated ostriches occurred over time, a direct comparison between the ELISA results obtained when the *Ms01* OppA protein was used as coating antigen and when the *Ms01* whole organism was used as coating antigen, was made by plotting these values against each other and investigating the relationship between them (Figures 4.19, 4.20, 4.21 and 4.22). The groups of birds that were vaccinated with the different vaccines were analyzed separately. The objective with this analysis was to detect whether vaccination of the ostriches caused an alteration in the ratio of the antibodies against *Ms01* (as selectively induced by the vaccine) as opposed to a general increase in the level of antibodies against the whole *Ms01* organism (as induced by infection with *Ms01* or other mycoplasmas prior to or during the vaccination trial).

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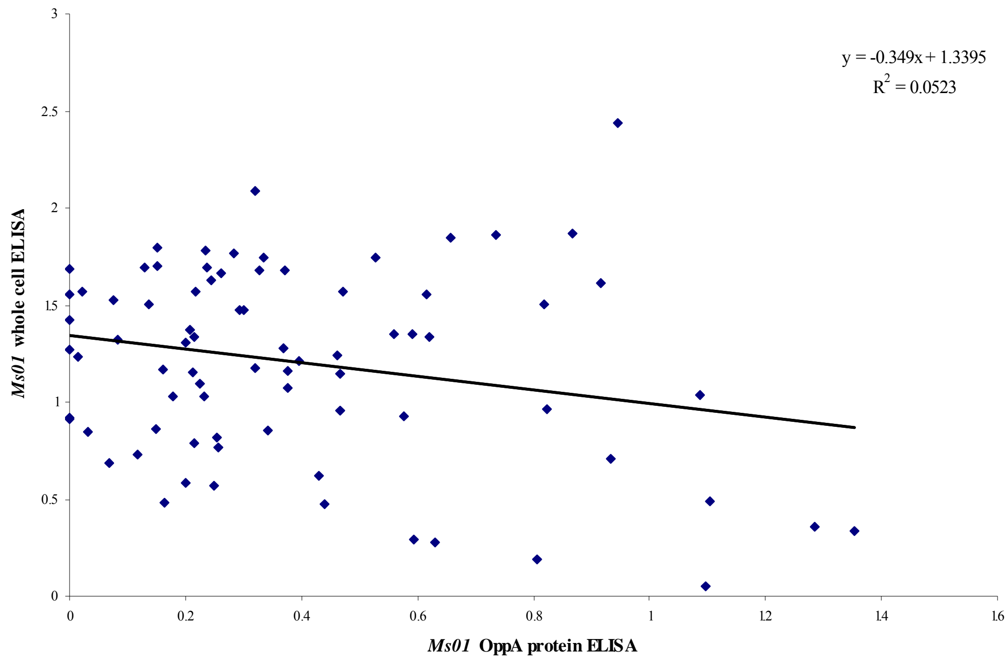


**Figure 4.19** A comparison of the antibody response curves generated following analyses of the pCIneo vaccinated ostriches by means of the *Ms01* OppA protein ELISA and *Ms01* whole organism ELISA. 100 µg of pCIneo DNA vaccine was administered to ostriches on day 0 and day 35 through intramuscular injection. Correlation statistics are shown on the graph.

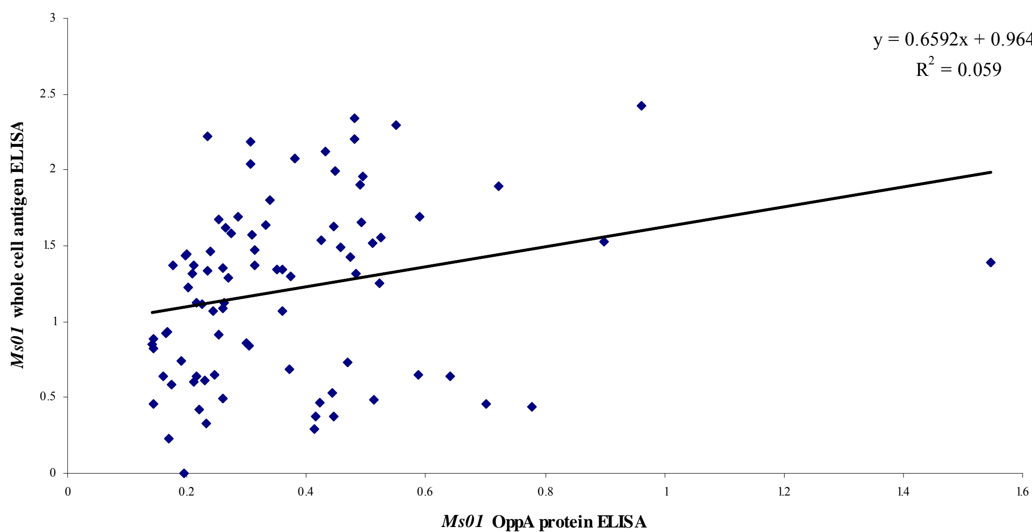


**Figure 4.20** A comparison of the antibody response curves generated following analyses of the VR1012 vaccinated ostriches by means of the *Ms01* OppA protein ELISA and *Ms01* whole organism ELISA. 100 µg of VR1012 DNA vaccine was administered to ostriches on day 0 and day 35 through intramuscular injection. The correlation statistics are shown on the graph.

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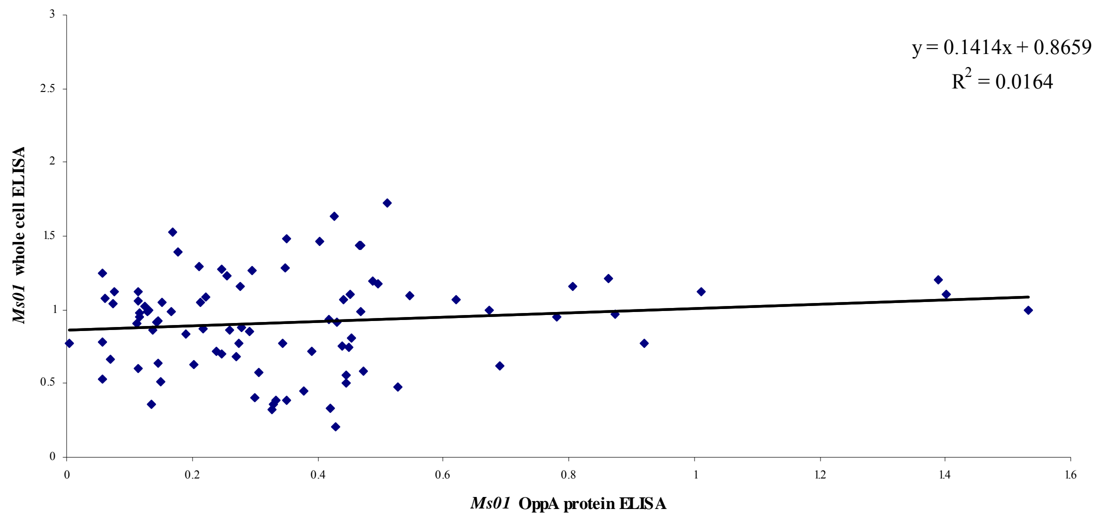


**Figure 4.21** A comparison of the average antibody response curves generated following analyses of the VR1020 vaccinated ostriches by means of the *Ms01* OppA protein ELISA and *Ms01* whole organism ELISA. 100 µg of VR1020 DNA vaccine was administered to ostriches on day 0 and day 35 through intramuscular injection. The correlation statistics are shown on the graph.

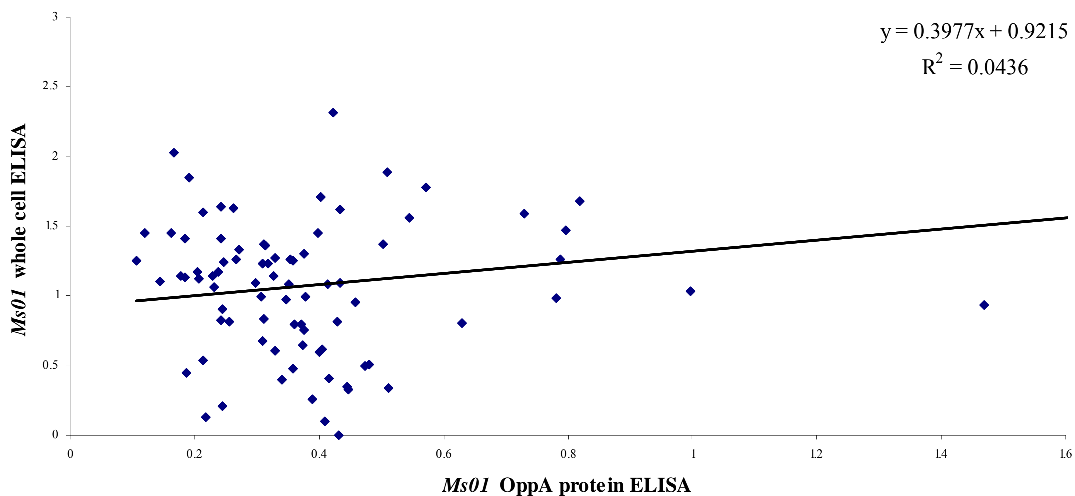


**Figure 4.22** A comparison of the antibody response curves generated following analyses of the control ostriches by means of the *Ms01* OppA protein ELISA and *Ms01* whole organism ELISA. No DNA vaccine was administered to the control ostriches. The correlation statistics are shown on the graph.

Although a negative relationship was observed between the results of the ELISA using the *Ms01* OppA protein as coating antigen and the ELISA using the *Ms01* whole cells as coating antigen for the pCIneo vaccinated group, a positive relationship between the two ELISAs was observed for all three of the remaining groups (the VR1012 and VR1020 vaccinated group and the control group). The y intercept of the three vaccinated groups and the control group was above 0, indicating that the ELISA using *Ms01*

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whole cells as coating antigen was detecting antibodies against a greater number of antigens, and not only the antibodies against the *Ms01* OppA protein as measured by the ELISA using the *Ms01* OppA protein as coating antigen. The gradient of the trend lines in all the vaccinated groups as well as the control groups was below 0.5, however, and thus failed to establish a direct relationship between the *Ms01* OppA antibody response measured by the ELISA using the *Ms01* OppA protein as coating antigen and that measured by the ELISA using the *Ms01* whole cells.

**4.4 Discussion**

The ELISA technique is commonly used in many laboratories to detect and diagnose various diseases in humans and animal species, including avian mycoplasmosis [50, 91]. However, before utilization, any ELISA must first be optimized as was done in this study. The optimal coating concentration was determined to be 1 µg/ml *of Ms01* OppA protein and $10^6$ *Ms01* cells/ml, while the optimal serum dilution was determined to be 1/160. The absorbance values obtained at a serum dilution of 1/160 resulted in the achievement of a high signal to background ratio and additionally, were mostly in the middle of linear portion of the serum dilution curve and would thus allow any increases or decreases in antibody titre to be easily observed. Specific binding of the respective antibodies to their antigen was confirmed with very low to negative absorbance values being observed in wells which did not contain any antigen (serum control wells) as well as in wells which were coated with antigen, but to which no serum was added (antigen control wells).

However, a basic flaw in these optimizations was the apparent lack of a negative signal indicating the absence of antibodies in any of the sera of the birds that were tested and particularly the general absence of antibodies in the birds at the start of the trial. The crucial question therefore really was if the ELISA was not showing nonspecific binding instead of indicating antibody levels to *Ms01* or whole mycoplasma cells. The ELISAs outlined above had not been developed at the time that the vaccination trial was started, and it was assumed that the ostriches that had not been vaccinated would test negative for antibody levels to the *Ms01* OppA protein or *Ms01* whole cells. However, the ELISA results indicated that significant antibody levels were present in the birds prior to the vaccination trial. In order to check that the ELISA was indeed specific and capable of measuring antibody levels against the *Ms01* OppA protein in animal sera, a test was performed by Amanda van Tonder, from our research group, in which ELISA plates were coated with the *Ms01* OppA protein at the same concentration as in the ELISAs described above. Two serum samples from a rabbit, one prior to vaccination with the recombinant OppA protein and one taken at the end of a series of vaccinations, were used with appropriate secondary antibodies and an equivalent detection system. In this trial it was found that the serum sample from the rabbit taken prior to vaccination gave a negative ELISA reading, i.e. an absorbance value of 0 at a serum dilution of 1:20 and greater, whilst the serum sample from the rabbit taken after vaccination gave a high absorbance value which was found to drop if the serum was serially diluted. This result can be explained because rabbits do not harbour ostrich mycoplasmas and should therefore not possess antibody levels against them, whilst after vaccination they produced antibodies

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which could be detected with the ELISA. From this result the deduction can therefore be made that the ELISA described above, in which the *Ms01* OppA protein was used as coating antigen, is capable of measuring antibody levels in ostrich serum and that the ostriches used in these trails already possessed significant antibody levels prior to the start of the vaccination trial.

The high antibody titre observed in the vast majority of samples on day 0 suggested that most of the ostriches used in the trial had already been exposed to the *Ms01* organism prior to the initiation of the vaccination trail, and that they possessed antibody levels against the *Ms01* organism. This appears likely, since no studies have yet been conducted to assess the prevalence of *Ms01* within South Africa, but a reportedly high prevalence of respiratory disease (probably due to mycoplasma infections) in South African feedlot ostriches suggests high prevalence [Pretorius, 2009]. The fact that at least one of the ostriches which died during the DNA vaccination trial (ostrich 207), displayed symptoms associated with ostrich mycoplasma infection, in addition to high antibody titres, indicates that prior exposure to the *Ms01* organism likely occurred.

It is, however, also possible that the ostriches used in the trial may have been infected with another ostrich-infecting mycoplasma prior to or during the trial, resulting in cross-reactivity in the ELISAs, as suggested by Abdelmoumen and Roy [Abdulmoumen and Roy, 1995]. According to these authors, the sharing of epitopes amongst avian mycoplasma species make serological diagnosis of avian mycoplasmas less than reliable, and that the mycoplasma ELISA is subject to nonspecificity of the reagents and cross-reactions amongst a variety of mycoplasma species. This view is, however, contradictory to what was found in an unpublished ostrich mycoplasma cross-reactivity study conducted at Onderstepoort Veterinary Institute [Pretorius, 2009]. Serological cross-reactivity tests conducted by the latter indicated that antibodies to the *Ms01* organism do not recognize *Ms02* and *Ms03*, and that although antibodies to *Ms02* partially cross-reacts with *Ms03*, they do not recognize *Ms01*. The fact that *Ms02* and *Ms03* are more closely related to each other than to *Ms01* support these findings. Some cross-reactivity can, however, not be discounted.

In the light of the above finding that the ostriches were likely exposed to the *Ms01* organism or alternatively the *Ms02* and/or *Ms03* organisms before the trial, the ELISA results should be interpreted somewhat differently. However, before interpreting the results, it is necessary to consider how an ostrich would typically respond to a successful vaccine. A primary antibody response, representing a slight increase in antibody titre, would initially be seen following vaccination on day 0. A peak in the antibody titre is usually observed during the primary antibody response, followed by a gradual decrease in titre. A boost vaccination is then administered a few weeks after the initial vaccination, resulting in a secondary antibody response. The antibody titre observed in the secondary immune response is typically many-fold higher than that seen in the primary immune response. This was also documented in the response to Newcastle Disease Virus vaccines and to two chicken mycoplasma vaccines in ostriches [Blignaut, 2000; Pretorius, 2009].

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As a result of the erratic antibody responses observed during the trial, no apparent primary and/or secondary antibody response could be detected against any of the DNA vaccines analyzed in this trial. The erratic immune responses and frequent outliers observed in all groups throughout the trial may be explained by the fact that the trial was coincidentally conducted during a period in which the ostrich mycoplasma infections are prevalent. The trial was conducted towards the end of winter and the beginning of spring, a period marked by weather conditions fluctuating between extremely cold, wet and windy weather, and more moderate sunny conditions. The incidence and severity of mycoplasma infections in ostriches are known to increase during such conditions [Pretorius, 2009]. The immunomodulatory capabilities of mycoplasmas consist of a "complex network of synergistic and antagonistic influences induced by mycoplasmas on the immune system" [Rottem, 2003], which allows mycoplasmas to either stimulate or suppress the immune system and probably accounts for the erratic immune responses observed in this trial [Rottem, 2003]. Mycoplasmas are able to induce the suppression or polyclonal stimulation of T and B lymphocytes (of which the B lymphocytes are responsible for antibody production), amongst other things [Razin *et al.,* 2009; Rottem, 2003; Bradbury, 2005]. Also, although no direct effect could be detected, the avian influenza virus most likely had an effect on the immune responses of ostriches infected with the virus, if for no other reason than the stress caused to the animal by the infection. Hence, one or the cumulative effect of the above possibilities may have resulted in the erratic immune responses observed during the trial. In addition, immunosuppression or a weakened immune system caused by an ostrich mycoplasma infection (in addition to the other stress-causing factors mentioned below) probably made the ostriches more vulnerable to other infections, and may thus be linked to the avian influenza outbreak which occurred during the trial. This possibility is supported by the fact that *M. gallisepticum* infections in chickens and turkeys have been linked to concurrent infections with viruses such as avian influenza, infectious pneumovirus, IBV and NDV [Dufour-Gesbert *et al.,* 2006; Mohammed *et al.,* 1987; Nili and Asasi, 2003; Yang *et al.,* 2009]. Moreover, the birds were very likely stressed due to the harsh weather conditions experienced during the trial, the weekly collection of blood samples, the new location to which the birds were moved prior to the initiation of the trial. Stress is known to not only be one of the contributing factors to mycoplasma infections, but to have a negative effect on the immune system by itself (which is why stressed birds are predisposed to mycoplasma infections) [Pretorius, 2009].

Since the *Ms01* organism has more epitopes than the *Ms01* OppA protein, it was expected that if the ostriches used in the trial were indeed exposed to the *Ms01* organism prior to or during the trial, that a Y-intercept of larger than zero would be obtained in a comparison between the results obtained using the *Ms01* whole cell ELISA and the *Ms01* OppA ELISA. This was indeed found to be the case. This meant that the ELISA in which *Ms01* whole cells were used as coating antigen was detecting antibodies against a greater number of antigens, than the ELISA in which *Ms01* OppA protein was used as coating antigen. In addition, since the *Ms01* OppA protein is one of the antigens present on the *Ms01* organism, a direct relationship was expected between the *Ms01* whole cell ELISA and the *Ms01* OppA ELISA. However, although a positive relationship could be observed in three of the four groups which took part in the

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vaccination trial, no direct relationship could be established in any of the groups. A possible explanation for this may be that the *Ms01* OppA protein, which is a membrane protein, is partially masked on the *Ms01* whole cell. Alternatively, this might be explained by the fact that the *Ms01* OppA protein antibody response is much more sensitive to change, since it represents only a portion of the entire antibody response to the *Ms01* organism. Any change which occurred in the *Ms01* OppA protein antibody response (whether it be in response to the DNA vaccines or for any other reason) may thus have been heavily diluted by other *Ms01*-specific antibodies in the serum, and perhaps not even detected by the *Ms01* whole cell ELISA, while the same response would easily be detected by the *Ms01* OppA ELISA and consequently cause differences in the antibody response trends to be observed between the two ELISAs. Finally, the finding that no correlation occurred between the weights of ostriches immunized and the antibody response obtained, suggests that: 1) the antibody responses elicited by the DNA vaccines are not dependant on the size of the vaccinated animal or 2) that no vaccine induced antibody response occurred in the ostriches.

The results obtained during the vaccination trial did not show conclusively that the pCIneo, VR1012 and VR1020 DNA vaccines were effective in eliciting antibodies against the *Ms01* organism and further studies will be required to evaluate whether they can be used to combat ostrich mycoplasma infections. It is imperative that in such studies it first be established that birds utilized during the vaccination trial were not previously exposed to the *Ms01* organism. The ELISA developed for use in this trial provides a means with which to do this. In addition, measures should be taken to ensure that birds are exposed to as little stress as possible by, for example, not relocating the birds prior to or during the trial, minimizing handling and blood collection times, and ensuring that birds are kept in optimal housing conditions. It is obvious that when ostriches are exposed to avian influenza other vaccination trials are immediately invalidated. However, although the results achieved in the vaccination trial of this study were jeopardized by the above mentioned factors, they were extremely valuable in establishing the conditions under which such trials will have to be undertaken in future. Future trials will have to include optimization studies in which the quantity of vaccine administered, the time of booster vaccination administration, and the use of adjuvants are investigated to establish whether the *Ms01 oppA* gene contained in the pCIneo, VR1012 and VR1020 DNA vaccines is effective in protecting ostriches against *Ms01* ostrich mycoplasma infections.

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## Chapter 5 - Conclusions and future perspectives

While mycoplasmas may still call to mind an image of a group of incapable or ambiguous organisms, mounting evidence have brought their pervasive and pathogenic potential to the fore as sophisticated, evolutionary advanced pathogens [Baseman and Tully, 1997]. The lack of effective antibiotic and vaccination therapies with which to combat these organisms bears testimony to this fact. Investigations into new vaccination strategies with which to combat mycoplasmas were inspired by the isolation of three ostrich-infecting mycoplasmas, *Ms01*, *Ms02* and *Ms03*, which cause huge losses to the South African ostrich industry each year. Vaccination using genes which encode immunogens, rather than the immunogen itself, has provided new possibilities for vaccine research, development and application, and thus stirred new hopes in the war against resilient pathogens [Gregersen, 2001]. Preliminary studies were conducted in which the ability of three such DNA vaccines, pCIneo, VR1012 and VR1020, to elicit antibody responses against the *Ms01* OppA protein in ostriches, was evaluated. Since adherence is considered an essential step in the pathogenesis of mycoplasmas, the cytoadherence-related OppA protein was considered a good candidate for vaccine development. However, in order to assess the efficacy of the DNA vaccination trial, the development of an ELISA which probes for antibodies against the *Ms01* OppA protein, was required. Thus, the *Ms01 oppA* gene was amplified, cloned, expressed and the resultant protein purified for use as coating antigen in the ELISA. Protein expression problems, as a result of the codon bias and large size of the *Ms01 oppA* gene, were overcome with the aid of a protein expression system utilizing a weaker promoter and a highly expressed GST purification tag. The identity of the expressed OppA protein was confirmed by means of SDS-PAGE and western blot, with the specific binding of serum antibodies to the antigen during the subsequent ELISA serving as additional confirmation.

The ability of the pCIneo, VR1012 and VR1020 DNA vaccines to elicit antibody responses against the *Ms01* OppA protein in ostriches could neither be confirmed nor rejected, due to the probable prior exposure of the ostriches used in the trial, to the *Ms01* and/or *Ms02* and *Ms03* organisms. An important deduction to be made from this trial, however, is that there is probably a high prevalence of the ostrich-infecting mycoplasmas in the South African ostrich industry. It might thus be a good idea to formally evaluate the prevalence of the *Ms01*, *Ms02* and *Ms03* organisms in South African ostriches. In addition, mycoplasma infected ostriches are probably predisposed to other infections such as avian influenza (as witnessed during this trial). Although there is no indication in the modest amount of literature currently available on the ostrich-infecting mycoplasmas that avian influenza has been linked to ostrich mycoplasma infections, avian influenza has previously been linked to mycoplasma infections of poultry [Bradury, 1984; Nili and Asasi, 2002; Nili and Asasi, 2003; Kleven, 2008]. Mycoplasmas and avian influenza both colonize the mucosal surfaces of birds (often causing respiratory infections) and thus occupy a similar niche. It might thus also prove useful to conduct a formal study to investigate whether or not mycoplasma infected ostriches are predisposed to avian influenza. Considering the results of the current avian influenza outbreak on the South African ostrich industry, as well as those in 2004 and 2006, such an investigation might not only serve to

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illuminate some obscurities about the ostrich infecting mycoplasmas, but may also be of aid in the battle against future avian influenza outbreaks in South Africa.

Furthermore, during vaccine development, the route of vaccine administration is known to be a key factor in the generation of immune responses. Since the mucosal surfaces serve not only as the portal of entry, but also as the target tissue of the ostrich infecting mycoplasmas, mucosal immunity in the host would be desirable [Oshop *et al.,* 2002]. In recent years, DNA vaccines have been efficiently delivered in attenuated gram positive and gram negative intracellular bacteria such as *Salmonella*, *Shigella* and *Listeria* [Yang *et al.,* 2009; Du *et al.,* 2005; Pan *et al.,* 2009]. This strategy enables administration of DNA vaccines via mucosal surfaces as well as delivery of the plasmid DNA directly to the antigen presenting cells which can elicit strong humoral and cellular immune responses against the pathogens from which the target gene was derived. *Salmonella*, in particular, has been successfully used to immunize against intracellular pathogens including NDV, hepatitis B virus and human immuno-deficiency virus (HIV). Moreover, secretory IgA (which is primarily responsible for mucosal immunity) specific for the antigens against which immunity was desired has been detected in the secretions of chickens vaccinated in this way. The possibility that *Ms01* may be an intracellular organism, which may require a cellular immune response, makes the use of *Salmonella* as carrier for *Ms01* DNA vaccines particularly promising. Also, a recent avian influenza vaccine study involving chickens found that the use of a *Salmonella*-delivered primary DNA vaccine and secondary (booster) inactivated vaccine induced strong immune responses and fully protected chickens from a high dose virus challenge [Pan *et al.,* 2009]. Similar studies conducted on mammals, involving the use of an intramuscularly-delivered primary DNA vaccine and secondary protein-based vaccine, obtained similarly positive results [Oshop *et al.,* 2002]. The use of a *Salmonella*-delivered primary *Ms01* OppA DNA vaccine and a secondary protein-based vaccine may thus serve as a good starting point for future *Ms01* vaccination trials.

However, before any future DNA vaccination trials are initiated, the ostriches utilized should first be serologically tested for prior exposure to the ostrich infecting mycoplasmas. If possible, only ostriches which test negative for previous exposure to ostrich infecting mycoplasmas should be utilized in such a trial. In addition, ostriches used in such a trial should be tested for additional infections such as *Salmonella* infections. Birds found to be infected with any organisms should then be treated with an appropriate antibiotic three weeks before initiation of the vaccination trial. In order to minimize stress on the birds, birds should not be moved to a testing facility prior to the vaccination trial, but tested on the farm on which they grew up. Additionally, handling of birds should be minimized by not collecting blood samples more than once in three weeks.

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## Addendum A

Alignment of the DNA sequences of the *oppA* gene cloned into the pET14b and pGEX-4T-1 prokaryotic expression vectors, with that of the modified *oppA* DNA sequence generated by Pretorius [2009].

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Sequence comparison of the original sequence of the Ms01 P100 gene, and the sequences of the same gene after cloning into the pEt14b and PGEX plasmid vectors

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                           10        20        30        40        50        60        70        80        90       100
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     --------ATGAAAAAAaGCGCAAGACTTTTATTATTAGGTGCTTTACCATTAGCAGCCTTAGCAGCTCCATTAGTTGCTGCGGCATGTAATAGTAAATC
pEt14b vector      CCATGGTTATGAAAAAAaGCGCAAGACTTTTATTATTAGGTGCTTTACCATTAGCAGCCTTAGCAGCTCCATTAGTTGCTGCGGCATGTAATAGTAAATC
pGEX-4T-1 vector   GTGGATCCATGAAAAAAaGCGCAAGACTTTTATTATTAGGTGCTTTACCATTAGCAGCCTTAGCAGCTCCATTAGTTGCTGCGGCATGTAATAGTAAATC

                          110       120       130       140       150       160       170       180       190       200
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     AGCCCCTTCGCAGAACACTGCTTTAGCTAAACAGCAGTTCGTTACTGAAATAAACGCAACACCAACATTTGATGCTTATACATATGATAGTTCAGCTTCA
pEt14b vector      AGCCCCTTCGCAGAACACTGCTTTAGCTAAACAGCAGTTCGTTACTGAAATAAACGCAACACCAACATTTGATGCTTATACATATGATAGTTCAGCTTCA
pGEX-4T-1 vector   AGCCCCTTCGCAGAACACTGCTTTAGCTAAACAGCAGTTCGTTACTGAAATAAACGCAACACCAACATTTGATGCTTATACATATGATAGTTCAGCTTCA

                          210       220       230       240       250       260       270       280       290       300
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     TATGGTGGATATTCTTCAAATGCTAGCTACCAACACACATCAGGTATGTTAGTTAGAGAACAAGGTGTTAATGAAATTCAAATTGATACAGTGACCTCAG
pEt14b vector      TATGGTGGATATTCTTCAAATGCTAGCTACCAACACACATCAGGTATGTTAGTTAGAGAACAAGGTGTTAATGAAATTCAAATTGATACAGTGACCTCAG
pGEX-4T-1 vector   TATGGTGGATATTCTTCAAATGCTAGCTACCAACACACATCAGGTATGTTAGTTAGAGAACAAGGTGTTAATGAAATTCAAATTGATACAGTGACCTCAG

                          310       320       330       340       350       360       370       380       390       400
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     ACACTGGAAAAGTTTCAAACTATATTACTAAACCAGCTTTCTCAAAATATACATTATCATTAGCAAAAGCTGTAGTTTTAACTTTAACAGATGGCACAGT
pEt14b vector      ACACTGGAAAAGTTTCAAACTATATTACTAAACCAGCTTTCTCAAAATATACATTATCATTAGCAAAAGCTGTAGTTTTAACTTTAACAGATGGCACAGT
pGEX-4T-1 vector   ACACTGGAAAAGTTTCAAACTATATTACTAAACCAGCTTTCTCAAAATATACATTATCATTAGCAAAAGCTGTAGTTTTAACTTTAACAGATGGCACAGT

                          410       420       430       440       450       460       470       480       490       500
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     TGTAGTTTACGATAATGATGATGCTGAAGTTGTTCCTGCACCAGATTTAACTTATGTAGATGCTGCAGGTGAAACTAAAAAAGCTTATTCATCAGCATAT
pEt14b vector      TGTAGTTTACGATAATGATGATGCTGAAGTTGTTCCTGCACCAGATTTAACTTATGTAGATGCTGCAGGTGAAACTAAAAAAGCTTATTCATCAGCATAT
pGEX-4T-1 vector   TGTAGTTTACGATAATGATGATGCTGAAGTTGTTCCTGCACCAGATTTAACTTATGTAGATGCTGCAGGTGAAACTAAAAAAGCTTATTCATCAGCATAT

                          510       520       530       540       550       560       570       580       590       600
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     CAAAGATTAAGTTCAGCAAATTCAAAATCAATTAATAGTCAAGAATTTGCAGAAAACTTGAAAAAAGCTAAAACATTACAATATGTACTTAAAGACAATT
pEt14b vector      CAAAGATTAAGTTCAGCAAATTCAAAATCAATTAATAGTCAAGAATTTGCAGAAAACTTGAAAAAAGCTAAAACATTACAATATGTACTTAAAGACAATT
pGEX-4T-1 vector   CAAAGATTAAGTTCAGCAAATTCAAAATCAATTAATAGTCAAGAATTTGCAGAAAACTTGAAAAAAGCTAAAACATTACAATATGTACTTAAAGACAATT

                          610       620       630       640       650       660       670       680       690       700
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     TAAAATGGGTAAATTCAAAAGGTGAAGAAACTAAATATCAAATTGTTCCTAAAGATTTCTATTATTCATGGCTAAGAACAAATCAAACAATTGGTAATGT
pEt14b vector      TAAAATGGGTAAATTCAAAAGGTGAAGAAACTAAATATCAAATTGTTCCTAAAGATTTCTATTATTCATGGCTAAGAACAAATCAAACAATTGGTAATGT
pGEX-4T-1 vector   TAAAATGGGTAAATTCAAAAGGTGAAGAAACTAAATATCAAATTGTTCCTAAAGATTTCTATTATTCATGGCTAAGAACAAATCAAACAATTGGTAATGT
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                            710       720       730       740       750       760       770       780       790       800
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene      TCGTCATGATGAAGAAAAAAGTGGAGGTTCAGAACAATTAGACAATGAAGTTAGAGATGCATTAGCAAGACCTAACAGTCGTGTATTTACAGATACAAGT
pEt14b vector       TCGTCATGATGAAGAAAAAAGTGGAGGTTCAGAACAATTAGACAATGAAGTTAGAGATGCATTAGCAAGACCTAACAGTCGTGTATTTACAGATACAAGT
pGEX-4T-1 vector    TCGTCATGATGAAGAAAAAAGTGGAGGTTCAGAACAATTAGACAATGAAGTTAGAGATGCATTAGCAAGACCTAACAGTCGTGTATTTACAGATACAAGT

                            810       820       830       840       850       860       870       880       890       900
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene      GAATACTCAAATGAATATGTTTTAAAAATCTTTGGTTTAGATACAGTAAAATTAAATGAAGAAAGTGAATTCGTTAAAAAAGTTGCTCCAAGTGCAAATT
pEt14b vector       GAATACTCAAATGAATATGTTTTAAAAATCTTTGGTTTAGATACAGTAAAATTAAATGAAGAAAGTGAATTCGTTAAAAAAGTTGCTCCAAGTGCAAATT
pGEX-4T-1 vector    GAATACTCAAATGAATATGTTTTAAAAATCTTTGGTTTAGATACAGTAAAATTAAATGAAGAAAGTGAATTCGTTAAAAAAGTTGCTCCAAGTGCAAATT

                            910       920       930       940       950       960       970       980       990      1000
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene      TAGGAGATGTAACAGCTGTAACCTTCCAAGGATTAACAGGTGAAGGTGCTAAAGTTCAAATGAATCAATTTTTTGATCAATTAATGCATGACTATACATT
pEt14b vector       TAGGAGATGTAACAGCTGTAACCTTCCAAGGATTAACAGGTGAAGGTGCTAAAGTTCAAATGAATCAATTTTTTGATCAATTAATGCATGACTATACATT
pGEX-4T-1 vector    TAGGAGATGTAACAGCTGTAACCTTCCAAGGATTAACAGGTGAAGGTGCTAAAGTTCAAATGAATCAATTTTTTGATCAATTAATGCATGACTATACATT

                           1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene      CTATCCAGCTCCATCACAATACATTGATGATATGAATGCAACAAATGGTTACAAATTAACTAATTACCAAGGCGATGTAACTGATAAAGTTTCTGCACTA
pEt14b vector       CTATCCAGCTCCATCACAATACATTGATGATATGAATGCAACAAATGGTTACAAATTAACTAATTACCAAGGCGATGTAACTGATAAAGTTTCTGCACTA
pGEX-4T-1 vector    CTATCCAGCTCCATCACAATACATTGATGATATGAATGCAACAAATGGTTACAAATTAACTAATTACCAAGGCGATGTAACTGATAAAGTTTCTGCACTA

                           1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene      GAAACTAAATCAAAAGCAATGGATAAAAGTAAATTAACTGCTAAATTAGGTGTTTACTGGTATGGTGTAACAGCAAATAGTACATTGTATTCAGGACCAT
pEt14b vector       GAAACTAAATCAAAAGCAATGGATAAAAGTAAATTAACTGCTAAATTAGGTGTTTACTGGTATGGTGTAACAGCAAATAGTACATTGTATTCAGGACCAT
pGEX-4T-1 vector    GAAACTAAATCAAAAGCAATGGATAAAAGTAAATTAACTGCTAAATTAGGTGTTTACTGGTATGGTGTAACAGCAAATAGTACATTGTATTCAGGACCAT

                           1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene      ACTATGCACAAGGCTTTGTAAGTGGTCAATCAGAAATATTTAAAAAGAATACTCACTTTGCAGAAAAAGCCTTTGCAGAATCTAAAAATACAGTTAATGA
pEt14b vector       ACTATGCACAAGGCTTTGTAAGTGGTCAATCAGAAATATTTAAAAAGAATACTCACTTTGCAGAAAAAGCCTTTGCAGAATCTAAAAATACAGTTAATGA
pGEX-4T-1 vector    ACTATGCACAAGGCTTTGTAAGTGGTCAATCAGAAATATTTAAAAAGAATACTCACTTTGCAGAAAAAGCCTTTGCAGAATCTAAAAATACAGTTAATGA

                           1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene      AaTTATTACAAACTATCAACAAAAAACCTTAAGCCCTGAAGAATTTAATACAAACATCTTTAACTTATATAGACAAGGTACTACATCAACTACTCCATAT
pEt14b vector       AaTTATTACAAACTATCAACAAAAAACCTTAAGCCCTGAAGAATTTAATACAAACATCTTTAACTTATATAGACAAGGTACTACATCAACTACTCCATAT
pGEX-4T-1 vector    AaTTATTACAAACTATCAACAAAAAACCTTAAGCCCTGAAGAATTTAATACAAACATCTTTAACTTATATAGACAAGGTACTACATCAACTACTCCATAT

                           1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene      TCATCATTAACTGAAGCTCAAAAACAAATCGTTAACCAAGACCCACAAGGATTTGGTATTAGATTATTCAAAAGAGAAAATACTAATTCAGCTCCTTATG
pEt14b vector       TCATCATTAACTGAAGCTCAAAAACAAATCGTTAACCAAGACCCACAAGGATTTGGTATTAGATTATTCAAAAGAGAAAATACTAATTCAGCTCCTTATG
pGEX-4T-1 vector    TCATCATTAACTGAAGCTCAAAAACAAATCGTTAACCAAGACCCACAAGGATTTGGTATTAGATTATTCAAAAGAGAAAATACTAATTCAGCTCCTTATG
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                          1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene    ATATAATCCAAACTCCATTTGTGTTTAACAATGTTACTGCAGATTACTCATTTAACGATGCTTATGCTCAATTAATGTATGGTAAAACAATAGAAGAATT
pEt14b vector     ATATAATCCAAACTCCATTTGTGTTTAACAATGTTACTGCAGATTACTCATTTAACGATGCTTATGCTCAATTAATGTATGGTAAAACAATAGAAGAATT
pGEX-4T-1 vector  ATATAATCCAAACTCCATTTGTGTTTAACAATGTTACTGCAGATTACTCATTTAACGATGCTTATGCTCAATTAATGTATGGTAAAACAATAGAAGAATT

                          1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene    AAAAGCCGGAAAAGGTACAGGAGATGCTTATATTTACGGAACAGGTTTAAGTTTTAGAACTTTATTACAAGCTGCAATTAACTGGAATACAGTAGCAGAT
pEt14b vector     AAAAGCCGGAAAAGGTACAGGAGATGCTTATATTTACGGAACAGGTTTAAGTTTTAGAACTTTATTACAAGCTGCAATTAACTGGAATACAGTAGCAGAT
pGEX-4T-1 vector  AAAAGCCGGAAAAGGTACAGGAGATGCTTATATTTACGGAACAGGTTTAAGTTTTAGAACTTTATTACAAGCTGCAATTAACTGGAATACAGTAGCAGAT

                          1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene    GTAAGAACAAACGGTGTTTCAGAAGCTTGGTTGGCGAAATTAGCCGATGGTGGTAATATTGGTGGAAAAGACCAAGAATCATCAGCAGAAAAAaCACCAT
pEt14b vector     GTAAGAACAAACGGTGTTTCAGAAGCTTGGTTGGCGAAATTAGCCGATGGTGGTAATATTGGTGGAAAAGACCAAGAATCATCAGCAGAAAAAaCACCAT
pGEX-4T-1 vector  GTAAGAACAAACGGTGTTTCAGAAGCTTGGTTGGCGAAATTAGCCGATGGTGGTAATATTGGTGGAAAAGACCAAGAATCATCAGCAGAAAAAaCACCAT

                          1810      1820      1830      1840      1850      1860      1870      1880      1890      1900
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene    TTGATGTAAAAGATAAAATTAATGCATTGAAAGCTGTAAATAAAGATAAACAATTAGTGGACTTCGGTGGCAATTTAGGAAAAGATCTAAACCCATCAGA
pEt14b vector     TTGATGTAAAAGATAAAATTAATGCATTGAAAGCTGTAAATAAAGATAAACAATTAGTGGACTTCGGTGGCAATTTAGGAAAAGATCTAAACCCATCAGA
pGEX-4T-1 vector  TTGATGTAAAAGATAAAATTAATGCATTGAAAGCTGTAAATAAAGATAAACAATTAGTGGACTTCGGTGGCAATTTAGGAAAAGATCTAAACCCATCAGA

                          1910      1920      1930      1940      1950      1960      1970      1980      1990      2000
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene    AAACGATGCTGCTGTTAGAGACAGATCTAATGTCAACGACAAAATAAAATCAGCTGGTTATGAAAAAATTAAAGAAGCTGTAAAAGCATTATTAGATGAG
pEt14b vector     AAACGATGCTGCTGTTAGAGACAGATCTAATGTCAACGACAAAATAAAATCAGCTGGTTATGAAAAAATTAAAGAAGCTGTAAAAGCATTATTAGATGAG
pGEX-4T-1 vector  AAACGATGCTGCTGTTAGAGACAGATCTAATGTCAACGACAAAATAAAATCAGCTGGTTATGAAAAAATTAAAGAAGCTGTAAAAGCATTATTAGATGAG

                          2010      2020      2030      2040      2050      2060      2070      2080      2090      2100
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene    TTTGAAAGAACACATCAAAATGTTAGACCGGCAGATGGTAAATATAGATTCACTTCATTCTATCCATTTATTAATCAATCAAAAGAATTTGGTGAATCAT
pEt14b vector     TTTGAAAGAACACATCAAAATGTTAGACCGGCAGATGGTAAATATAGATTCACTTCATTCTATCCATTTATTAATCAATCAAAAGAATTTGGTGAATCAT
pGEX-4T-1 vector  TTTGAAAGAACACATCAAAATGTTAGACCGGCAGATGGTAAATATAGATTCACTTCATTCTATCCATTTATTAATCAATCAAAAGAATTTGGTGAATCAT

                          2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene    TAAAATTTGTTAAAGAGGCTATAGAAGGATTAGATTCTAGAATTCAATTAGATTTAGTATTCTTTACTGATAATAAAGATCCTAATTATGTTGCATATAT
pEt14b vector     TAAAATTTGTTAAAGAGGCTATAGAAGGATTAGATTCTAGAATTCAATTAGATTTAGTATTCTTTACTGATAATAAAGATCCTAATTATGTTGCATATAT
pGEX-4T-1 vector  TAAAATTTGTTAAAGAGGCTATAGAAGGATTAGATTCTAGAATTCAATTAGATTTAGTATTCTTTACTGATAATAAAGATCCTAATTATGTTGCATATAT

                          2210      2220      2230      2240      2250      2260      2270      2280      2290      2300
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene    AAACCAAGGAGCAAATGGAACAAGAAACGTTGGTTGGAGTTATGACTATAACTCAATAGGTTCAGGTTATGATGGTTTATCATGGAATTGGCCATTATTC
pEt14b vector     AAACCAAGGAGCAAATGGAACAAGAAACGTTGGTTGGAGTTATGACTATAACTCAATAGGTTCAGGTTATGATGGTTTATCATGGAATTGGCCATTATTC
pGEX-4T-1 vector  AAACCAAGGAGCAAATGGAACAAGAAACGTTGGTTGGAGTTATGACTATAACTCAATAGGTTCAGGTTATGATGGTTTATCATGGAATTGGCCATTATTC
```

```
                            2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     CCAACTCTAATTAAAATTGGTGTTGAAAAAGATAGTCATCCAGAATTTGCTACTGCATTTCCAAGAATCGCTAAATTAGCAGAAGATTTATTAGCTTATC
pEt14b vector      CCAACTCTAATTAAAATTGGTGTTGAAAAAGATAGTCATCCAGAATTTGCTACTGCATTTCCAAGAATCGCTAAATTAGCAGAAGATTTATTAGCTTATC
pGEX-4T-1 vector   CCAACTCTAATTAAAATTGGTGTTGAAAAAGATAGTCATCCAGAATTTGCTACTGCATTTCCAAGAATCGCTAAATTAGCAGAAGATTTATTAGCTTATC

                            2410      2420      2430      2440      2450      2460      2470      2480      2490      2500
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     AAGAACAACCAGGTCACGAATTTGTATCTTCAGTACCATTTAAAGAATTATACAAAGTAGAACCAAGAAGATACACAGTATTGCCTACTCTATTAGCTTC
pEt14b vector      AAGAACAACCAGGTCACGAATTTGTATCTTCAGTACCATTTAAAGAATTATACAAAGTAGAACCAAGAAGATACACAGTATTGCCTACTCTATTAGCTTC
pGEX-4T-1 vector   AAGAACAACCAGGTCACGAATTTGTATCTTCAGTACCATTTAAAGAATTATACAAAGTAGAACCAAGAAGATACACAGTATTGCCTACTCTATTAGCTTC

                            2510      2520      2530      2540      2550      2560      2570      2580      2590      2600
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     AAATGTTACAAAAAATTCTGTAACAGATAAATATGAGCTTGTTTTAACAGAAAAAAATAGACCAATACCTTATAAACCACAAGGTAATAAGCAAGTAACT
pEt14b vector      AAATGTTACAAAAAATTCTGTAACAGATAAATATGAGCTTGTTTTAACAGAAAAAAATAGACCAATACCTTATAAACCACAAGGTAATAAGCAAGTAACT
pGEX-4T-1 vector   AAATGTTACAAAAAATTCTGTAACAGATAAATATGAGCTTGTTTTAACAGAAAAAAATAGACCAATACCTTATAAACCACAAGGTAATAAGCAAGTAACT

                            2610      2620      2630      2640      2650      2660      2670      2680      2690      2700
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     GATATTTATCAATACTCAGCGGTTTTCTGGAACCAATACGTAGCAGACAAAACAAATGATTATTTAACTGAATTAATGGAAGAACTAACAACATTTTTAG
pEt14b vector      GATATTTATCAATACTCAGCGGTTTTCTGGAACCAATACGTAGCAGACAAAACAAATGATTATTTAACTGAATTAATGGAAGAACTAACAACATTTTTAG
pGEX-4T-1 vector   GATATTTATCAATACTCAGCGGTTTTCTGGAACCAATACGTAGCAGACAAAACAAATGATTATTTAACTGAATTAATGGAAGAACTAACAACATTTTTAG

                            2710      2720      2730      2740      2750      2760      2770      2780      2790      2800
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MS01 OppA gene     GTATTGAATATTCATCAGCAACTATAACAAAAGCAAAAGATTCATTTGTTAACGTTTTAGTACAAAAAGGTTATGTAGCACCTTACACAGTAAATAATAG
pEt14b vector      GTATTGAATATTCATCAGCAACTATAACAAAAGCAAAAGATTCATTTGTTAACGTTTTAGTACAAAAAGGTTATGTAGCACCTTACACAGTAAATAATAG
pGEX-4T-1 vector   GTATTGAATATTCATCAGCAACTATAACAAAAGCAAAAGATTCATTTGTTAACGTTTTAGTACAAAAAGGTTATGTAGCACCTTACACAGTAAATAATAG

                            2810      2820      2830      2840
                   ....|....|....|....|....|....|....|....|....|.
MS01 OppA gene     TGTTGACATGTATGTTGATTGGAGAATCAATAAATAA
pEt14b vector      TGTTGACATGTATGTTGATTGGAGAATCAATAAATAATAACCATGG
pGEX-4T-1 vector   TGTTGACATGTATGTTGATTGGAGAATCAATAAATAAGAATTCCCG
```



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## Addendum B

The *Ms01 oppA* gene sequence. Rare codons are highlighted.

```
atg aaa aaa agc gca AGA ctt tta tta tta ggt gct tta CCA tta gca gcc tta
gca gct CCA tta gtt gct gcg gca TGT aat AGT aaa TCA gcc CCT TCG cag aac
act gct tta gct aaa cag cag ttc gtt act gaa ATA aac gca ACA CCA ACA ttt
gat gct tat ACA tat gat AGT TCA gct TCA tat ggt GGA tat tct TCA aat gct
agc tac caa cac ACA TCA ggt atg tta gtt AGA gaa caa ggt gtt aat gaa att
caa att gat ACA gtg acc TCA gac act GGA aaa gtt TCA aac tat att act aaa
CCA gct ttc TCA aaa tat ACA tta TCA tta gca aaa gct gta gtt tta act tta
ACA gat ggc ACA gtt gta gtt tac gat aat gat gat gct gaa gtt gtt CCT gca
CCA gat tta act tat gta gat gct gca ggt gaa act aaa aaa gct tat TCA TCA
gca tat caa AGA tta AGT TCA gca aat TCA aaa TCA att aat AGT caa gaa ttt
gca gaa aac ttg aaa aaa gct aaa ACA tta caa tat gta ctt aaa gac aat tta
aaa tgg gta aat TCA aaa ggt gaa gaa act aaa tat caa att gtt CCT aaa gat
ttc tat tat TCA tgg CTA AGA ACA aat caa ACA att ggt aat gtt cgt cat gat
gaa gaa aaa AGT GGA ggt TCA gaa caa tta gac aat gaa gtt AGA gat gca tta
gca AGA CCT aac AGT cgt gta ttt ACA gat ACA AGT gaa tac TCA aat gaa tat
gtt tta aaa atc ttt ggt tta gat aca gta aaa tta aat gaa gaa AGT gaa ttc
gtt aaa aaa gtt gct CCA AGT gca aat tta GGA gat gta ACA gct gta acc ttc
caa GGA tta ACA ggt gaa ggt gct aaa gtt caa atg aat caa ttt ttt gat caa
tta atg cat gac tat ACA ttc tat CCA gct CCA TCA caa tac att gat gat atg
aat gca ACA aat ggt tac aaa tta act aat tac caa ggc gat gta act gat aaa
gtt tct gca CTA gaa act aaa TCA aaa gca atg gat aaa AGT aaa tta act gct
aaa tta ggt gtt tac tgg tat ggt gta ACA gca aat AGT ACA ttg tat TCA GGA
CCA tac tat gca caa ggc ttt gta AGT ggt caa TCA gaa ATA ttt aaa aag aat
act cac ttt gca gaa aaa gcc ttt gca gaa tct aaa aat ACA gtt aat gaa att
att ACA aac tat caa caa aaa acc tta agc CCT gaa gaa ttt aat ACA aac atc
ttt aac tta tat AGA caa ggt act ACA TCA act act CCA tat TCA TCA tta act
gaa gct caa aaa caa atc gtt aac caa gac CCA caa GGA ttt ggt att AGA tta
ttc aaa AGA gaa aat act aat TCA gct CCT tat gat ATA atc caa act CCA ttt
gtg ttt aac aat gtt act gca gat tac TCA ttt aac gat gct tat gct caa tta
atg tat ggt aaa ACA ATA gaa gaa tta aaa gcc GGA aaa ggt ACA GGA gat gct
tat att tac GGA ACA ggt tta AGT ttt AGA act tta tta caa gct gca att aac
tgg aat ACA gta gca gat gta AGA ACA aac ggt gtt TCA gaa gct tgg ttg gcg
aaa tta gcc gat ggt ggt aat att ggt GGA aaa gac caa gaa TCA TCA gca gaa
aaa ACA CCA ttt gat gta aaa gat aaa att aat gca ttg aaa gct gta aat aaa
gat aaa caa tta gtg gac ttc ggt ggc aat tta GGA aaa gat CTA aac CCA TCA
gaa aac gat gct gct gtt AGA gac AGA tct aat gtc aac gac aaa ATA aaa TCA
gct ggt tat gaa aaa att aaa gaa gct gta aaa gca tta tta gat gag ttt
gaa AGA ACA cat caa aat gtt AGA ccg gca gat ggt aaa tat AGA ttc act TCA
ttc tat CCA ttt att aat caa TCA aaa gaa ttt ggt gaa TCA tta aaa ttt gtt
aaa gag gct ATA gaa GGA tta gat tct AGA att caa tta gat tta gta ttc ttt
act gat aat aaa gat CCT aat tat gtt gca tat ATA aac caa GGA gca aat GGA
ACA AGA aac gtt ggt tgg AGT tat gac tat aac TCA ATA ggt TCA ggt tat gat
ggt tta TCA tgg aat tgg CCA tta ttc CCA act CTA att aaa att ggt gtt gaa
aaa gat AGT cat CCA gaa ttt gct act gca ttt CCA AGA atc gct aaa tta gca
gaa gat tta tta gct tat caa gaa caa CCA ggt cac gaa ttt gta tct TCA gta
CCA ttt aaa gaa tta tac aaa gta gaa CCA AGA AGA tac ACA gta ttg CCT
act CTA tta gct TCA aat gtt ACA aaa aat tct gta ACA gat aaa tat gag ctt
gtt tta ACA gaa aaa aat AGA CCA ATA CCT tat aaa CCA caa ggt aat aag caa
gta act gat att tat caa tac TCA gcg gtt ttc tgg aac caa tac gta gca gac
aaa ACA aat gat tat tta act gaa tta atg gaa gaa CTA ACA ACA ttt tta ggt
att gaa tat TCA TCA gca act ATA ACA aaa gca aaa gat TCA ttt gtt aac gtt
tta gta caa aaa ggt tat gta gca CCT tac ACA gta aat aat agt gtt gac atg
tat gtt gat tgg AGA atc aat aaa taa
```

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## Addendum C

Ostrich weights as measured on day 35 of the DNA vaccination trial.

| **Ostriches vaccinated with the pCIneo DNA vaccine** | | **Ostriches vaccinated with the VR1012 DNA vaccine** | | **Ostriches vaccinated with the VR1020 DNA vaccine** | | **Ostriches which did not receive any DNA vaccine (negative control group)** | |
|---|---|---|---|---|---|---|---|
| *Ostrich* | *Weight (kg)* | *Ostrich* | *Weight (kg)* | *Ostrich* | *Weight (kg)* | *Ostrich* | *Weight (kg)* |
| 204 | dead | 208 | 62 | 205 | 58.5 | 201 | 55 |
| 343 | 79.5 | 209 | 56 | 206 | 57 | 202 | 79 |
| 363 | 92 | 322 | 65 | 207 | dead | 203 | 46.8 |
| 427 | 58 | 434 | 58.5 | 321 | 93 | 329 | 64 |
| 461 | 85.5 | 475 | 62.5 | 424 | 48.5 | 421 | dead |
| 468 | 63.5 | 478 | 67 | 443 | 62.5 | 465 | 69.5 |
| 477 | 85 | 487 | 72 | 451 | 56.5 | 469 | 48.2 |
| 490 | 76.5 | 481 | dead | 467 | 56 | 474 | 63.5 |
| 406 | 63.5 | 401 | dead | 491 | 53.5 | 456 | 56 |
| 416 | 49.5 | 454 | 96 | 404 | 70 | 483 | 54 |

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## Addendum D

The raw data obtained during the serological analyses of the ostriches in the DNA vaccination trial, as determined by the ELISA using the Ms01 OppA protein as coating antigen, follows on the next page. This is directly followed by the raw data obtained when the ELISA using Ms01 whole cells as coating antigen, was used for the serological analyses. The data are arranged in three columns, each containing the ostrich identification number, treatment (Trt), time and response (Rsp) on each page. Columns are read from top to bottom and left to right.

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ELISA using the OppA protein as antigen:

| Ostrich | Trt | Time | Resp |
|---|---|---|---|
| 204 | 1 | 0 | 0.395 |
| 343 | 1 | 0 | 0.214 |
| 363 | 1 | 0 | 0.527 |
| 427 | 1 | 0 | 2.139 |
| 461 | 1 | 0 | 0.284 |
| 468 | 1 | 0 | 0.866 |
| 477 | 1 | 0 | - |
| 490 | 1 | 0 | - |
| 406 | 1 | 0 | 0.319 |
| 416 | 1 | 0 | 0.481 |
| 209 | 2 | 0 | 0.830 |
| 322 | 2 | 0 | 0.275 |
| 434 | 2 | 0 | 0.625 |
| 475 | 2 | 0 | 0.266 |
| 478 | 2 | 0 | 0.234 |
| 487 | 2 | 0 | 0.288 |
| 481 | 2 | 0 | 0.340 |
| 401 | 2 | 0 | 0.635 |
| 454 | 2 | 0 | 0.470 |
| 208 | 2 | 0 | 0.061 |
| 205 | 3 | 0 | 0.307 |
| 206 | 3 | 0 | 0.178 |
| 321 | 3 | 0 | 0.653 |
| 424 | 3 | 0 | 0.395 |
| 443 | 3 | 0 | 0.772 |
| 451 | 3 | 0 | 1.225 |
| 467 | 3 | 0 | 0.960 |
| 491 | 3 | 0 | 0.250 |
| 404 | 3 | 0 | 0.052 |
| 201 | 4 | 0 | 0.440 |
| 202 | 4 | 0 | 0.379 |
| 203 | 4 | 0 | 0.089 |
| 329 | 4 | 0 | 0.563 |
| 465 | 4 | 0 | 0.029 |
| 469 | 4 | 0 | 0.475 |
| 474 | 4 | 0 | 0.279 |
| 456 | 4 | 0 | 0.166 |
| 483 | 4 | 0 | 0.195 |
| 204 | 1 | 7 | 0.000 |
| 343 | 1 | 7 | 0.201 |
| 363 | 1 | 7 | 0.327 |
| 427 | 1 | 7 | 0.192 |
| 461 | 1 | 7 | 0.467 |
| 468 | 1 | 7 | 0.152 |
| 477 | 1 | 7 | 0.377 |
| 490 | 1 | 7 | 0.372 |
| 406 | 1 | 7 | 0.262 |
| 416 | 1 | 7 | 0.302 |
| 209 | 2 | 7 | 0.182 |
| 322 | 2 | 7 | 0.322 |
| 434 | 2 | 7 | 0.253 |
| 475 | 2 | 7 | 0.122 |
| 478 | 2 | 7 | 0.000 |
| 487 | 2 | 7 | 0.106 |
| 481 | 2 | 7 | 0.438 |
| 401 | 2 | 7 | 0.423 |
| 454 | 2 | 7 | 0.485 |
| 208 | 2 | 7 | 0.000 |
| 205 | 3 | 7 | 0.248 |
| 206 | 3 | 7 | 0.000 |
| 321 | 3 | 7 | 0.000 |
| 424 | 3 | 7 | 0.150 |
| 443 | 3 | 7 | 0.140 |
| 451 | 3 | 7 | 0.115 |
| 467 | 3 | 7 | 0.000 |
| 491 | 3 | 7 | 0.114 |
| 404 | 3 | 7 | 0.133 |
| 201 | 4 | 7 | 0.376 |
| 202 | 4 | 7 | 0.373 |
| 203 | 4 | 7 | 0.209 |
| 329 | 4 | 7 | 0.081 |
| 465 | 4 | 7 | 0.057 |
| 469 | 4 | 7 | 0.000 |
| 474 | 4 | 7 | 0.195 |
| 456 | 4 | 7 | 0.120 |
| 483 | 4 | 7 | 0.101 |
| 204 | 1 | 14 | - |
| 343 | 1 | 14 | 0.224 |
| 363 | 1 | 14 | 0.300 |
| 427 | 1 | 14 | 0.061 |
| 461 | 1 | 14 | 0.824 |
| 468 | 1 | 14 | 0.472 |
| 477 | 1 | 14 | 0.000 |
| 490 | 1 | 14 | 0.032 |
| 406 | 1 | 14 | 0.245 |
| 416 | 1 | 14 | 0.399 |
| 209 | 2 | 14 | 0.000 |
| 322 | 2 | 14 | 0.168 |
| 434 | 2 | 14 | 0.153 |
| 475 | 2 | 14 | 0.047 |
| 478 | 2 | 14 | 0.000 |
| 487 | 2 | 14 | 0.191 |
| 481 | 2 | 14 | 0.265 |
| 401 | 2 | 14 | 0.436 |
| 454 | 2 | 14 | 0.288 |
| 208 | 2 | 14 | 0.077 |
| 205 | 3 | 14 | 0.000 |
| 206 | 3 | 14 | 0.282 |
| 321 | 3 | 14 | 0.000 |
| 424 | 3 | 14 | 0.050 |
| 443 | 3 | 14 | 0.229 |
| 451 | 3 | 14 | 0.133 |
| 467 | 3 | 14 | 0.000 |
| 491 | 3 | 14 | 0.190 |
| 404 | 3 | 14 | 0.000 |
| 201 | 4 | 14 | 0.394 |
| 202 | 4 | 14 | 0.309 |
| 203 | 4 | 14 | 0.111 |
| 329 | 4 | 14 | 0.017 |
| 465 | 4 | 14 | 0.020 |
| 469 | 4 | 14 | 0.000 |
| 474 | 4 | 14 | 0.310 |
| 456 | 4 | 14 | 0.162 |
| 483 | 4 | 14 | 0.170 |
| 204 | 1 | 21 | - |
| 343 | 1 | 21 | 0.342 |
| 363 | 1 | 21 | 0.136 |
| 427 | 1 | 21 | 0.000 |
| 461 | 1 | 21 | 0.439 |
| 468 | 1 | 21 | 0.466 |
| 477 | 1 | 21 | 0.000 |
| 490 | 1 | 21 | 0.199 |
| 406 | 1 | 21 | 0.207 |
| 416 | 1 | 21 | 0.258 |
| 209 | 2 | 21 | 0.000 |
| 322 | 2 | 21 | 0.116 |
| 434 | 2 | 21 | 0.145 |
| 475 | 2 | 21 | 0.051 |
| 478 | 2 | 21 | 0.000 |
| 487 | 2 | 21 | 0.071 |
| 481 | 2 | 21 | - |
| 401 | 2 | 21 | 0.396 |
| 454 | 2 | 21 | 0.291 |
| 208 | 2 | 21 | 0.091 |
| 205 | 3 | 21 | 0.000 |
| 206 | 3 | 21 | 0.000 |
| 321 | 3 | 21 | 0.000 |
| 424 | 3 | 21 | 0.000 |
| 443 | 3 | 21 | 0.060 |
| 451 | 3 | 21 | 0.113 |
| 467 | 3 | 21 | 0.290 |
| 491 | 3 | 21 | 0.232 |
| 404 | 3 | 21 | 0.025 |
| 201 | 4 | 21 | 0.467 |
| 202 | 4 | 21 | 0.416 |

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| | | | |
|---|---|---|---|
| 203 | 4 | 21 | 0.340 |
| 329 | 4 | 21 | 0.087 |
| 465 | 4 | 21 | 0.062 |
| 469 | 4 | 21 | 0.000 |
| 474 | 4 | 21 | 0.229 |
| 456 | 4 | 21 | 0.185 |
| 483 | 4 | 21 | 0.148 |
| 204 | 1 | 28 | - |
| 343 | 1 | 28 | 0.257 |
| 363 | 1 | 28 | 0.335 |
| 427 | 1 | 28 | 0.000 |
| 461 | 1 | 28 | 0.068 |
| 468 | 1 | 28 | 0.319 |
| 477 | 1 | 28 | 0.162 |
| 490 | 1 | 28 | 0.215 |
| 406 | 1 | 28 | 0.370 |
| 416 | 1 | 28 | 0.281 |
| 209 | 2 | 28 | 0.000 |
| 322 | 2 | 28 | 0.083 |
| 434 | 2 | 28 | 0.145 |
| 475 | 2 | 28 | 0.167 |
| 478 | 2 | 28 | 0.000 |
| 487 | 2 | 28 | 0.066 |
| 481 | 2 | 28 | - |
| 401 | 2 | 28 | - |
| 454 | 2 | 28 | 0.357 |
| 208 | 2 | 28 | 0.202 |
| 205 | 3 | 28 | 1.197 |
| 206 | 3 | 28 | 0.019 |
| 321 | 3 | 28 | 0.000 |
| 424 | 3 | 28 | 0.000 |
| 443 | 3 | 28 | 0.129 |
| 451 | 3 | 28 | 0.051 |
| 467 | 3 | 28 | 0.000 |
| 491 | 3 | 28 | 0.154 |
| 404 | 3 | 28 | 0.079 |
| 201 | 4 | 28 | 0.403 |
| 202 | 4 | 28 | 0.214 |
| 203 | 4 | 28 | 0.125 |
| 329 | 4 | 28 | 0.085 |
| 465 | 4 | 28 | 0.217 |
| 469 | 4 | 28 | 0.000 |
| 474 | 4 | 28 | 0.672 |
| 456 | 4 | 28 | 0.191 |
| 483 | 4 | 28 | 0.185 |
| 204 | 1 | 35 | - |
| 343 | 1 | 35 | 0.237 |
| 363 | 1 | 35 | 0.151 |
| 427 | 1 | 35 | 0.000 |
| 461 | 1 | 35 | 0.616 |
| 468 | 1 | 35 | 0.817 |
| 477 | 1 | 35 | 0.000 |
| 490 | 1 | 35 | 0.253 |
| 406 | 1 | 35 | 0.233 |
| 416 | 1 | 35 | 0.176 |
| 209 | 2 | 35 | 0.000 |
| 322 | 2 | 35 | 0.205 |
| 434 | 2 | 35 | 0.215 |
| 475 | 2 | 35 | 0.123 |
| 478 | 2 | 35 | 0.000 |
| 487 | 2 | 35 | 0.075 |
| 481 | 2 | 35 | - |
| 401 | 2 | 35 | - |
| 454 | 2 | 35 | 0.260 |
| 208 | 2 | 35 | 0.000 |
| 205 | 3 | 35 | 0.008 |
| 206 | 3 | 35 | 0.031 |
| 321 | 3 | 35 | 0.211 |
| 424 | 3 | 35 | 0.000 |
| 443 | 3 | 35 | 0.105 |
| 451 | 3 | 35 | 0.093 |
| 467 | 3 | 35 | 0.438 |
| 491 | 3 | 35 | 0.167 |
| 404 | 3 | 35 | 0.000 |
| 201 | 4 | 35 | 0.337 |
| 202 | 4 | 35 | 0.188 |
| 203 | 4 | 35 | 0.152 |
| 329 | 4 | 35 | 0.122 |
| 465 | 4 | 35 | 0.128 |
| 469 | 4 | 35 | 0.000 |
| 474 | 4 | 35 | 0.606 |
| 456 | 4 | 35 | 0.106 |
| 483 | 4 | 35 | 0.009 |
| 204 | 1 | 42 | - |
| 343 | 1 | 42 | 0.213 |
| 363 | 1 | 42 | 0.129 |
| 427 | 1 | 42 | 0.000 |
| 461 | 1 | 42 | 0.118 |
| 468 | 1 | 42 | 0.462 |
| 477 | 1 | 42 | 0.014 |
| 490 | 1 | 42 | 0.164 |
| 406 | 1 | 42 | 0.218 |
| 416 | 1 | 42 | 0.121 |
| 209 | 2 | 42 | 0.000 |
| 322 | 2 | 42 | 0.303 |
| 434 | 2 | 42 | 0.097 |
| 475 | 2 | 42 | 0.080 |
| 478 | 2 | 42 | 0.000 |
| 487 | 2 | 42 | 0.031 |
| 481 | 2 | 42 | - |
| 401 | 2 | 42 | - |
| 454 | 2 | 42 | 0.266 |
| 208 | 2 | 42 | 0.134 |
| 205 | 3 | 42 | 0.000 |
| 206 | 3 | 42 | 0.088 |
| 321 | 3 | 42 | 0.000 |
| 424 | 3 | 42 | 0.000 |
| 443 | 3 | 42 | 0.431 |
| 451 | 3 | 42 | 0.305 |
| 467 | 3 | 42 | 0.048 |
| 491 | 3 | 42 | 0.181 |
| 404 | 3 | 42 | 0.000 |
| 201 | 4 | 42 | 0.383 |
| 202 | 4 | 42 | 0.401 |
| 203 | 4 | 42 | 0.452 |
| 329 | 4 | 42 | 0.205 |
| 465 | 4 | 42 | 0.000 |
| 469 | 4 | 42 | 0.000 |
| 474 | 4 | 42 | 0.310 |
| 456 | 4 | 42 | 0.232 |
| 483 | 4 | 42 | 0.000 |
| 204 | 1 | 49 | - |
| 343 | 1 | 49 | 0.376 |
| 363 | 1 | 49 | 0.075 |
| 427 | 1 | 49 | 0.000 |
| 461 | 1 | 49 | 0.148 |
| 468 | 1 | 49 | 0.558 |
| 477 | 1 | 49 | 0.021 |
| 490 | 1 | 49 | 1.086 |
| 406 | 1 | 49 | - |
| 416 | 1 | 49 | 0.242 |
| 209 | 2 | 49 | 0.000 |
| 322 | 2 | 49 | 0.007 |
| 434 | 2 | 49 | 0.045 |
| 475 | 2 | 49 | 0.067 |
| 478 | 2 | 49 | 0.000 |
| 487 | 2 | 49 | 0.043 |
| 481 | 2 | 49 | - |
| 401 | 2 | 49 | - |
| 454 | 2 | 49 | 0.055 |
| 208 | 2 | 49 | 0.244 |
| 205 | 3 | 49 | 0.000 |
| 206 | 3 | 49 | 0.075 |
| 321 | 3 | 49 | 0.000 |
| 424 | 3 | 49 | 0.011 |
| 443 | 3 | 49 | 0.368 |
| 451 | 3 | 49 | 0.128 |
| 467 | 3 | 49 | 0.347 |
| 491 | 3 | 49 | 0.212 |
| 404 | 3 | 49 | 0.000 |

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| | | | |
|---|---|---|---|
| 201 | 4 | 49 | 0.459 |
| 202 | 4 | 49 | 0.480 |
| 203 | 4 | 49 | 1.350 |
| 329 | 4 | 49 | 0.147 |
| 465 | 4 | 49 | 0.813 |
| 469 | 4 | 49 | 0.000 |
| 474 | 4 | 49 | 0.658 |
| 456 | 4 | 49 | - |
| 483 | 4 | 49 | - |
| 204 | 1 | 58 | - |
| 343 | 1 | 58 | 0.179 |
| 363 | 1 | 58 | 0.000 |
| 427 | 1 | 58 | 0.000 |
| 461 | 1 | 58 | 0.656 |
| 468 | 1 | 58 | 0.619 |
| 477 | 1 | 58 | 0.000 |
| 490 | 1 | 58 | 0.248 |
| 406 | 1 | 58 | 0.293 |
| 416 | 1 | 58 | 0.188 |
| 209 | 2 | 58 | 0.000 |
| 322 | 2 | 58 | 0.027 |
| 434 | 2 | 58 | 0.112 |
| 475 | 2 | 58 | 0.070 |
| 478 | 2 | 58 | 0.000 |
| 487 | 2 | 58 | 0.138 |
| 481 | 2 | 58 | - |
| 401 | 2 | 58 | - |
| 454 | 2 | 58 | 0.314 |
| 208 | 2 | 58 | 0.532 |
| 205 | 3 | 58 | 0.000 |
| 206 | 3 | 58 | 0.000 |
| 321 | 3 | 58 | 0.000 |
| 424 | 3 | 58 | 0.201 |
| 443 | 3 | 58 | 0.261 |
| 451 | 3 | 58 | 0.059 |
| 467 | 3 | 58 | 0.233 |
| 491 | 3 | 58 | 0.309 |
| 404 | 3 | 58 | 0.000 |
| 201 | 4 | 58 | 0.494 |
| 202 | 4 | 58 | 0.359 |
| 203 | 4 | 58 | 0.232 |
| 329 | 4 | 58 | 0.404 |
| 465 | 4 | 58 | 0.114 |
| 469 | 4 | 58 | 0.000 |
| 474 | 4 | 58 | 0.695 |
| 456 | 4 | 58 | - |
| 483 | 4 | 58 | - |
| 204 | 1 | 64 | - |
| 343 | 1 | 64 | 0.429 |
| 363 | 1 | 64 | 0.575 |
| 427 | 1 | 64 | 0.414 |
| 461 | 1 | 64 | 0.915 |
| 468 | 1 | 64 | 0.735 |
| 477 | 1 | 64 | 0.234 |
| 490 | 1 | 64 | 0.591 |
| 406 | 1 | 64 | 0.083 |
| 416 | 1 | 64 | 0.104 |
| 209 | 2 | 64 | 0.000 |
| 322 | 2 | 64 | 0.129 |
| 434 | 2 | 64 | 0.159 |
| 475 | 2 | 64 | 0.097 |
| 478 | 2 | 64 | 0.000 |
| 487 | 2 | 64 | 0.094 |
| 481 | 2 | 64 | - |
| 401 | 2 | 64 | - |
| 454 | 2 | 64 | 0.431 |
| 208 | 2 | 64 | 0.608 |
| 205 | 3 | 64 | 0.042 |
| 206 | 3 | 64 | 0.022 |
| 321 | 3 | 64 | 0.000 |
| 424 | 3 | 64 | 0.000 |
| 443 | 3 | 64 | 0.049 |
| 451 | 3 | 64 | 0.279 |
| 467 | 3 | 64 | 0.100 |
| 491 | 3 | 64 | 0.230 |
| 404 | 3 | 64 | 0.035 |
| 201 | 4 | 64 | 0.533 |
| 202 | 4 | 64 | 0.431 |
| 203 | 4 | 64 | 0.608 |
| 329 | 4 | 64 | 0.042 |
| 465 | 4 | 64 | 0.022 |
| 469 | 4 | 64 | 0.000 |
| 474 | 4 | 64 | 0.000 |
| 456 | 4 | 64 | - |
| 483 | 4 | 64 | - |

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ELISA using Mso1 whole cells as antigen:

| Ostrich | Trt | Time | Resp |
|---|---|---|---|
| 204 | 1 | 0 | 1.214 |
| 343 | 1 | 0 | 1.336 |
| 363 | 1 | 0 | 1.741 |
| 427 | 1 | 0 | 0.946 |
| 461 | 1 | 0 | 1.767 |
| 468 | 1 | 0 | 1.868 |
| 477 | 1 | 0 | 1.659 |
| 490 | 1 | 0 | 2.16 |
| 406 | 1 | 0 | 2.088 |
| 416 | 1 | 0 | 2.034 |
| 209 | 2 | 0 | 1.393 |
| 322 | 2 | 0 | 1.625 |
| 434 | 2 | 0 | 2.427 |
| 475 | 2 | 0 | 1.066 |
| 478 | 2 | 0 | 2.038 |
| 487 | 2 | 0 | 1.491 |
| 481 | 2 | 0 | 1.535 |
| 401 | 2 | 0 | 1.893 |
| 454 | 2 | 0 | - |
| 208 | 2 | 0 | 0.612 |
| 205 | 3 | 0 | 1.727 |
| 206 | 3 | 0 | 1.286 |
| 321 | 3 | 0 | 0.968 |
| 424 | 3 | 0 | 0.618 |
| 443 | 3 | 0 | 0.948 |
| 451 | 3 | 0 | 1.206 |
| 467 | 3 | 0 | 0.996 |
| 491 | 3 | 0 | 1.194 |
| 404 | 3 | 0 | 0.561 |
| 201 | 4 | 0 | 0.996 |
| 202 | 4 | 0 | 0.1 |
| 203 | 4 | 0 | 1.121 |
| 329 | 4 | 0 | 1.257 |
| 465 | 4 | 0 | 1.599 |
| 469 | 4 | 0 | 2.625 |
| 474 | 4 | 0 | 1.71 |
| 456 | 4 | 0 | 2.027 |
| 483 | 4 | 0 | 2.316 |
| 204 | 1 | 7 | 0.923 |
| 343 | 1 | 7 | 1.307 |
| 363 | 1 | 7 | 1.68 |
| 427 | 1 | 7 | 1.104 |
| 461 | 1 | 7 | 0.959 |
| 468 | 1 | 7 | 1.793 |
| 477 | 1 | 7 | 1.16 |
| 490 | 1 | 7 | 1.678 |
| 406 | 1 | 7 | 1.662 |
| 416 | 1 | 7 | 1.963 |
| 209 | 2 | 7 | 1.525 |
| 322 | 2 | 7 | 1.655 |
| 434 | 2 | 7 | 1.693 |
| 475 | 2 | 7 | 1.122 |
| 478 | 2 | 7 | 1.636 |
| 487 | 2 | 7 | 1.579 |
| 481 | 2 | 7 | 1.253 |
| 401 | 2 | 7 | 1.52 |
| 454 | 2 | 7 | 0.643 |
| 208 | 2 | 7 | 0.231 |
| 205 | 3 | 7 | 1.107 |
| 206 | 3 | 7 | 0.531 |
| 321 | 3 | 7 | 0.914 |
| 424 | 3 | 7 | 0.507 |
| 443 | 3 | 7 | 0.51 |
| 451 | 3 | 7 | 0.884 |
| 467 | 3 | 7 | 0.638 |
| 491 | 3 | 7 | 1.48 |
| 404 | 3 | 7 | 0.473 |
| 201 | 4 | 7 | 0.649 |
| 202 | 4 | 7 | 0.611 |
| 203 | 4 | 7 | 1.146 |
| 329 | 4 | 7 | 0.989 |
| 465 | 4 | 7 | 1.408 |
| 469 | 4 | 7 | 1.253 |
| 474 | 4 | 7 | 1.233 |
| 456 | 4 | 7 | 1.451 |
| 483 | 4 | 7 | 1.271 |
| 204 | 1 | 14 | - |
| 343 | 1 | 14 | 1.096 |
| 363 | 1 | 14 | 1.477 |
| 427 | 1 | 14 | 1.285 |
| 461 | 1 | 14 | 0.96 |
| 468 | 1 | 14 | 1.571 |
| 477 | 1 | 14 | 0.912 |
| 490 | 1 | 14 | 0.849 |
| 406 | 1 | 14 | 1.625 |
| 416 | 1 | 14 | 1.995 |
| 209 | 2 | 14 | 1.092 |
| 322 | 2 | 14 | 1.806 |
| 434 | 2 | 14 | 1.906 |
| 475 | 2 | 14 | 0.853 |
| 478 | 2 | 14 | 1.575 |
| 487 | 2 | 14 | 1.348 |
| 481 | 2 | 14 | 1.34 |
| 401 | 2 | 14 | 1.551 |
| 454 | 2 | 14 | 0.535 |
| 208 | 2 | 14 | 0.645 |
| 205 | 3 | 14 | 1.525 |
| 206 | 3 | 14 | 0.804 |
| 321 | 3 | 14 | 0.874 |
| 424 | 3 | 14 | 0.776 |
| 443 | 3 | 14 | 0.718 |
| 451 | 3 | 14 | 1.265 |
| 467 | 3 | 14 | 0.924 |
| 491 | 3 | 14 | 1.635 |
| 404 | 3 | 14 | 0.383 |
| 201 | 4 | 14 | 0.674 |
| 202 | 4 | 14 | 0.393 |
| 203 | 4 | 14 | 1.142 |
| 329 | 4 | 14 | 0.823 |
| 465 | 4 | 14 | 1.169 |
| 469 | 4 | 14 | 1.63 |
| 474 | 4 | 14 | 1.09 |
| 456 | 4 | 14 | 1.448 |
| 483 | 4 | 14 | 1.454 |
| 204 | 1 | 21 | - |
| 343 | 1 | 21 | 0.853 |
| 363 | 1 | 21 | 1.504 |
| 427 | 1 | 21 | 0.63 |
| 461 | 1 | 21 | 0.471 |
| 468 | 1 | 21 | 1.147 |
| 477 | 1 | 21 | 1.269 |
| 490 | 1 | 21 | 0.583 |
| 406 | 1 | 21 | 1.372 |
| 416 | 1 | 21 | 1.91 |
| 209 | 2 | 21 | 0.912 |
| 322 | 2 | 21 | 1.691 |
| 434 | 2 | 21 | 2.343 |
| 475 | 2 | 21 | 0.883 |
| 478 | 2 | 21 | 1.223 |
| 487 | 2 | 21 | 1.459 |
| 481 | 2 | 21 | - |
| 401 | 2 | 21 | 1.317 |
| 454 | 2 | 21 | 0.374 |
| 208 | 2 | 21 | 0.492 |
| 205 | 3 | 21 | 1.393 |
| 206 | 3 | 21 | 1.125 |
| 321 | 3 | 21 | 0.771 |
| 424 | 3 | 21 | 1.023 |
| 443 | 3 | 21 | 0.665 |
| 451 | 3 | 21 | 1.162 |
| 467 | 3 | 21 | 1.213 |
| 491 | 3 | 21 | 1.437 |
| 404 | 3 | 21 | 0.33 |
| 201 | 4 | 21 | 0.404 |
| 202 | 4 | 21 | 0.323 |

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| | | | |
|---|---|---|---|
| 203 | 4 | 21 | 0.952 |
| 329 | 4 | 21 | 0.837 |
| 465 | 4 | 21 | 1.24 |
| 469 | 4 | 21 | 1.102 |
| 474 | 4 | 21 | 1.262 |
| 456 | 4 | 21 | 1.411 |
| 483 | 4 | 21 | 1.3 |
| 204 | 1 | 28 | - |
| 343 | 1 | 28 | 0.765 |
| 363 | 1 | 28 | 1.741 |
| 427 | 1 | 28 | 0.593 |
| 461 | 1 | 28 | 0.683 |
| 468 | 1 | 28 | 1.172 |
| 477 | 1 | 28 | 1.168 |
| 490 | 1 | 28 | 0.791 |
| 406 | 1 | 28 | 1.274 |
| 416 | 1 | 28 | 1.853 |
| 209 | 2 | 28 | 1.472 |
| 322 | 2 | 28 | 1.672 |
| 434 | 2 | 28 | 2.202 |
| 475 | 2 | 28 | 1.357 |
| 478 | 2 | 28 | 1.29 |
| 487 | 2 | 28 | 2.22 |
| 481 | 2 | 28 | - |
| 401 | 2 | 28 | - |
| 454 | 2 | 28 | 0.481 |
| 208 | 2 | 28 | 0.685 |
| 205 | 3 | 28 | 1.107 |
| 206 | 3 | 28 | 0.834 |
| 321 | 3 | 28 | 1.249 |
| 424 | 3 | 28 | 0.906 |
| 443 | 3 | 28 | 0.858 |
| 451 | 3 | 28 | 1.05 |
| 467 | 3 | 28 | 0.985 |
| 491 | 3 | 28 | 0.722 |
| 404 | 3 | 28 | 0.588 |
| 201 | 4 | 28 | 0.538 |
| 202 | 4 | 28 | 0.211 |
| 203 | 4 | 28 | 0.907 |
| 329 | 4 | 28 | 1.229 |
| 465 | 4 | 28 | 1.707 |
| 469 | 4 | 28 | 1.643 |
| 474 | 4 | 28 | 1.472 |
| 456 | 4 | 28 | 1.851 |
| 483 | 4 | 28 | 1.078 |
| 204 | 1 | 35 | - |
| 343 | 1 | 35 | 1.693 |
| 363 | 1 | 35 | 1.701 |
| 427 | 1 | 35 | 1.354 |
| 461 | 1 | 35 | 1.552 |
| 468 | 1 | 35 | 1.507 |
| 477 | 1 | 35 | 1.421 |
| 490 | 1 | 35 | 0.82 |
| 406 | 1 | 35 | 1.027 |
| 416 | 1 | 35 | 1.717 |
| 209 | 2 | 35 | 1.618 |
| 322 | 2 | 35 | 1.296 |
| 434 | 2 | 35 | 2.295 |
| 475 | 2 | 35 | 0.636 |
| 478 | 2 | 35 | 1.317 |
| 487 | 2 | 35 | 1.066 |
| 481 | 2 | 35 | - |
| 401 | 2 | 35 | - |
| 454 | 2 | 35 | 0.379 |
| 208 | 2 | 35 | 0.457 |
| 205 | 3 | 35 | 1.294 |
| 206 | 3 | 35 | 0.625 |
| 321 | 3 | 35 | 0.919 |
| 424 | 3 | 35 | 0.952 |
| 443 | 3 | 35 | 0.599 |
| 451 | 3 | 35 | 1.228 |
| 467 | 3 | 35 | 1.124 |
| 491 | 3 | 35 | 1.461 |
| 404 | 3 | 35 | 0.404 |
| 201 | 4 | 35 | 0.444 |
| 202 | 4 | 35 | 0.128 |
| 203 | 4 | 35 | 1.329 |
| 329 | 4 | 35 | 0.978 |
| 465 | 4 | 35 | 1.362 |
| 469 | 4 | 35 | 1.368 |
| 474 | 4 | 35 | 1.585 |
| 456 | 4 | 35 | 1.255 |
| 483 | 4 | 35 | 1.168 |
| 204 | 1 | 42 | - |
| 343 | 1 | 42 | 1.156 |
| 363 | 1 | 42 | 1.696 |
| 427 | 1 | 42 | 1.096 |
| 461 | 1 | 42 | 0.728 |
| 468 | 1 | 42 | 1.243 |
| 477 | 1 | 42 | 1.235 |
| 490 | 1 | 42 | 0.484 |
| 406 | 1 | 42 | 1.569 |
| 416 | 1 | 42 | 1.503 |
| 209 | 2 | 42 | 0.425 |
| 322 | 2 | 42 | 1.424 |
| 434 | 2 | 42 | 2.126 |
| 475 | 2 | 42 | 0.581 |
| 478 | 2 | 42 | 0.93 |
| 487 | 2 | 42 | 1.443 |
| 481 | 2 | 42 | - |
| 401 | 2 | 42 | - |
| 454 | 2 | 42 | 0.466 |
| 208 | 2 | 42 | 0.841 |
| 205 | 3 | 42 | 1.048 |
| 206 | 3 | 42 | 0.865 |
| 321 | 3 | 42 | 0.982 |
| 424 | 3 | 42 | 0.771 |
| 443 | 3 | 42 | 0.754 |
| 451 | 3 | 42 | 0.991 |
| 467 | 3 | 42 | 1.073 |
| 491 | 3 | 42 | 0.938 |
| 404 | 3 | 42 | 0.383 |
| 201 | 4 | 42 | 0.592 |
| 202 | 4 | 42 | 0 |
| 203 | 4 | 42 | 1.777 |
| 329 | 4 | 42 | 0.81 |
| 465 | 4 | 42 | 1.136 |
| 469 | 4 | 42 | 1.371 |
| 474 | 4 | 42 | 1.62 |
| 456 | 4 | 42 | 1.061 |
| 483 | 4 | 42 | 1.138 |
| 204 | 1 | 49 | - |
| 343 | 1 | 49 | 1.071 |
| 363 | 1 | 49 | 1.529 |
| 427 | 1 | 49 | 0.532 |
| 461 | 1 | 49 | 0.86 |
| 468 | 1 | 49 | 1.35 |
| 477 | 1 | 49 | 1.57 |
| 490 | 1 | 49 | 1.035 |
| 406 | 1 | 49 | 1.584 |
| 416 | 1 | 49 | 1.508 |
| 209 | 2 | 49 | 0.329 |
| 322 | 2 | 49 | 1.374 |
| 434 | 2 | 49 | 2.075 |
| 475 | 2 | 49 | 0.637 |
| 478 | 2 | 49 | 1.114 |
| 487 | 2 | 49 | 1.374 |
| 481 | 2 | 49 | - |
| 401 | 2 | 49 | - |
| 454 | 2 | 49 | 0.606 |
| 208 | 2 | 49 | 0.295 |
| 205 | 3 | 49 | 1.063 |
| 206 | 3 | 49 | 0.698 |
| 321 | 3 | 49 | 1.121 |
| 424 | 3 | 49 | 0.574 |
| 443 | 3 | 49 | 0.446 |
| 451 | 3 | 49 | 0.854 |
| 467 | 3 | 49 | 0.768 |
| 491 | 3 | 49 | 0.743 |
| 404 | 3 | 49 | - |

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| | | | |
|---|---|---|---|
| 201 | 4 | 49 | 0.511 |
| 202 | 4 | 49 | 0.333 |
| 203 | 4 | 49 | 0.93 |
| 329 | 4 | 49 | 0.795 |
| 465 | 4 | 49 | 1.035 |
| 469 | 4 | 49 | 0.819 |
| 474 | 4 | 49 | 0.986 |
| 456 | 4 | 49 | - |
| 483 | 4 | 49 | - |
| 204 | 1 | 58 | - |
| 343 | 1 | 58 | 1.026 |
| 363 | 1 | 58 | 1.685 |
| 427 | 1 | 58 | 0.806 |
| 461 | 1 | 58 | 1.848 |
| 468 | 1 | 58 | 1.334 |
| 477 | 1 | 58 | 1.554 |
| 490 | 1 | 58 | 0.572 |
| 406 | 1 | 58 | 1.475 |
| 416 | 1 | 58 | 1.802 |
| 209 | 2 | 58 | 0.82 |
| 322 | 2 | 58 | 1.434 |
| 434 | 2 | 58 | 1.994 |
| 475 | 2 | 58 | 0.924 |
| 478 | 2 | 58 | 1.368 |
| 487 | 2 | 58 | 2.19 |
| 481 | 2 | 58 | - |
| 401 | 2 | 58 | - |
| 454 | 2 | 58 | 0.734 |
| 208 | 2 | 58 | 0.453 |
| 205 | 3 | 58 | 1.046 |
| 206 | 3 | 58 | 0.359 |
| 321 | 3 | 58 | 1.079 |
| 424 | 3 | 58 | 1.177 |
| 443 | 3 | 58 | 0.686 |
| 451 | 3 | 58 | 1.089 |
| 467 | 3 | 58 | 1.163 |
| 491 | 3 | 58 | 1.099 |
| 404 | 3 | 58 | 0.32 |
| 201 | 4 | 58 | 0.48 |
| 202 | 4 | 58 | 0.257 |
| 203 | 4 | 58 | 1.084 |
| 329 | 4 | 58 | 0.801 |
| 465 | 4 | 58 | 1.089 |
| 469 | 4 | 58 | 0.796 |
| 474 | 4 | 58 | 1.679 |
| 456 | 4 | 58 | - |
| 483 | 4 | 58 | - |
| 204 | 1 | 64 | - |
| 343 | 1 | 64 | 0.624 |
| 363 | 1 | 64 | 0.93 |
| 427 | 1 | 64 | 0.933 |
| 461 | 1 | 64 | 1.611 |
| 468 | 1 | 64 | 1.859 |
| 477 | 1 | 64 | 1.779 |
| 490 | 1 | 64 | 1.348 |
| 406 | 1 | 64 | 1.319 |
| 416 | 1 | 64 | 1.578 |
| 209 | 2 | 64 | 0 |
| 322 | 2 | 64 | 0.861 |
| 434 | 2 | 64 | 1.961 |
| 475 | 2 | 64 | 0.738 |
| 478 | 2 | 64 | 1.337 |
| 487 | 2 | 64 | 1.124 |
| 481 | 2 | 64 | - |
| 401 | 2 | 64 | - |
| 454 | 2 | 64 | 0.651 |
| 208 | 2 | 64 | 0.44 |
| 205 | 3 | 64 | 1.272 |
| 206 | 3 | 64 | - |
| 321 | 3 | 64 | 0.991 |
| 424 | 3 | 64 | 0.993 |
| 443 | 3 | 64 | 0.781 |
| 451 | 3 | 64 | 1.069 |
| 467 | 3 | 64 | 0.999 |
| 491 | 3 | 64 | 1.437 |
| 404 | 3 | 64 | 0.209 |
| 201 | 4 | 64 | 0.345 |
| 202 | 4 | 64 | 0.498 |
| 203 | 4 | 64 | 1.264 |
| 329 | 4 | 64 | 0.759 |
| 465 | 4 | 64 | 1.562 |
| 469 | 4 | 64 | 0.603 |
| 474 | 4 | 64 | 1.886 |

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