Identification and molecular characterization of immunogenic antigens in *Mycoplasma mycoides* subsp. *mycoides* small colony type

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Dedicated to my beloved Parents

(Especially to the cherished memories of my father)
This study has been published in part:

Abstract:

Identification of immunogenic polypeptides from Mycoplasma mycoides subsp. mycoides SC genomic library using phage display.
89th Conference of Research Workers in Animal Diseases (CRWAD)
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1 Introduction

Mycoplasma mycoides subsp. mycoides small colony type (MmmSC) is the etiological agent of Contagious Bovine Pleuropneumonia (CBPP), a highly contagious respiratory disease in cattle. MmmSC belongs to the genus Mycoplasma, a member of the class Mollicutes, which has evolved from Gram-positive bacteria that possess genomes with low G+C contents. The clinical appearance of CBPP can vary from peracute and acute forms with 70% mortality to subacute, chronic, and latent forms. The clinical symptoms of acute CBPP involve respiratory distress, coughing, cessation of rumination, anorexia, and severe pleuritic pain. Since the disease is transmitted solely by direct animal-to-animal contact clinically healthy or inconspicuous carriers are the major cause for disease distribution by trade over long distances.

CBPP is mainly present in subsaharan Africa. Currently it is responsible for major losses in livestock production in Africa and, therefore, has serious socioeconomic consequences. In most other parts of the world, CBPP was eradicated using drastic policies of stamping-out, control of cattle movement, and quarantine measures, which are not applicable in nomadic African societies. The more recent re-emerging outbreak of CBPP in some European countries (outbreaks between 1993 and 1994) required expensive eradication measures and demonstrated the constant threat of the disease to industrialized countries.

Vaccination using life vaccine strains is currently the main method to control the spread of CBPP. Drawbacks of life vaccine strains are adverse reactions, possible failures to elicit a protective immune response and a significantly retained virulence in some cases leading to clinical CBPP. CBPP-testing is done by serology using a Complement Fixation Test (CFT) or an Enzyme Linked Immuno Sorbant Assay (ELISA) based on the species-specific LppQ protein. Due to limited specificity of the serology, culture with subsequent confirmation by PCR is required in areas of low prevalence.

Recently, the complete genome sequence of MmmSC type strain PG1 has been determined. Therefore, rational whole genome-based approaches to identify new species-specific immunogenic MmmSC proteins are now possible, and the phage
display technology has been shown to be a suitable tool for this purpose. This technology which is based on i) the construction of an expression library in the filamentous bacteriophage M13, ii) the expression of the putative foreign antigens as fusions with the minor coat protein III (gIII), and iii) the identification of immunogenic epitopes by an antibody-based screening was used in the work presented here.
2 Literature review

2.1.1 Mycoplasma

The genus *Mycoplasma* is a member of the class *Mollicutes* which has evolved from Gram-positive bacteria that lack a cell wall (SIRAND-PUGNET et al. 2007). They can be parasitic or saprophytic and, not having a cell wall, they are unaffected by penicillin or other beta-lactam antibiotics that target cell wall synthesis. As a group, *Mollicutes* have small genomes (580 to 1380 kb) with a low G+C-content (18-40 mol%) and reduced biosynthetic capabilities which explains their dependence on the host (DYBVIG and VOELKER 1996; SIRAND-PUGNET et al. 2007). Most *Mycoplasma* species require sterols for the stability of their cytoplasmic membrane. Sterols are acquired from the environment, usually as cholesterol from the animal host. Additionally they use an alternate genetic code where the codon UGA is encoding the amino acid tryptophan instead of the usual opal stop codon.

2.1.2 Contagious Bovine Pleuropneumonia (CBPP)

In 1896 Nocard and Roux reported the cultivation of *Mycoplasma mycoides* subspecies *mycoides* SC (*Mmm*SC) as the causative agent of Contagious Bovine Pleuropneumonia (CBPP). CBPP is a highly contagious respiratory disease in cattle and the only bacterial disease included in the former List A of the World Organization of Animal Health (http://www.oie.int) of prioritized infectious animal diseases. CBPP appears in different forms, ranging from a peracute and acute variant with 70% mortality to subacute and chronic forms. The clinical symptoms of acute CBPP involve respiratory distress, coughing, cessation of rumination, anorexia, and severe pleuritic pain (WESTBERG et al. 2004). Since the disease is transmitted by direct contact between infected and susceptible individuals only, subacute and chronic forms are associated with a high risk of introducing the infectious agent into previously uninfected herds (FAO 2000).
2.1.3 Prevalence

Contagious Bovine Pleuropneumonia (CBPP) has sporadically occurred in all regions of the world (SCHNEIDER et al. 1994). Currently, CBPP is mainly present in Sub-Saharan Africa, and annual losses of US$ 2 billion have been ascribed to the disease although the reliability of this figure is uncertain (MASIGA and DOMENECH 1995; ROEDER et al. 1999). It is responsible for major losses in livestock production in Africa and, therefore, has serious socioeconomic consequences and is of major importance in the international trade of animals and animal products (FAO 2003).

CBPP is widespread in Africa and is also assumed to be prevalent in some countries of Asia (WESTBERG et al. 2004). During the 1980s and 1990s, there have also been several outbreaks of CBPP in southern Europe. In Italy the disease reappeared in 1990 but was eliminated in 1993 (FAO 1997). The last case in Spain was in 1994. In Portugal, after a period without cases being diagnosed, the disease reappeared in 1983; following the implementation of an eradication programme the number of cases has declined rapidly in recent years, from 2818 in 1996, 64 in 1997, 12 in 1998 with a single case in 1999 (OIE 2001). No cases have been reported in 2000. These outbreaks, which required expensive eradication measures, demonstrated the constant threat of the disease even to industrialized countries (BRUDERER et al. 2002).

2.1.4 Virulence factors

In spite of the fact that MmmSC has been identified more than 100 years ago, its molecular mechanisms of pathogenicity and its virulence factors are still poorly known (RAZIN et al. 1998). In contrast to other pathogenic bacteria, where virulence is mainly determined by toxins, cytolysins and invasins, no such typical primary virulence factors have been identified in genomes of completely sequenced Mycoplasma species (PILO et al. 2007). This might be due to the small genomes of Mycoplasma species with the genome of MmmSC having a size of 1211 Kb (WESTBERG et al. 2004). This minimal genome may have led the Mycoplasma species to radically economise genetic resources and biosynthetic capacities and
adapt to an obligate parasitic life style (RAZIN et al. 1998). Instead of the expression of virulence factors pathogenicity of *Mycoplasma* species appears to be due to intrinsic metabolic and catabolic functions which also ensure the microbe’s survival in the host (FREY and NICOLET 1997; PILO et al. 2007).

### 2.1.5 Control measures

Progress was made in controlling CBPP in Africa during the colonial era and during the first two decades following independence. Large parts of Southern, Western and Eastern Africa were cleared using slaughter and movement control as well as testing strategies based on the complement fixation test (CFT) (HAMMOND and BRANAGAN 1965). However, the CFT (CAMPBELL and TURNER 1953) as well as the more recently developed competitive ELISA (LE GOFF and THIAUCOURT 1998) do not reliably detect carrier animals. Furthermore, with a sensitivity of 70% these tests – although well suited for the diagnosis of infected herds - but are not reliable as a means of establishing freedom from infection in individual animals.

As commercial vaccines became available, control programmes increasingly relied upon vaccination combined with movement control. However, it appears that the quality of vaccines used in recent times may have declined (WAITE and MARCH 2001). There are a number of factors that may have contributed to this situation such as the lack of independent quality control measures in some manufacturing facilities in Africa and poorly maintained cold-chains (THIAUCOURT et al. 2003). The incomplete protective efficacy, the occurrence of post-vaccination reactions in up to 1% of vaccinated animals as well as occasional deaths have contributed to owner reluctance to use existing vaccines in some areas. Furthermore, the serological tests available do not detect vaccinated animals effectively so that sero-monitoring as a means of monitoring vaccination and establishing levels of herd immunity resulting from vaccination cannot be conducted effectively (THIAUCOURT et al. 2003).

In the 1980s and 1990s, economic crises afflicted many African countries, and the subsequent structural adjustment programmes resulted in a decline of funding for public veterinary services. This had an inevitable effect on surveillance and control.
programmes in Africa, including those for CBPP (WINDSOR 2000). Other factors such as increasing public empowerment, recognition of the negative effects of movement control on pastoral livelihoods and a decline in the ability of veterinary services to enforce policies has decreased the effectiveness of measures adopted against CBPP. As a result, the disease is again present throughout most parts of Africa (FAO 2000; MASIGA et al. 1996).

2.1.6 Serological tests

For serological diagnosis the complement fixation test (CFT) (CAMPBELL and TURNER 1953) is still the most widely used test. Some authors report it as highly sensitive (70%) in the acute phase, with lower sensitivity in later stages, other authors report an overall sensitivity of only 63.6% (BELLINI et al. 1998). Specificity is reported to be 98% and, therefore, some authors report false positive results leading to the misclassification of up to one third of the herds investigated (STARK et al. 1995).

Since the CFT is labor-intensive and difficult to standardize, an indirect ELISA was developed but found to show many non-specific reactions. Consequently a more specific (100% in vaccinated animals) and sensitive (80 to 93% in different cases) competitive ELISA was developed and introduced into 11 African countries through an FAO/IAEA Co-ordinated Research Project (CRP) on the “Monitoring of Contagious Bovine Pleuropneumonia in Africa using enzyme immunoassays” (LE GOFF and LEFEVRE 1989; LE GOFF and THIAUCOURT 1998). The main objective of this CRP was to compare and validate the main serological tests for the diagnosis of CBPP, in particular the CFT and the competitive ELISA (cELISA). The relative sensitivity of the cELISA vs. CFT was determined to be 0.96 and the relative specificity 0.97. Conversely, the relative sensitivity of the CFT vs. the cELISA was determined to be 0.9 (90%) and the relative specificity 0.99 (LE GOFF and THIAUCOURT 1998).

Lipoprotein Q (LppQ) appears to be specific for *MmmSC*. The aminoterminal domain of mature LppQ was shown to be surface-exposed. It induced a strong, specific,
early, and persistent immune response in naturally and in experimentally infected animals. The carboxyterminal end of LppQ contains an integral membrane domain consisting of repeated units rich in hydrophobic and aromatic amino acids, which have a pore forming capacity (ABDO et al. 2000). Using the LppQ protein as solid phase antigen 97% percent of the sera diagnosed negative for CBPP by CFT (titers lower than 50% of complement fixation at a serum dilution of 1:10) or suspicious (titers with 50% to 75% of complement fixation at a serum dilution of 1:10) were negative while 79% of the CFT-positive sera (titers with 100% of complement fixation at a dilution of 1:10) reacted positive (BRUDERER et al. 2002).

The good correlation of the LppQ-based ELISA with the CFT and the strong antigenicity of LppQ have been exploited for the development of a robust indirect ELISA for serological diagnosis and for epidemiological investigations of CBPP (BRUDERER et al. 2002). This ELISA showed a high level of diagnostic sensitivity (88.5) and specificity (97.8) when used in one country at different temperatures but not enough data are available for reliable estimates under different epidemiological conditions (BRUDERER et al. 2002).

2.1.7 CBPP vaccines

All effective CBPP vaccines are live vaccines; current vaccine preparations are based on strains T1 44 and T1 SR and contain freeze-dried broth culture material (ROEDER et al. 1999). The vaccines are generally considered to exhibit poor stability and protective efficacy (RWEYEMAMU et al. 1995; THIAUCOURT et al. 2000). Live vaccine strains have drawbacks of adverse reactions and a significantly retained virulence in some cases leading to clinical CBPP (MBULU et al. 2004). A severe drawback is that attenuated vaccines produce local as well as systemic toxic reactions (PIERCY 1970) and sometimes they may potentiate the disease (PROVOST 1996). They relatively have short duration of post-vaccinal immunity (MASIGA and DOMENECH 1995).

In Australia CBPP was successfully eradicated using the MmmSC strain V5 broth vaccine, and no problems concerning both, thermostability and protective efficacy
were observed under field conditions and found to be as hostile as those likely to be encountered in Africa (NEWTON and NORRIS 2000). Thus, keeping the liquid vaccine wrapped in a damp cloth and protecting it from direct sunlight was adequate to maintain protective efficacy (HUDSON 1968b). Indeed, even vaccine strain KH3J was successfully used as a vaccine strain in Australia during the early 1960s (HUDSON 1968a).

2.1.8 Phage display technology

The phage display technology is based on the groundbreaking work of G.P Smith (SMITH 1985). In this system genotype and phenotype of a polypeptide are linked by fusing short gene fragments to the minor coat protein III gene (gIII) of the filamentous bacteriophage M13. This results in the expression of a fusion protein on the phage surface, allowing affinity purification of the protein of interest and its encoding gene by binding of the polypeptide for example to a specific antibody (SMITH and SCOTT 1993). The in vitro procedure for isolating polypeptides or peptides by their binding activity was called panning (PARMLEY and SMITH 1988). At first, the gene fragments were directly inserted into the phage genome fused to the wildtype gIII gene (MCCAFFERTY et al. 1990). Today, most successful phage display systems uncouple polypeptide expression from phage propagation by providing the genes encoding the polypeptide::pIII fusion proteins on a separate plasmid, termed as phagemid that contains a phage morphogenetic signal for packaging into the assembled phage particles (BREITLING et al. 1990). Hyperphage (RONDOT et al. 2001; SOLTES et al. 2007) has a truncated gIII gene on the phage genome and the only source of pIII - an essential M13 coat protein - is the oligopeptide::pIII fusion protein encoded on the phagemid. Infective phage particles can only be produced if cloned DNA inserts are in-frame behind the pelB leader sequence and the pIII ORF downstream. The produced phage particles, containing foreign DNA and displaying the corresponding foreign polypeptide on the phage surface, can be used directly for panning.
In general, the phage display technology is widely used for display and selection of peptides (SIDHU et al. 2003; SIDHU and KOIDE 2007), antibodies (HUST and DÜBEL 2004; HUST and DÜBEL 2005; TAUSSIG et al. 2007); alternative scaffolds (SKERRA 2007) and further oligopeptides. The display and selection of oligopeptides by phage display is a crucial tool for the identification of protein-ligand interactions (COCHRANE et al. 2000), selection of lectins (YANG et al. 2007), identification of allergic proteins (KODZIUS et al. 2003; RHYNER et al. 2004; CRAMERI et al. 2001) or the profiling of Multiple Sclerosis-associated autoantibodies (GOVARTS et al. 2007) from cDNA or genomic libraries.

For the development of diagnostic tools and vaccines new immunogenic oligopeptides have to be identified. To date, only a few immunogenic oligopeptides in Mycoplasma are known (FUTO et al. 1995; HSU et al. 1997; KIM et al. 1990; MEENS et al. 2006; STRASSER et al. 1991). This is due to the usage of UGA codons encoding tryptophan and the low GC content which cause problems in identifying immunogenic oligopeptides by recombinant DNA technology.

### 2.1.9 Bacteriophage M13

The bacteriophage M13 belongs to the filamentous phages and possesses a circular, single stranded positive strand DNA of 6407 nucleotides in length. The phage has a length of 700-900 nm and a width of approximately 6.5 nm with a relative molecular mass of approximately 16.3 MD (RUSSEL and MODEL 1988). The DNA is encapsulated in a coat containing approximately 2700 copies of major coat protein pVIII, and capped with 5 copies each of there different minor coat proteins (pIV, pVI, pIII) on the ends (Fig. 1). The minor coat protein pIII attaches to the receptor at the tip of the F pilus of the host *E.coli* (VIEIRA and MESSING 1987). For replication M13 attaches to its host and the DNA is taken up via the F-pilus. Once inside the cell the single stranded DNA molecule acts as the template for synthesis of a complementary strand resulting in the double stranded replicative form (RF-form).
The replication of the plus strands takes place via the rolling circle mechanism using the bacterial DNA replication machinery. Phage envelope proteins are synthesized as integral membrane proteins of the bacterial host. The bacteria do not lyse during phage production, but experience an extension of generation time. Each bacterial cell infected produces approximately 100-200 phages (BREITLING and S.ĐÜBEL 1997).

2.1.10 Phage display vector pHORF3

For the presentation of polypeptides on the surface of the phage M13, a fusion of the polypeptide gene fragment with the coat protein gene \textit{gIII} of the phage is necessary (CRAMERI et al 2003). Fig. 2 shows a schematic diagram of the phagemid pHORF3. The phagemid contains necessary components of a plasmid such as a bacterial origin of replication, an antibiotic resistance determinant and a unique Pmel restriction endonuclease site for cloning located in between a signal sequence and the \textit{gIII} gene encoding protein pIII. Using the Hyperphage – encoding a truncated pIII protein - as a helper phage the only source of pIII (an essential M13 coat protein) is the polypeptide:pIII fusion protein encoded on the phagemid. Infective phage particles can only be produced if cloned DNA inserts are in-frame with both the \textit{pelB} leader sequence and \textit{gIII}. (HUST et al. 2006).
Fig. 2: Schematic diagram of phagemid pHORF3. Lac Pr., *lacZ*-promoter; RBS, ribosomal binding site; *pel*β, signal sequence for periplasmatic secretion of bacterial protein; Pmel, restrictions endonuclease site; *tag*, His-Tag; amber, stop-codon; *gIII*, gene coding for phage envelope protein pIII; M13 ori, origin of replication for M13-Phage; *bla*, ß-lactamase gene; ColE1 ori, origin of replication of *E. coli* ColE1 plasmids.

2.1.11 Panning

The combination of genotype and phenotype in the phage display vectors permits the selection of specific binders out of large libraries over the affinity to a target protein (RHYNER et al. 2004). This type of selection is very common especially in the area of recombinant antibody generation (HUST et al. 2007), it is named “panning” and, at the time, was a novel procedure for isolating proteins by their binding activity in vitro (PARMLEY and SMITH 1988). In the panning procedure, the targeted proteins (e.g. antigen or antibody) are immobilized and incubated with a phage display library. During this panning specific pIII fusion proteins bind to the respective target protein, and unspecifically bound phages are removed by repeated washing steps. The specifically bound phages are eluted and reamplified by infection of *E. coli*. For enrichment of specific binders from highly complex libraries (10^6 to 10^12 different phages) two to six panning rounds are carried out (BREITLING and S.DÜBEL 1997).
2.1.12 Importance of the development of diagnostic tools

Reliable and efficient diagnostic tests for the serological diagnosis of infectious diseases are the cornerstone of any disease control strategy. The requirements for diagnostic tests depend on the purpose of the diagnostic testing and the epidemiological needs. The monitoring of a region-wide vaccination programme has different needs compared to the needs for export certification of individual animals. In the case of CBPP countries adjacent to infected areas may want to confirm absence of the disease relying on serology and slaughterhouse inspections. In this case, in order to minimize the risk of introducing the disease, tests with high diagnostic sensitivity are required in order to detect close to 100% of infected animals. Countries operating a disease control programme based on stamping out of positive herds and compensation of the farmers require tests with a high diagnostic specificity; here, the diagnostic sensitivity is not crucial as it can be increased to acceptable levels by increasing the number of animals tested in the herds under investigation. In countries where the disease is present and control programmes include vaccinations a suitable diagnostic test must facilitate the differentiation of infected and vaccinated animals. This goal could be achieved following the concept of DIVA (Differentiating Infected and Vaccinated Animals) vaccines (VAN OIRSCHOT 2001), which is based on the absence of one immunogenic protein in the vaccine strain which is present in the wild-type. The proof of concept for field applications has been given for viral DIVA vaccines such as pseudorabies virus (PRV) and bovine herpesvirus 1 (BHV1) vaccines (VAN OIRSCHOT et al. 1996), which are being used successfully to eradicate the diseases.

2.2 Aim of the project

Since the nucleotide sequence of complete genome of MmmSC type strain PG1 has recently been determined (WESTBERG et al. 2004), more rational whole genome-based approaches to identify new species-specific immunogenic MmmSC proteins have become possible. Efficient expression systems allow the production of substantial amounts of recombinant proteins from MmmSC in heterologous bacterial
hosts for use as novel diagnostics or in subunit vaccines (ABDO et al. 2000; BRUDERER et al. 2002)

The aim of the project presented here was i) the identification of immunogenic oligopeptides of *MmmSC* using the phage display technique, ii) to confirm the discriminatory efficacy and species specificity, iii) express promising peptides as fusion proteins in *E. coli*, and iv) investigate their applicability as solid-phase antigens for improved diagnosis of *MmmSC*. 
3 Material and Methods

3.1 Chemicals, reagents and equipment

Chemicals and reagents used in this study were purchased from Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany) or Roth (Karlsruhe, Germany) unless stated otherwise. Media and media ingredients were purchased from Difco (Augsburg, Germany) and Oxoid (Wesel, Germany). Equipment and specific biologicals are indicated in the text.

3.2 Bacterial cultures

3.2.1 Growth conditions, media, antibiotic solutions and supplements

*Escherichia coli* (*E. coli*) strains were cultured in Luria-Bertani (LB) medium at 37°C in an incubator (Heraeus T6320 3PH, Heraeus Instruments GmbH Labortechnik, Hanau, Germany) or a shaking incubator (Incubator shaker Series 25, New Brunswick Scientific Co., Inc., Edison, NJ, U.S.A); if necessary, the medium was supplemented with the appropriate antibiotics (ampicillin 100 μg/ml). Media used in this study are listed in Table 1.
### Material and Methods

**Table 1: Media used in this study**

<table>
<thead>
<tr>
<th>Media</th>
<th>Concentration</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB broth</td>
<td>10 g Bacto\textsuperscript{®} tryptone, 5 g yeast extract, 5 g NaCl, add distilled water to 1 litre</td>
<td>Bacto-yeast extract</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacto-Tryptone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
</tr>
<tr>
<td>LB agar</td>
<td>LB broth with 1.5 % agar (w/v)</td>
<td>Bacto-Agar in LB-medium</td>
</tr>
<tr>
<td>PH Medium</td>
<td>20% horse serum</td>
<td>WDT, Garbsen, Germany</td>
</tr>
<tr>
<td>Supplemented blood agar</td>
<td>40g Columbia blood agar base\textsuperscript{®}, add distilled water to 800 ml, autoclave, cool to 55°C, add 200 ml bovine blood and 0.7% nicotine amide adenine dinucleotide (NAD)</td>
<td>Columbia blood agar base\textsuperscript{®}</td>
</tr>
<tr>
<td>2xTY-Agar</td>
<td>1.5 % (w/v)</td>
<td>Bacto-Agar-Agar in 2xTY-medium</td>
</tr>
<tr>
<td>2xTY-Medium (pH 7.0)</td>
<td>1.0 % (w/v) 1.6 % (w/v) 0.05 % (w/v)</td>
<td>Bacto-Yeast extract</td>
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<tr>
<td></td>
<td></td>
<td>Bacto-Tryptone</td>
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<tr>
<td></td>
<td></td>
<td>NaCl</td>
</tr>
<tr>
<td>2xTY-GA</td>
<td>100 mM 100 µg/ml</td>
<td>2xTY-medium</td>
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<tr>
<td></td>
<td></td>
<td>Glucose</td>
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<td></td>
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<td>Ampicillin</td>
</tr>
<tr>
<td>2xTY-AK</td>
<td>100 µg/ml 500 µg/ml</td>
<td>2xTY-Medium</td>
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<td>Ampicillin</td>
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<td>Kanamycin</td>
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<tr>
<td>2xTY-T</td>
<td>10 µg/ml</td>
<td>2xTY-medium</td>
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<tr>
<td></td>
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<td>Tetracycline</td>
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<tr>
<td>SOB-Medium (pH 7.0)</td>
<td>2 % (w/v) 0.5 % (w/v) 0.05 % (w/v) 1%</td>
<td>Tryptone</td>
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<tr>
<td></td>
<td></td>
<td>Yeast extract</td>
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<td>NaCl</td>
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<tr>
<td></td>
<td></td>
<td>2M MgCl\textsubscript{2}-solution</td>
</tr>
<tr>
<td>SOB-Agar</td>
<td>1.5 % (w/v)</td>
<td>Bacto-Agar in SOB-medium</td>
</tr>
<tr>
<td>SOC-Medium (pH 7.0)</td>
<td>2 % (w/v) 0.5 % (w/v) 0.05 % (w/v) 10 mM 20 mM</td>
<td>Tryptone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yeast extract</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MgCl\textsubscript{2}-solution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
</tbody>
</table>
3.2.2 List of bacterial strains, plasmids and primers

Bacterial strains used in this study are listed in Table 2, plasmids and phagemids are listed in Table 3, primers are listed in Table 4.

Table 2: Bacterial strains

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycoplasma mycoides</em> subsp. mycoides SC</td>
<td>MmmSC 237</td>
<td>(JORES et al. 2008)</td>
</tr>
<tr>
<td><em>Mycoplasma mycoides</em> subsp. mycoides SC</td>
<td>PG1</td>
<td>(WESTBERG et al. 2004)</td>
</tr>
<tr>
<td><em>E. coli</em> XL1 Blue MRF’</td>
<td>K12 strain: (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F’ proAB lacIqZΔM15 Tn10 (TetR)]</td>
<td>Stratagene (Amsterdam, Niederlande)</td>
</tr>
<tr>
<td><em>E. coli</em> Top 10 F’</td>
<td>K12 strain:[F’lacIq Tn10(TetR)] mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG</td>
<td>Invitrogen (Karlsruhe)</td>
</tr>
<tr>
<td><em>E. coli</em> DH5αF</td>
<td></td>
<td>(SAMBRook et al. 1989)</td>
</tr>
<tr>
<td>M13K07 Helperphage</td>
<td></td>
<td>(VIEIRA and MESSING 1987)</td>
</tr>
<tr>
<td><em>Hyperphage</em> Helperphage</td>
<td></td>
<td>(RONDOT et al. 2001)</td>
</tr>
</tbody>
</table>
### 3.2.3 Plasmids and phagemids

#### Table 3: Plasmids and phagemids

<table>
<thead>
<tr>
<th>Plasmids / phagemids</th>
<th>Characteristic(s)</th>
<th>Source and/or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHORF3</td>
<td>pHORF3 used for insertion of foreign DNA</td>
<td>Kügler, 2008</td>
</tr>
<tr>
<td>pHORF3X</td>
<td>pHORF3X was modified such that the singular PmeI site used for insertion of foreign DNA is shifted by 1bp, allowing the in-frame fusion with gIII in an additional reading frame</td>
<td>This study</td>
</tr>
<tr>
<td>pHORF3XX</td>
<td>pHORF3X was modified such that the singular PmeI site used for insertion of foreign DNA is shifted by 2bp, allowing the in-frame fusion with gIII in an additional reading frame</td>
<td>This study</td>
</tr>
<tr>
<td>pGEX5x2</td>
<td><em>E. coli</em> expression vector carrying an <em>bla</em> resistance determinant, for construction of GST fusion proteins</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>pMSC0029-500</td>
<td><em>E.coli</em> expression vector for MmmSC peptide MSC_0029, for construction of GST fusion protein</td>
<td>This study</td>
</tr>
<tr>
<td>pMSC0108-500</td>
<td><em>E.coli</em> expression vector for MmmSC peptide MSC_0108, for construction of GST fusion protein</td>
<td>This study</td>
</tr>
<tr>
<td>pMSC0636-500</td>
<td><em>E.coli</em> expression vector for MmmSC peptide MSC_0636, for construction of GST fusion protein</td>
<td>This study</td>
</tr>
</tbody>
</table>
### Table 4: Primers

<table>
<thead>
<tr>
<th>Oligonucleotide primer</th>
<th>DNA-Sequence (5´- 3´)</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHLacZPro_f</td>
<td>GGCTCGTATGTTGTGTGG</td>
<td>Control for phagemids; bind with LacZ promoter</td>
</tr>
<tr>
<td>MHgIII_r1</td>
<td>CTAAAGTTTTTGCCTCTTTCC</td>
<td>Control for Phagemids; bind with pIII</td>
</tr>
<tr>
<td>oMHORF3_Pme_rX1</td>
<td>CTTTGGATCCCTAATGATGATG GTGATGATGGTTAAAACCTCGCC</td>
<td>Primer for modification of pHORF3 by adding 1bp (pHORF3X)</td>
</tr>
<tr>
<td>oMHORF3_Pme_rXX2</td>
<td>CTTTGGATCCCTAATGATGATG GTGATGATGGTTAAAACCTCGC C</td>
<td>Primer for modification of pHORF3 by adding 2bp (pHORF3XX)</td>
</tr>
<tr>
<td>oMSC0029A</td>
<td>GATCGAATTCAATGGGGACAC ACACTCAATAT</td>
<td>Forward primer to clone MSC0029</td>
</tr>
<tr>
<td>oMSC0029B</td>
<td>CGATGCGGCCGCTTTAAAAAGTT CTAGCAGCTTTCTAT</td>
<td>Reverse primer to clone MSC0029</td>
</tr>
<tr>
<td>oMSC0108A</td>
<td>GATAATCGAATTCAATGGATTTC AAGTGCTTATTCAATTCAA</td>
<td>Forward primer to clone MSC0108</td>
</tr>
<tr>
<td>oMSC0108B</td>
<td>TCCGATGCGGCCGCTCATCAAT TAGTTTG</td>
<td>Reverse primer to clone MSC0108</td>
</tr>
<tr>
<td>oMSC0636A</td>
<td>AGACGAATTCAATGGTTTATTA TGCAAAACAATGAACCG</td>
<td>Forward primer to clone MSC0636</td>
</tr>
<tr>
<td>oMSC0636B</td>
<td>ATATGCGGCGCTCATGATTTT AATTGTTCTTTTAATTTG</td>
<td>Reverse primer to clone MSC0636</td>
</tr>
<tr>
<td>GEX5*</td>
<td>GCTGGCA AGCCAC GTTTGGT</td>
<td>Control forward primer for vector pGEX</td>
</tr>
<tr>
<td>GEX3*</td>
<td>GGGAGCTGCATCTGTCAGAG</td>
<td>Control reverse primer for vector pGEX</td>
</tr>
<tr>
<td>M13-fw</td>
<td>GTAAGACGACGCGCCAG</td>
<td>Control forward primer for M13</td>
</tr>
<tr>
<td>M13-rev</td>
<td>CAGGAAACAGCTATGAC</td>
<td>Control reverse primer for M13</td>
</tr>
</tbody>
</table>
### 3.2.4 Buffers and solutions

#### Table 5: Buffers and solutions used in this study

<table>
<thead>
<tr>
<th>Buffer, solution</th>
<th>Concentration</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Solution</td>
<td>2 M</td>
<td>Glucose in dH₂O</td>
</tr>
<tr>
<td>Ampicillin stock solution</td>
<td>100 mg/ml</td>
<td>Ampicillin 100 mg/ml in 70% ethanol, a few drops of concentrated HCl added until all substance was completely dissolved</td>
</tr>
<tr>
<td>Kanamycin stock solution</td>
<td>50 mg/ml</td>
<td>(50 mg/ml) 50% glycerol</td>
</tr>
<tr>
<td>Tetracycline stock solution</td>
<td>10 mg/ml</td>
<td>Tetracyclin in 100% ethanol</td>
</tr>
<tr>
<td>IPTG stock solution</td>
<td>10 mg/ml</td>
<td>IPTG</td>
</tr>
<tr>
<td>Acrylamide stock solution</td>
<td>30% (w/v) 0.8% (w/v)</td>
<td>Acrylamid  Bisacrylamid</td>
</tr>
<tr>
<td>APS-solution.</td>
<td>10% (w/v)</td>
<td>Ammoniumpersulfate in dH₂O</td>
</tr>
<tr>
<td>BCIP-solution.</td>
<td>100% (v/v) 1.5% (w/v)</td>
<td>Dimethylformamid  BCIP</td>
</tr>
<tr>
<td>Block buffer</td>
<td>2% (w/v)</td>
<td>Milk powder or gelatine in PBST 0.1%</td>
</tr>
<tr>
<td>Carbonate-buffer (pH 9.6)</td>
<td>1.59 g/l 2.94 g/l</td>
<td>Na₂CO₃  NaHCO₃ in dH₂O</td>
</tr>
<tr>
<td>DAB-solution</td>
<td>25 mg/ml</td>
<td>DAB (3.3’-Diaminobenzidin) in dH₂O</td>
</tr>
<tr>
<td>Ethidium bromide solution</td>
<td>0.01% (w/v)</td>
<td>Ethidium bromide in dH₂O</td>
</tr>
<tr>
<td>H₂O₂-solution</td>
<td>30%</td>
<td>H₂O₂ in dH₂O</td>
</tr>
<tr>
<td>H₂SO₄-(stop)-solution</td>
<td>0.5 M</td>
<td>H₂SO₄ in dH₂O</td>
</tr>
<tr>
<td>Cobalt-solution</td>
<td>0.02% (w/v)</td>
<td>CoCl₂ in dH₂O</td>
</tr>
<tr>
<td>Laemilli-buffer</td>
<td>10% (w/v) 50% (v/v) 0.02% (w/v) 15% (v/v)</td>
<td>SDS  Glycerine  Bromphenole blue  β-Mercaptoethanol</td>
</tr>
<tr>
<td>Sodium acetate (pH 5.2)</td>
<td>3 M</td>
<td>Sodium acetate</td>
</tr>
<tr>
<td>Buffer, solution</td>
<td>Concentration</td>
<td>Components</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------------</td>
<td>------------</td>
</tr>
<tr>
<td>NBT-solution</td>
<td>70 % (v/v)</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td></td>
<td>3.0 % (w/v)</td>
<td>NBT</td>
</tr>
<tr>
<td>PBS (pH 7.4)</td>
<td>0.8 % (w/v)</td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>0.02 % (w/v)</td>
<td>KCl</td>
</tr>
<tr>
<td></td>
<td>0.144 % (w/v)</td>
<td>Na₂HPO₄ x 2H₂O</td>
</tr>
<tr>
<td></td>
<td>0.024 % (w/v)</td>
<td>KH₂PO₄</td>
</tr>
<tr>
<td>PBST</td>
<td>0.05% or 0,1%</td>
<td>Tween 20 in PBS</td>
</tr>
<tr>
<td>PEG/NaCl-solution</td>
<td>20 % (w/v)</td>
<td>Polyethylene glycol 6000</td>
</tr>
<tr>
<td></td>
<td>2.5 M</td>
<td>NaCl</td>
</tr>
<tr>
<td>Phage dilution buffer (pH 7.5)</td>
<td>10 mM</td>
<td>Tris-HCl</td>
</tr>
<tr>
<td></td>
<td>20 mM</td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td>EDTA</td>
</tr>
<tr>
<td>Separating gel buffer</td>
<td>1.0 M</td>
<td>Tris-HCl (pH 6.8)</td>
</tr>
<tr>
<td>SDS-solution</td>
<td>10 % (w/v)</td>
<td>SDS in dH₂O</td>
</tr>
<tr>
<td>SDS-PAGE-running buffer</td>
<td>25 mM</td>
<td>Tris-HCl (pH 8.0)</td>
</tr>
<tr>
<td></td>
<td>192 mM</td>
<td>Glycine</td>
</tr>
<tr>
<td></td>
<td>0.1 % (w/v)</td>
<td>SDS</td>
</tr>
<tr>
<td>Substrate buffer (pH 9.5)</td>
<td>100 mM</td>
<td>Tris-HCl (pH 9.5)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM</td>
<td>MgCl₂</td>
</tr>
<tr>
<td>Solution S1 for alkaline lysis</td>
<td>60.06 g/L</td>
<td>Tris</td>
</tr>
<tr>
<td></td>
<td>2 % (v/v)</td>
<td>500 mM EDTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNase</td>
</tr>
<tr>
<td>Solution S2 for alkaline lysis</td>
<td>2 % (v/v)</td>
<td>10 M NaOH</td>
</tr>
<tr>
<td></td>
<td>5 % (v/v)</td>
<td>SDS (20 %)</td>
</tr>
<tr>
<td>Solution S3 for alkaline lysis</td>
<td>29.5 % (v/v)</td>
<td>Conc. Acetic acid</td>
</tr>
<tr>
<td>TAE-buffer</td>
<td>4 mM</td>
<td>Tris-HCl</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td>Acetic acid</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>EDTA (pH 8.0)</td>
</tr>
<tr>
<td>TMB-(substrate)-solution</td>
<td>20 parts</td>
<td>Sol. A</td>
</tr>
<tr>
<td></td>
<td>1 part</td>
<td>Sol. B</td>
</tr>
<tr>
<td>Solution A (pH 4.1) for ABTS</td>
<td>30 mM</td>
<td>Calcium citrate</td>
</tr>
<tr>
<td></td>
<td>1 % (w/v)</td>
<td>Citric acid</td>
</tr>
<tr>
<td>Solution B for ABTS</td>
<td>10 mM</td>
<td>Tetramethyl benzidine</td>
</tr>
<tr>
<td></td>
<td>10 % (v/v)</td>
<td>Acetone</td>
</tr>
<tr>
<td></td>
<td>90 % (v/v)</td>
<td>Ethanol</td>
</tr>
<tr>
<td></td>
<td>0.3 % (v/v)</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>Running gel-buffer</td>
<td>1.5 M</td>
<td>Tris-HCl (pH 8.8) in dH₂O</td>
</tr>
<tr>
<td>Trypsin-solution</td>
<td>10 µg/mL</td>
<td>Trypsin in PBS</td>
</tr>
<tr>
<td>TE-buffer:</td>
<td>pH 8.0</td>
<td>10 mM Tris-HCl (pH 8.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM Na₂-EDTA pH 8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adjust pH with HCl</td>
</tr>
</tbody>
</table>
Table 6: Ready-made buffers and solutions used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Companies</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTPs</td>
<td>Fermentas (St. Leon-Rot)</td>
</tr>
<tr>
<td>Injection solution</td>
<td>Millipore (Schwalbach)</td>
</tr>
<tr>
<td>Buffers for restriction endonucleases</td>
<td>NEB (Bad Schwalbach)</td>
</tr>
<tr>
<td>Red Taq buffer</td>
<td>Sigma (Deisenhofen)</td>
</tr>
<tr>
<td>T4 DNA ligase buffer</td>
<td>Promega (Mannheim)</td>
</tr>
<tr>
<td>T4 DNA polymerase buffer</td>
<td>NEB (Bad Schwalbach)</td>
</tr>
</tbody>
</table>

3.2.5 Enzymes

Table 7: Enzyme used in this study

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Companies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf intestinal phosphatase (CIP)</td>
<td>NEB (Bad Schwalbach)</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>NEB (Bad Schwalbach)</td>
</tr>
<tr>
<td>Restriction endonucleases</td>
<td>NEB (Bad Schwalbach)</td>
</tr>
<tr>
<td>T4 DNA-ligase</td>
<td>Promega (Mannheim)</td>
</tr>
<tr>
<td>T4 DNA-polymerase</td>
<td>NEB (Bad Schwalbach)</td>
</tr>
</tbody>
</table>
3.2.6 Sera and Antibodies

Table 8: Different Antibodies and Sera

<table>
<thead>
<tr>
<th>Antibodies, Serum</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-bovine IgG-AP</td>
<td>Dianova (Hamburg)</td>
</tr>
<tr>
<td>Goat anti-bovine IgG-HRP</td>
<td>Dianova (Hamburg)</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG-AP</td>
<td>Sigma (Deisenhofen)</td>
</tr>
<tr>
<td>Rabbit anti-bovine IgG</td>
<td>Sigma (Deisenhofen)</td>
</tr>
<tr>
<td>Rabbit anti GST</td>
<td>Sigma (Deisenhofen)/ Dianova (Hamburg)</td>
</tr>
<tr>
<td>Mouse anti-pIII</td>
<td>MoBiTec (Göttingen)</td>
</tr>
<tr>
<td>Mouse anti-M13</td>
<td>Progen (Heidelberg)</td>
</tr>
<tr>
<td>Rabbit-hyperimmune sera raised against different mycoplasmal species</td>
<td>Dr. M. Heller, FLI Jena</td>
</tr>
<tr>
<td>Preimmune sera obtained from uninfected cattle</td>
<td>Joerg Jores, ILRI Nairobi Kenya and Dr. M. Heller, FLI Jena</td>
</tr>
<tr>
<td>Sera from CBPP infection trials</td>
<td>Joerg Jores, ILRI Nairobi Kenya</td>
</tr>
</tbody>
</table>

3.2.7 Bacterial strains and culture conditions

The MmmsC field isolate 237 was grown as stationary culture for 3 days at 37°C in 1000 ml bottles in modified PH media, supplemented with 20% horse serum (WDT, Garbsen, Germany). The cells were then centrifuged at 20,000×g for 30 min at 4°C and washed three times in phosphate-buffered saline (PBS, 150 mM NaCl, 1.5 mM KH$_2$PO$_4$, 9 mM Na$_2$HPO$_4$ x 12H$_2$O, 2.5 mM KCl [pH 7.2]). Washed cell pellets were stored at −20°C.

3.2.8 Quantification of bacterial growth by optical density

Five ml overnight culture were inoculated in 45 ml LB broth, grown to an optical density at 600 nm (OD$_{600}$) of approximately 0.3 in the shaking incubator at 37°C and 180 rpm and then stored on ice for 10 min. An equivalent of 20 ml broth culture with
an OD$_{600}$ of 0.3 was inoculated in 180 ml LB broth, and the OD$_{600}$ was determined every 15 min until stationary phase was reached.

3.3 Manipulation of nucleic acids

3.3.1 Plasmids

The plasmids used in this study are summarized in Table 3. Restriction endonuclease digests, ligations, generation of blunt ends using different restriction endonucleases, alkaline phosphatase treatments and agarose gel electrophoresis were done according to standard protocols and the respective manufacturer’s instructions. Restriction endonucleases, other enzymes, DNA size standards and buffers were purchased from New England Biolabs, Frankfurt, Germany, unless stated otherwise.

3.3.2 Primers

The primers used in this study are summarized in Table 4. Primers were synthesized by Invitrogen, Karlsruhe, Germany.

3.4 Isolation of DNA

3.4.1 Plasmid DNA

Plasmid DNA was either prepared by alkaline lysis (BIRNBOIM and DOLY 1979) following standard procedures (SAM BROOK et al. 1989) or by using the Midi Plasmid Preparation kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer’s instructions. DNA cleanup following alkaline lysis was performed by phenol-chloroform extraction according to standard procedures (SAM BROOK et al. 1989) or by using the Gene Clean® kit (Q-BIOgene, Heidelberg, Germany) according to the manufacturer’s instructions. Centrifugation steps were carried out in a micro centrifuge.
3.4.2 Total genomic DNA of \textit{MmmSC}

The \textit{MmmSC} field isolate 237 pellet stored at $-20^\circ$C (described in 3.2.7) was used for total genomic DNA of \textit{MmmSC} extraction. Bacterial cells were lysed by the addition of 10 mM EDTA (pH 8.0), 1% SDS, and 0.5 mg/ml proteinase K; mixing of the solution in a 10 ml polypropylene tube was achieved by gentle inversion of the tube. The mixture was incubated at 55°C for 1 h. RNA contamination was removed by the addition of RNase to a final concentration of 100 µg/ml and further incubation at 37°C for 20 min. DNA was purified by adding 1/5 volume of phenol equilibrated in TE buffer (pH 7.8) to the solution and mixing by careful shaking. Afterwards the mixture was stored for 1 h at -20°C, followed by thawing in a 55°C water bath. Then 1/5 volume of chloroform: isoamylalcohol (24:1) was added, mixed, and centrifuged at 12,000 rpm for 10 min. The aqueous (top) phase containing DNA was carefully removed using a plastic Pasteur pipette and transferred to a new tube. Chloroform-isoamyl extraction was repeated until no interphase was visible. The upper phase was transferred into a new tube, and DNA was precipitated by adding 0.1 volumes of 3M Na-acetate (pH 5.2) and 1 volume of isopropanol. The DNA threat generated by careful inversion of the tube was collected with a small pipette tip, washed twice in 70% ethanol for 5 min and finally in 96% ethanol for 5 min. DNA was dissolved in 200 µl A. bidest overnight at 4°C. Five µl of the DNA were analysed by gel electrophoresis.

3.4.3 Polymerase chain reaction (MULLIS et al. 1992)

PCR was performed in a thermal cycler in a 25 or 50 µl total reaction volume using \textit{Taq} DNA polymerase (NEB, Bad Schwalbach, Germany). The reaction mixtures were prepared on ice by addition of the reagents in the order described in Table 9.
Table 9: PCR reaction mixture

<table>
<thead>
<tr>
<th>reaction components</th>
<th>Stock solution</th>
<th>Final concentration</th>
<th>volume per reaction (μl)</th>
<th>volume per reaction (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. bidest.</td>
<td>-</td>
<td>-</td>
<td>11.15</td>
<td>22.3</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>50 mM</td>
<td>1.5 mM</td>
<td>0.75</td>
<td>1.5</td>
</tr>
<tr>
<td>PCR-buffer</td>
<td>10 x</td>
<td>1 x</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 mM</td>
<td>0.2 mM</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>forward primer</td>
<td>5 pmol/μl</td>
<td>0.5 pmol/μl</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>reverse primer</td>
<td>5 pmol/μl</td>
<td>0.5 pmol/μl</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td><em>Taq</em> DNA polymerase</td>
<td>5 U/μl</td>
<td>0.5 U/μl</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Template</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>final volume</td>
<td></td>
<td></td>
<td>25</td>
<td>50</td>
</tr>
</tbody>
</table>

The DNA template was prepared by boiling a single colony. Amplification conditions are listed in Table10. PCR products were analysed by gel electrophoresis on a 1.5% agarose gel.
### Table 10: PCR conditions

<table>
<thead>
<tr>
<th>Primers</th>
<th>PCR protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 for, M13 rev</td>
<td>3’ 94°C, (1’ 94°C, 1’ 55°C, 1’ 72°C) x 32; 10’ 72°C</td>
</tr>
<tr>
<td>MHLacZPro_f, MHgIII_r1</td>
<td>3’ 94°C, (30” 94°C, 1’ 45°C, 3’30” 72°C) x 31; 10’ 72°C</td>
</tr>
<tr>
<td>MHLacZPro_f, oMHORF3_Pme_rX1</td>
<td>3’ 94°C, (30” 94°C, 1’ 50°C, 3’30” 72°C) x 31; 10’ 72°C</td>
</tr>
<tr>
<td>MHLacZPro_f, oMHORF3_Pme_rXX2</td>
<td>3’ 94°C, (30” 94°C, 1’ 50°C, 3’30” 72°C) x 31; 10’ 72°C</td>
</tr>
<tr>
<td>oGEX5, oGEX3</td>
<td>3’ 94°C, (30” 94°C, 1’ 55°C, 3’30” 72°C) x 31; 10’ 72°C</td>
</tr>
<tr>
<td>oMSC0029A, oMSC0029B</td>
<td>3’ 94°C, (30” 94°C, 1’ 55°C, 3’30” 72°C) x 31; 10’ 72°C</td>
</tr>
<tr>
<td>oMSC0108A, oMSC0108B</td>
<td>3’ 94°C, (30” 94°C, 1’ 55°C, 3’30” 72°C) x 31; 10’ 72°C</td>
</tr>
<tr>
<td>oMSC0636A, oMSC0636B</td>
<td>3’ 94°C, (30” 94°C, 1’ 55°C, 3’30” 72°C) x 31; 10’ 72°C</td>
</tr>
</tbody>
</table>

#### 3.4.4 Preparation of DNA template by colony boiling

A single colony was touched with a 2-200 µl pipette tip picking up part of the colony. The material was resuspended in 50 µl TE buffer or A. bidest. in a microtiter plate and boiled in a microwave oven for 8 min at 180 W. Five µl of this suspension served as template in a 25 µl and 50 µl PCR reactions, respectively. As negative control, 50 µl TE buffer were boiled at the same conditions, and 5 µl were used in the same volume of premix.

#### 3.4.5 Nucleotide sequencing and sequence analysis

Nucleotide sequencing was done by SEQLAB, Göttingen, Germany. Sequence analysis were performed using **Basic Local Alignment Search Tool (BLAST)**.
(ALTSCHUL et al. 1990) and the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/).

3.5 Transformation

3.5.1 Preparation of *E. coli* competent cells for transformation

Competent *E. coli* were prepared using the method described by HANAHAN et al. (1991). *Escherichia coli* cells were grown in 250 ml LB broth with 20 mM MgCl$_2$ to an OD$_{600}$ of 0.3 to 0.4 and then chilled on ice. Cells were harvested by centrifugation at 4,500 $\times$ g and 4°C for 10 min, resuspended in 30 ml ice-cold TFB1 and kept on ice for a minimum of 90 min before centrifugation again as described above. The pellet was resuspended in 5 ml of ice-cold TFB2; aliquots of approximately 250 µl were transferred (using a pre-chilled pipette) to pre-chilled eppendorf tubes and stored at -70°C until use.

**TFB1:**

- 30 mM potassium acetate,
- 100 mM RbCl,
- 10 mM CaCl$_2$,
- 50 mM MnCl$_2$,
- 15% glycerine (pH 5.8)

**TFB2:**

- 10 mM MOPS,
- 75 mM CaCl$_2$,
- 10 mM RbCl,
- 15% glycerine (pH 6.5)

Both solutions were sterilized by filtration (FP30/0.2 CA-S, pore size 0.2 µM cellulose acetate, Schleicher & Schuell, Dassel, Germany) and stored at 4°C.

3.5.2 Transformation of *E. coli* by heat shock

The competent cells were thawed on ice, split into 100 µl aliquots and approximately 0.5 µg DNA or 12.5 µl of a ligation reaction were added. After incubation for a minimum of 30 min on ice the cells were placed into a 42°C temperature block (Multi-Block Heater, Lab-Line, Kleinfeld-Labortechnik GmbH, Gehrden, Germany) for 3 min and then chilled on ice for 2 min. Two hundred µl of LB broth were added, and the cells were allowed to regenerate at 37°C for 1 hour before spreading the mixture on LB agar plates containing the appropriate antibiotic.
3.5.3 Preparation of electro-competent *E. coli* cells for transformation

Competent *E. coli* cells were prepared using the method described by TUNG and CHOW 1995. Two Erlenmeyer flasks each with 250 ml LB broth were inoculated with 2.5 ml each of an overnight culture of *E. coli* cells, grown to an OD_{600} of 0.6 with vigorous agitation and chilled on ice for 30 min. Cells were harvested by centrifugation at 2,600 x g at 4 °C for 15 min. The obtained pellets were resuspended in 5 ml pre-chilled washing buffer (10 % [v/v] glycerol in A. bidest.), and 100 ml of additional washing buffer was added. Cells were again pelleted by centrifugation at 9,200 x g and 4°C for 15 min, and the washing step was repeated twice. After the last washing step, both pellets were suspended in a total volume of 5 ml pre-chilled GYTT medium (see below). Cells were transferred to pre-chilled 1.5 ml reaction tubes and stored in 200 µl aliquots at -70 °C until use.

**GYTT medium**

10 % [v/v] glycerol, 0.125 % [w/v] yeast extract, 0.25 % [w/v] Bacto® tryptone, 0.02 % [v/v] Tween®80

3.5.4 Electrotransformation of *E. coli*

For transformation of electro-competent *E. coli* cells were thawed on ice, and 30 to 50 µl were mixed with 10 µl of a dialysed ligation reaction. The Gene Pulser (Bio-Rad, Munich, Germany) was set to 2.5 kV and 25 µF, the pulse controller was set on 200 Ohm. Transformation of the DNA-cell-mix was performed according to the manufacturer's instructions in a 0.2 cm electroporation cuvette (Electrotransformation cuvette, 0.2 cm electrode gap, Cellprojects, Harrietsham, United Kingdom). After transformation 500 µl prewarmed LB medium was added, mixed well and transferred to a 1.5 ml reaction tube. In order to express the respective antibiotic resistance determinant, the cells were incubated at 37°C for 1 h with shaking and then plated on LB plates with the supplemented antibiotic.
3.6 Phage Display Methods

3.6.1 Construction of pHORF3X and pHorf3XX

*E. coli* culture and standard cloning procedures were performed according to Sambrook and Russell (SAMBROOK and RUSSELL 2001). The phage vector pHORF3 (KÜGLER et al. 2008) was kindly provided by Michael Hust, Technical University Braunschweig, Braunschweig, Germany. Using primers (Table 4) vector pHORF3 was then modified such that the unique *Pmel* site used for insertion of foreign DNA is shifted by 1 and 2 bp resulting in plasmids designated as pHORF3X and pHORF3XX, respectively. This allows the successful cloning of additional ORFs from the same DNA fragment pool used in initial experiments (Fig. 3).

3.6.2 Construction of a *MmmSC* genomic phage display library

A schematic overview of the construction and enrichment of open reading frames (ORFs) is given in Fig. 5. The genomic DNA of *MmmSC* African strain 237 was isolated according to Meens (MEENS et al. 2006). Genomic DNA was digested by using five different blunt end-cutting restriction endonucleases. The restricted DNA was separated by 1.5% agarose gel electrophoresis. DNA fragments with a size of 100-1000 bp were isolated using the Qiagen kit (Qiagen PCR DNA and gel band purification kit) according to the manufacturer's instructions and pooled. In parallel, vectors pHORF3, pHORF3X and pHORF3XX were digested using NEB buffer 4, BSA and 30 U *Pmel* at 37°C for two hrs in a 60 µl reaction volume. The dephosphorylation of the digested vector was performed by addition of 0.5 U calf intestine phosphatase (CIP) and incubation for 30 min to one hr at 37°C. The linearized vector fragment was purified using the GFX kit (GE Healthcare). One hundred ng of vector DNA were ligated with 35 ng *MmmSC* genomic DNA fragments using 3 µl T4 DNA ligase (Promega, Mannheim, Germany) and ligase buffer in 60 µl reaction volume at 16°C overnight. The ligation mix was dialyzed for 20 min against distilled water on a floating 0.025 µm filter membrane; then 7.5 µl ligation solution were mixed with 40 µl electrocompetent TOP10F' (Invitrogen,
Karlsruhe, Germany), incubated on ice for 1 min and transferred into prechilled 0.2 cm electroporation cuvettes. The transformation was performed using a 2.5 kV pulse in a BIORAD micropulser (München, Germany). Immediately, 300 µl LB medium (SAMBROOK and RUSSELL 2001) were added, and cells were incubated in a shaking incubator at 37°C and 200 rpm for 1 h. One 10 µl aliquot was used for titration to calculate the amount of independent transformants (HUST et al. 2007). The remaining 337.5 µl were plated on LB agar plates with 100 µg/ml ampicillin and incubated overnight at 37°C. Colonies were harvested by suspending in 2 ml of 2 x TY medium with a Drigalsky spatula. The libraries were either used directly for Hyperphage packaging or an equal volume of 50% glycerine was added, and the libraries were stored at -70°C in 1 ml aliquots.

3.6.3 Enrichment of ORFs using Hyperphage

To enrich ORFs in the MmmSC genomic library required a display of the corresponding oligopeptides on phage particles for the panning; the libraries were packaged using Hyperphage, a helperphage not encoding pIII protein on its genome (RONDOT et al. 2001; HUST et al. 2006 and SOLTES et al. 2007). Two hundred ml of 2 x TY medium containing 100 µg/ml ampicillin and 100 mM glucose were inoculated with 1 ml of library stock suspension. The bacteria were grown to an OD₆0₀ of 0.4 - 0.5 at 37°C and 250 rpm. Twenty five ml bacterial culture (~1.25x10¹⁰ bacteria) were infected with 2.5x10¹¹ Hyperphage, incubated at 37°C for 30 min without shaking, followed by 30 min at 250 rpm. The infected cells were harvested by centrifugation for 10 min at 3,220 x g. The pellet was resuspended in 250 ml of 2 x TY containing 100 µg/ml ampicillin and 50 µg/ml kanamycin (2 x TY-AK). The phages were produced upon growth at 30°C and 250 rpm over night. On the following day cells were centrifuged for 20 min at 3,220 x g. The phage in the supernatant were precipitated with 1/5 volume of 20% (w/v) polyethylene glycol (PEG)/2.5 M NaCl solution for 1 h on ice with gentle shaking and pelleted by centrifugation for 1 h at 3,220 x g at 4°C. The precipitated phages were resuspended in 300 µl PBS (phosphate buffered saline, (SAMBROOK and RUSSELL 2001). Residual bacteria
and cell debris were removed by additional centrifugation for 5 min at 15,000 x g at 20°C. The supernatants containing the oligopeptide-containing phage were stored at 4°C, and phage titration was performed according to HUST et al. (2007). The panning procedure was performed as shown schematically in Fig. 7.

3.6.4 Colony PCR

_E. coli_ clones containing pHORF3 and derivatives were analysed by colony PCR using primers MHLacZPro_f (5' GGCTC GTATGTTGTGG 3') and MHgIII_r (5' GGAAAGACGACAAAAACTTTAG 3') and the following protocol: 94°C 1 min, 45°C 1 min, 72°C 2 min, 30 cycles. The DNA was separated by 1 or 1.5% agarose gel electrophoresis.

3.6.5 Selection of phage encoding immunogenic oligopeptides of _MmmSC_

Six wells of a MaxiSorb® 96 well MTP plate (Nunc, Wiesbaden, Germany) were coated with 150 µl of rabbit anti-bovine IgG (5 µg/ml) in PBS overnight, washed and blocked with PBST supplemented with 2% (w/v) skim milk powder (2% M-PBST) for 1.5 h. All washing steps between incubation steps were performed using PBST buffer and an ELISA washer (Tecan Columbus, Crailsheim, Germany). The serum from chronically _MmmSC_-infected animals was diluted 1:10 in PBST supplemented with 2% M-PBST and pre-incubated in Maxisorb® Microtiter plates (MTP) wells coated with 150 µl of Hyperphage (1 x 10^{11} cfu/ml) for 1 h at room temperature to remove serum IgG unspecifically binding to the helperphage. This pre-incubation procedure was repeated twice by transferring the serum into new Hyperphage-coated wells. After preincubation the bovine serum was bound to the plate by incubating it on the rabbit anti-bovine IgG-coated MTP wells for 2 h. After washing, 4 x 10^{10} cfu oligopeptide-encoding phage particles of the Hyperphage-packaged _MmmSC_ genomic library were incubated onto the captured bovine IgGs for 2 h (for the 1^{st} panning round). For the following panning rounds 1x10^{12} cfu of the previous panning rounds were used. The non-binding oligopeptide-encoding phage particles were
removed by ten washing steps with PBST using an ELISA washer. In the second, third and fourth panning rounds the number of washing steps were increased to 20, 30 and 40, respectively. Elution of bound phage particles was performed using 200 µl of trypsin solution (10 µg/ml) for 30 min at 37°C. Ten µl of the eluted phage solution were used for titration according to HUST et al. (2007). Twenty ml of TOP10F' E. coli cells were grown to an OD$_{600}$ of 0.4-0.5, infected with the remaining 190 µl of the eluted phage solution and incubated for 30 min at 37°C. Afterwards, the cells were pelleted by centrifugation for 10 min at 3,220 x g. The bacterial pellet was dissolved in 250 µl of 2 x TY medium containing 100 mM glucose and 100 µg/ml ampicillin (2 x TY-GA), plated onto 15 cm 2 x TY-GA agar plates, and incubated overnight at 37°C. The grown colonies were harvested in 5 ml of 2 x TY-GA using a Drigalsky spatula. Fifty ml of 2 x TY-GA were inoculated with 200 µl of bacterial culture and grown to an OD$_{500}$ of 0.4 - 0.5 at 37°C and 250 rpm. Five ml of bacterial culture corresponding to about ~2.5x10$^9$ cells were infected with 5 x 10$^{10}$ cfu of Hyperphage, incubated at 37°C for 30 min without shaking and another 30 min with shaking at 250 rpm. The infected cells were harvested by centrifugation for 10 min at 3,220 x g. The pellet was resuspended in 30 ml of 2 x TY-AK, and the phages were produced in a shaking incubator at 30°C and 250 rpm over night. On the following day the phage particles were precipitated.

**3.6.6 Production of individual phage clones for screening**

Polypropylene 96-well U bottom plates (Greiner bio-one, Frickenhausen, Germany) containing 175 µl of 2 x TY-GA per well were inoculated with single E. coli colonies from the phage titration plates of the panning rounds and incubated at 37°C with constant shaking at 1000 rpm (thermo shaker PST60-HL4, lab4you, Berlin, Germany) overnight. Then, 10 ml glass tubes each containing 1.5 ml of 2xTY-GA were inoculated with 10-20 µl of the overnight cultures and incubated at 37°C and 250 rpm for 2 h to 5 h. The bacteria were infected with 5 x 10$^9$ cfu Hyperphage/tube and incubated at 37°C without shaking for 30 min, followed by 30 min incubation with shaking at 250 rpm. Cells were centrifuged at 3,220 x g for 10 min and the
supernatants were discarded. The bacteria pellets were dissolved in 1.5 ml of 2 x TY containing 100 µg/ml ampicillin and 30 µg/ml kanamycin (2 x TY-AK) and incubated at 30°C at 250 rpm overnight for phage production. Bacteria were pelleted as described above and the supernatants were transferred to a new 2 ml eppendorf tube. Phages were precipitated with 1/5 volume of a 20% PEG/2.5 M NaCl solution at 4°C for 1 h and centrifuged at 3,220 x g for 1 h. The phage pellet were dissolved in 150 µl PBS, and residual clumps were removed by another centrifugation at 3,220 x g for 5 min. The supernatants were stored at 4°C.

3.6.7 Identification of immunogenic oligopeptides by monoclonal phage ELISA

The wells of a PolySorb® plates were coated overnight with 10 µl of phages in 100 µl of coating buffer and washed with 2% M-PBST thrice; between each incubation step the wells were washed with PBST. The serum from MmmSC-infected animals was diluted 1:250 in 2%-MPBST and incubated for 1 h in the phage-coated wells. The bound bovine IgGs were detected using rabbit anti-bovine IgG conjugated with horseradish peroxidase (HRP) (1:2,000) for 1.5 h and the ELISAs were developed using 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) (Roche Diagnostics, Mannheim, Germany) as a substrate. The staining reaction was stopped by adding 100 µl of 50% methanol. The absorbance at 405 nm and scattered light at 492 nm were measured using a SUNRISE microtiter plate reader (Tecan, Crailsheim, Germany).

3.6.8 Phage titration

The titration of phage was performed as follows:
Inoculate 5 ml 2 x TY-T in a 100 ml Erlenmeyer flask with E. coli XL1-Blue MRF' and grow overnight in a shaking incubator at 37°C and 250 rpm. Inoculate 50 ml of 2 x TY-T with 500 µl overnight culture and grow at 250 rpm and 37°C to an OD_{600} of 0.5.
Note: If the bacteria have reached an OD$_{600}$ of 0.5 before they are needed, store the culture immediately on ice to maintain the F pili on the *E. coli* cells for several hours. M13K07 helperphage (kan$^+$) or other scFv-phage (amp$^+$) can be used as positive control to check the infectibility of the *E. coli* cells.

Make serial dilutions of the phage suspension in PBS. The number of eluted phage depends on several parameters (e.g. antigen, library, panning round, washing stringency etc.). In case of a successful enrichment, the titre of eluted phage usually is $10^3$ – $10^5$ phage per well after the first panning round and increases two to three orders in magnitude per additional panning round. The phage preparations after reamplification of the eluted phage should have a titre of about $10^{12}$ – $10^{14}$ phage/ml. Infect 50 µl bacterial suspension with 10 µl of the phage dilution and incubate 30 min at 37°C. Plate the 60 µl of infected bacteria on 2 x TY-GA agar plates (9 cm petri dishes). Incubate the plates overnight at 37°C. Count the colonies and calculate the cfu or cfu/ml titre according to the dilution.

### 3.7 Manipulation of proteins

#### 3.7.1 Preparation of proteins

**Preparation of proteins from *Mmm*SC by whole cell lysis**

*Mmm*SC was grown as described in 3.2.7. Bacteria were harvested by centrifugation at 4°C and 7,000 × g for 10 min. The supernatant was removed; the cells were resuspended in 500 µl of Tris-HCl (50 mM, pH 7.3) and stored at -70°C. The cells were ruptured using a FastPrep instrument (FastPrep® FP120, B101 Thermo Savant, Qbiogene, Heidelberg, Germany), using a setting of 3 x 40 sec with the intensity 5.0. Unbroken cells were removed by centrifugation at 16,000 × g in a benchtop centrifuge (Centrifuge 5415D, Eppendorf AG, Hamburg, Germany) for 10 min. The supernatant was transferred into a new microcentrifuge tube and stored at -70°C. It was used as a positive control in initial ELISA experiments.
3.7.2 Generation of recombinant proteins using the glutathione-S-transferase (GST) translational fusion technology

The recombinant expression of the MSC_0029, MSC_0108 and MSC_0636 encoding genes in *E. coli* required the identification of TAG codons; then the DNA-fragment encoding the peptide detected in the phage-peptide library and located between two stop codons was amplified by PCR using primer pairs with an EcoRI-(upstream primer) or NotI-restriction endonuclease sites (downstream primer) at their 5´ends. The corresponding PCR fragments were digested with EcoRI and NotI, ligated into pGEX5x2, and then transformed into DH5αF´. The expression plasmids were designated as pMSC0029-500, pMSC0108-500 and pMSC0636-500. The calculated values for the molecular mass and the IP of the fusion protein are 32.5, 32.0, and 45.0 kDa, and 5.72, 5.91, and 6.0 respectively. Successful construction of expression vectors was confirmed by small-scale expression of GST fusion proteins as described by BERENSMEIER et al. (2004).

3.7.3 Preparation of aggregated fusion protein

High-level expression of GST-oligopeptide in *E. coli* led to the formation of inclusion bodies, which comprise dense, insoluble aggregates. Single colonies of freshly transformed *E. coli* DH5αF´/pMSC029-500, DH5αF´/pMSC108-500, and DH5αF´/pMSC636-500 were cultured overnight in 5 ml LB broth supplemented with 100 µg/ml ampicillin and 1% glucose. For preparation of protein aggregates, 50 ml of LB broth supplemented with ampicillin (100 µg/ml) were inoculated with 0.5 ml overnight culture of the *E. coli* carrying the respective expression plasmid. The culture was grown to an OD<sub>600</sub> of 0.3 to 0.5, then induced with IPTG (1 mM) and further incubated for 2 h with shaking. Cells harvested upon IPTG-induced protein synthesis contained the GST-fusion proteins as an insoluble aggregate for all three clones.

Bacterial cells were harvested by centrifugation at 6,000 x g for 10 min. The pellet was resuspended in 2.5 ml of lysis buffer (25 % sucrose in TrisHCl [50 mM, pH 8.0]). The solution was frozen at –70°C for 30 min and thawed, then 0.25 ml lysozyme (10
mg/ml in 250 mM Tris [pH 8.0]) was added and the mixture was kept on ice for 10
min. Another incubation step on ice for 10 min followed after addition of 10 ml 2 x
RIPA/TET (see below; mixed in a ratio of 5:4). The solution was sonicated (Sonic
Cell Disruptor, Branson Sonifer, Branson Power, Dannbury, U.S.A) for 3 min using
the maxi tip with output-setting 5 and 50 % duty cycle; sonication was repeated until
the solution appeared clear and opalescent. Sonication was followed by
centrifugation at 32,500 x g for 20 min. The resulting pellet was resuspended in
500 μl of A. dest. The pellet was diluted 1:2 in 2 x sample buffer and 10 μl of each
protein preparation were separated on a 10.8 % SDS PAGE.

2X RIPA
20 mM Tris, pH 7.4
300 mM NaCl
2 % Na-deoxycholic acid sodium salt
2 % NP-40® (Tergitol)

TET
100 mM Tris, pH 8.0
50 mM EDTA, pH 8.0
2 % Triton-X 100

Sample buffer (2x)
1.5 ml 0.5 M Tris pH 6.8
6.0 ml 10 % SDS
3.0 ml 50 % glycerole
10.0 μl 1 % bromophenol blue
1.0 ml 2-mercaptoethanol

3.7.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
PAGE)
Discontinuous SDS-PAGE was performed following standard procedures
(SAMBROOK et al. 1989) using a Protean II Minigel system (BioRad Inc, Munich,
Germany).
3.7.5 Determination of protein concentration

Protein concentration was estimated using SDS PAGE with both the protein preparation in question and BSA (1mg/ml) loaded in serial twofold dilutions, Coomassie blue staining, and subsequent comparison of band intensities.

3.7.6 Protein detection

Protein stains
SDS-PAGE gels were stained with Coomassie Brilliant Blue R250.

Immunoblotting
Western blotting of the SDS-PAGE minigels was done using the Mini Trans-Blot® system (BioRad Inc., Munich, Germany). Proteins were transferred to a nitrocellulose membrane (Protran BA85 0.45 µM, Schleicher and Schuell, Dassel, Germany) as described by SAMBROOK et al. (1989) for 30 min at 50 V. As a primary antibody a pool of sera obtained from 10 chronically CBPP-infected cattle (Table.8) or rabbit hyper-immune sera were used. Blots were developed using an alkaline phosphatase-conjugated goat anti-rabbit or goat anti-bovine IgG antibody (Jackson ImmunoResearch Laboratories, Dianova, Hamburg, Germany), diluted 1:2,000, as conjugate, and BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium) as substrate for visualization of protein bands (SAMBROOK et al. 1989).

3.7.7 ELISA with recombinant fusion protein as solid phase antigen

One hundred µl of recombinant fusion protein-solution (2.5 µg fusion protein/ml), prepared as described above, were coated on 96-well Polysorp® ELISA plates (Nunc GmbH & Co. KG, Wiesbaden, Germany) using carbonate coating buffer (50 mM [pH 9.6]) overnight at 4°C and stored at -20°C until usage. Incubation with primary antibodies was performed for 1 h at room temperature (Hyperimmune sera raised in rabbits against \textit{MmmSC}, CBPP-infected animals sera and corresponding negative sera). Then goat anti-rabbit or goat anti-bovine peroxidase conjugates (Dianova,
Hamburg, Germany) were each incubated for 1 h at room temperature. The ELISAs were developed using 2, 2-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) (Roche Diagnostics, Mannheim, Germany) as a substrate.
4 Results

4.1 Construction of pHORF3X and pHORF3XX from pHORF3

The phage vector pHORF3 (KÜGLER et al. 2008) was kindly provided by Michael Hust, Technical University Braunschweig. It was constructed by replacing the NotI restriction sites in pHORF2 (HUST et al. 2006) with a unique PmeI site in order to allow the introduction of blunt-end genomic DNA fragments between the 5' pelB signal sequence and the 3' gIII gene fragment. If signal peptide, inserted genomic DNA fragment, and gIII encode an intact ORF, the peptide encoded by the inserted DNA fragment is displayed as gIII fusion protein on the phage surface (KÜGLER et al. 2008).

The vector pHORF3 provided was modified such that the singular PmeI site used for insertion of foreign DNA was shifted by 1 and 2 bp, respectively, and designated as pHORF3X and pHORF3XX. This allows the successful cloning of additional ORFs from the same DNA fragment pool used in initial experiments (Fig. 3, Fig. 4).
Results

Fig. 3: Schematic diagram of vector pHORF3 and derivatives. (A) circular map of pHORF3; (B) linear map of pHORF3 showing the relative position of the singular Pmel restriction site used for cloning; (C) DNA and amino acid sequence of pHORF3 and derivatives pHORF3X and pHORF3XX showing that three of nine possible reading frames can be expressed using the three vectors and that only vector pHORF3X will result in a functional pIII protein if it does not contain an insert.

Lac Pr., promoter of the bacterial lac operon; RBS, ribosome binding site; pelB, sequence encoding the signal peptide of bacterial pectate lyase, mediating protein transport into the periplasmic space; tag, hexahistidine tag for detection and purification of soluble proteins; amber, amber stop codon allowing production of soluble proteins; terminator, sequence terminating transcription; bla, β-lactamase gene encoding ampicillin resistance; colE1, origin of replication for E.coli; M13 ori, intergenic region of filamentous phage (M13) for phage packaging.
Fig. 4: Expression vector pHORF3. Circular map of pHORF3, enlargement of the region restricted by Pmel, and ethidium bromide-stained gel of restriction endonuclease digests of vectors pHORF3 (2), pHORF3X (2) and pHORF3XX (3) using Pmel, the cloning site for MmmSC DNA inserts. Lane 1 shows the 5 kb size marker.

4.2 Construction of the MmmSC genomic library and enrichment of ORFs

Genomic DNA of MmmSC was restricted using five different restriction endonucleases (Alul, Psil, Sspl, HpyCH4V, Rsal) generating blunt ends; the fragments were pooled and ligated into the Pmel site of pHORF3, pHORF3X and pHORF3XX resulting in three different phagemid libraries; the cloning scheme is shown in Fig. 5. The complexity of the phagemid libraries as determined by dilution and counting of the colony-forming units (cfu) was found to be between 1.6-2.4 x 10^7,
and about 90% of the clones contained DNA inserts of 100 - 800 bp as assessed by colony PCR before packaging. (Fig. 6A).
The phagemid libraries were packaged into Hyperphage in order to produce phage and to enrich for ORFs. Due to this process the complexity of the individual libraries was statistically reduced by a factor of 6 and the maximum size of DNA inserts decreased to 500 bp as assessed by infection of *E. coli* and colony PCR (Fig. 6B).
Results

Fig. 5: Diagramm depicting the construction of the MmmSC phage library. DNA prepared from MmmSC (ethidium bromide-stained gel of purified DNA alongside a 5 kb size marker) is restricted with 5 different blunt end-cutting restriction endonucleases (AluI, PsiI, SspI, HpyCH4V and RsaI; ethidium bromide-stained gel of DNA restricted with AluI alongside a 5 kb size marker). Fragments of 200 to 800 bp derived from the different restriction endonuclease digests were pooled and ligated into vectors pHORF3, pHORF3X and pHorf3XX thereby constructing three individual libraries. The libraries were than packaged into Hyperphage facilitating selection of in-frame inserts since expression of pIII depends on translational in-frame fusion with the peptide encoded by the MmmSC-derived insert.
Fig. 6: PCR analysis of randomly selected clones by PCR before (A) and after packaging (B). (A) PCR products of individual transformants of the MmmSC libraries with size marker (5Kb), 24 randomly selected colonies (lanes 1 – 24) and the control pHORF3 (lane 25) showing that approximately 70% of the clones contain DNA inserts of 100 - 800 bp. (B) PCR products of the phage display library after packaging into hyperphage with size marker (1Kb), randomly selected colonies (lanes 1 – 22) and the control pHORF3 (lane 23) showing that after enrichment of ORFs by packaging into Hyperphage the maximum size of DNA inserts decreased to 500 bp and the percentage of clones with sizable insert was increased.

4.3 Selection of immunogenic oligopeptides

The panning procedure for identifying immunogenic oligopeptides of MmmSC was performed in microtitre plates (MTP) using pooled sera obtained from chronically MmmSC-infected animals (positive control serum) as depicted in Fig. 7. Initial experiments had revealed that the positive control serum mediated binding of phage particles (i.e. Hyperphage without MmmSC-derived insert) thereby counteracting the
specific isolation of immunogenic oligopeptides encoded by phage libraries. Therefore, the serum used for the panning procedure was preabsorbed to Hyperphage-coated microtitre wells. The serum IgG was then immobilized to the microtiter plate surface by rabbit anti-bovine IgG coated to the plate surface. Subsequently, the Hyperphage-packaged ORF-enriched genomic MmmSC library was incubated on the captured bovine serum IgG, and non-binding phage particles were removed by repeated washing. Binders were eluted, amplified in *E. coli* and packed again with Hyperphage before starting another panning round; in total four panning rounds were performed. The titre after each panning round was determined (Fig. 8). In the third and fourth panning round the titre of eluted phage increased three to four orders of magnitude; this documented the enrichment of specific phage.
Results

Fig. 7: Schematic overview of the panning process. The phage library was used for panning on a pool of 10 sera derived from CBPP-infected animals. Phage binding to the serum were selected by multiple panning rounds and plaque-purified to identify monoclonal binders by ELISA.
Fig. 8: Phage titers (cfu/ml) obtained from each panning round after elution. The increasing phage titre documents the enrichment of specific binders and coincides with a decreasing complexity.

4.4 Identification of immunogenic oligopeptides

*Escherichia coli* clones infected with the eluted phage were isolated from panning rounds 2, 3 and 4 and used for monoclonal phage production in glass tubes and screening by phage ELISA (i. e. an ELISA using phage as solid phase antigen and the positive control serum as detecting antibody) to identify phage displaying immunogenic *MmmSC*-derived oligopeptides (Fig. 9). A serial two-fold dilution of mycoplasmal whole cell antigen of known concentration served as positive and Hyperphage particles as negative control.
Fig. 9: Identification of individual phage encoding an immunogenic polypeptide showing the ELISA results (A) and a schematic of the coating and development procedure used (B). Individual phage were used as solid-phase antigen (A3, A4), Hyperphage without insert served as negative control (A2) and MmmSC whole cell lysate served as positive control (A1). The schematic depicts the phage as solid phase antigen (a), the MmmSC-positive serum (b) and the anti-bovine conjugate (c). The phage clone in A3 was identified as negative, the phage clone in A4 as positive.

4.5 Identification of immunogenic peptides

After having performed four panning rounds with the positive control serum, a total of 147 clones (49 from each of the three libraries based on vectors with the cloning site in three different reading frames with respect to the 3’ pIII protein-encoding ORF) were sequenced. DNA sequencing of 147 clones revealed that 90% of the clones
contained an ORF consisting of a MmmSC genomic DNA fragment in-frame with the *pelB* signal sequence and the pIII protein-encoding ORF. One hundred and twenty clones revealed in an analyzable sequence, and twenty-two different clones could be identified by NCBI Blast analysis (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) with all clones present at least twice (Table 11).

The individual clones were amplified and the relative strength of antigenicity was determined by phage ELISA. Eight clones showing a reactivity of more than 30 OD\% with respect to the positive control (whole *MmmSC* lysate) were considered to be of potential diagnostic value and were subjected to further investigation (Fig. 10).
Table 11: Overview of the immunogenic polypeptides identified in *MmmSC*.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Clone No.</th>
<th>Mycoplasma DNA Segment</th>
<th>Product from Mycoplasma mycoides</th>
<th>Insert Length bp</th>
<th>Locus tag</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>23668-23767</td>
<td>Mannitol-1-phosphate 5-dehydrogenase</td>
<td>99</td>
<td>MSC_0017</td>
<td>99%</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>32410-32351</td>
<td>Acyl carrier protein phosphodiesterase</td>
<td>60</td>
<td>MSC_0029</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>44289-44393</td>
<td>D-Lactate dehydrogenase</td>
<td>105</td>
<td>MSC_0034</td>
<td>100%</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>77529-77641</td>
<td>Transcriptional regulator involved in nitrogen regulation NifR3 family</td>
<td>184</td>
<td>MSC_0068</td>
<td>100%</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>97781-98005</td>
<td>Prolipoprotein putative phosphate ABC transport</td>
<td>224</td>
<td>MSC_0079</td>
<td>99%</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>127879-127937</td>
<td>Glucosyl transferase</td>
<td>59</td>
<td>MSC_0108</td>
<td>100%</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>159499-159643</td>
<td>Hypothetical transmembrane protein</td>
<td>144</td>
<td>MSC_0135</td>
<td>100%</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>218688-218814</td>
<td>Oligopeptide ABC transporter, substrate binding component, oppA</td>
<td>147</td>
<td>MSC_0184</td>
<td>100%</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>304438-304508</td>
<td>Pyruvate dehydrogenase (Lipoamide betachain pdh)</td>
<td>71</td>
<td>MSC_0266</td>
<td>99%</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>385965-386234</td>
<td>tRNA pseudouridine synthase B</td>
<td>269</td>
<td>MSC_0336</td>
<td>98%</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>696996-697122</td>
<td>ATP dependent protease ClpB</td>
<td>128</td>
<td>MSC_0613</td>
<td>100%</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>698434-698492</td>
<td>Putative hydrolase of HAD family</td>
<td>89</td>
<td>MSC_0814</td>
<td>100%</td>
</tr>
<tr>
<td>13</td>
<td>4</td>
<td>701239-701317</td>
<td>Prolipoprotein</td>
<td>78</td>
<td>MSC_0817</td>
<td>100%</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>733298-733109</td>
<td>Conserved hypothetical protein</td>
<td>159</td>
<td>MSC_0836</td>
<td>100%</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>750456-750516</td>
<td>Conserved hypothetical protein</td>
<td>80</td>
<td>MSC_0856</td>
<td>100%</td>
</tr>
<tr>
<td>16</td>
<td>3</td>
<td>940581-940693</td>
<td>Conserved hypothetical protein</td>
<td>112</td>
<td>MSC_0826</td>
<td>98%</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>1032859-1032922</td>
<td>Amino acid permease</td>
<td>64</td>
<td>MSC_0908</td>
<td>100%</td>
</tr>
<tr>
<td>18</td>
<td>19</td>
<td>1117031-1117172</td>
<td>UDP glucose 4-epimerase</td>
<td>141</td>
<td>MSC_0978</td>
<td>100%</td>
</tr>
<tr>
<td>19</td>
<td>7</td>
<td>1153378-1153506</td>
<td>DNA directed RNA polymerase beta subunit (rPoc)</td>
<td>199</td>
<td>MSC_1009</td>
<td>98%</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>1160317-1160401</td>
<td>Glucose inhibited division protein A</td>
<td>85</td>
<td>MSC_1017</td>
<td>98%</td>
</tr>
<tr>
<td>21</td>
<td>2</td>
<td>1205636-1205659</td>
<td>AAA family ATPase</td>
<td>124</td>
<td>MSC_1062</td>
<td>100%</td>
</tr>
<tr>
<td>22</td>
<td>6</td>
<td>1210760-1210841</td>
<td>Conserved hypothetical transmembrane protein</td>
<td>82</td>
<td>MSC_1066</td>
<td>100%</td>
</tr>
</tbody>
</table>

The protein IDs and locus tags are given for *MmmSC* type strain PG1 (NCBI Genbank accession no. BX293980). All proteins are identified repeatedly among 147 highly positive clones analysed individually by nucleotide sequencing indicating a sufficient selection within the four high success rate of panning rounds.
Fig. 10: Individual testing of the 22 different seroreactive phage clones in a phage ELISA. The phage clones were used as solid phase antigen and developed using the positive control serum at a dilution of 1:200; the optical density was expressed as % positive control (MmmSC whole cell lysate). The bars on top of the columns depict the standard deviation from four independent experiments. Eight of the clones resulting in a reaction of OD% >30 were selected for further experiments.
4.6 Selection of clones with diagnostic potential

The eight clones with potential diagnostic value were individually amplified, and the discrimination between positive and negative control serum was investigated by ELISA using serial two-fold dilutions. For six of the eight clones (MSC_0029, 0108, 0636, 0656, 1009 and 1062) an at least 4-fold difference in titre was observed between the positive and the negative control serum (Fig. 11). These six clones were tested with respect to their discriminatory potential using the ten individual positive sera and the six individual negative sera contained in the respective control sera. Four of the clones (MSC_029, 108, 636 and 1062) reacted with all individual positive sera; no overlap in OD%-values was seen between positive and negative sera (Fig. 12).

These four clones were investigated for cross reactivity to other mycoplasmal species using rabbit hyperimmune sera directed against 17 different mycoplasmal species. All four clones (MSC_0029, 0108, 0636, 1062) were highly positive with the hyperimmune serum directed against MmmSC. All clones showed the strongest cross-reactivity with the serum directed against M. canadense; for one clone (MmmSC1062) it reached 85 OD% and, therefore, this clone was excluded from further analyses (Table 12). The three remaining clones (MSC_0029, MSC_0108 and MSC_0636) were expressed as GST fusion proteins.
Fig. 11: Selection of individual immunogenic phage with high discriminatory power by ELISA. The eight phage clones (MSC_0029, MSC_0108, MSC_0613, MSC_0636, MSC_0656, MSC_0908, MSC_1009 and MSC_1062) selected based on their reactivity with the positive serum were investigated for their discriminatory ability by simultaneous ELISA with the negative control serum. Individual phage were used as solid phase antigen and the ELISA was developed using serial two-fold dilutions (1:250 to 1:16,000) of positive (green) and negative serum (blue). The bars depict the standard deviation obtained in three independent experiments. MmmSC whole cell lysate served as positive control and Hyperphage as negative control. Six clones (MSC_0029, MSC_0108, MSC_0636, MSC_0656, MSC_1009 and MSC_1062) showed an at least 4-fold difference in titer between positive and negative serum. These clones were selected for further investigation.
Fig. 12: Selection of individual immunogenic phage with high discriminatory power by ELISA with individual bovine sera. The six clones showing discriminatory power using the positive and negative control serum were tested using 10 individual positive and 6 individual negative sera. The columns indicate the arithmetic mean of the individual sera and the bars depict the standard deviation. For four of the six clones (MSC_0029, MSC_0108, MSC_0636, and MSC_1062) no overlap in OD%-values was seen between positive and negative sera. These four clones were selected for further investigation.
Table 12: ELISA cross reactivity of four clones using rabbit sera directed against 17 different *Mycoplasma* species.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Sera</th>
<th>MSC_0029</th>
<th>MSC_0108</th>
<th>MSC_0636</th>
<th>MSC_1062</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Mmm</em>SC</td>
<td>100±4.6</td>
<td>100±12.1</td>
<td>100±2.5</td>
<td>100±8.9</td>
</tr>
<tr>
<td>2</td>
<td>Negative serum</td>
<td>27±3.0</td>
<td>30±2.6</td>
<td>29±1.0</td>
<td>36±0.7</td>
</tr>
<tr>
<td>3</td>
<td><em>M. akalesens</em></td>
<td>17±0.6</td>
<td>21±2.1</td>
<td>43±1.3</td>
<td>41±0.5</td>
</tr>
<tr>
<td>4</td>
<td><em>M. argini</em></td>
<td>21±1.6</td>
<td>21±0.3</td>
<td>26±1.6</td>
<td>32±0.3</td>
</tr>
<tr>
<td>5</td>
<td><em>M. californicum</em></td>
<td>21±2.5</td>
<td>22±0.9</td>
<td>32±0.6</td>
<td>32±2.2</td>
</tr>
<tr>
<td>6</td>
<td><em>M. bovigenitalium</em></td>
<td>17±0.3</td>
<td>17±1.9</td>
<td>22±1.1</td>
<td>25±0.3</td>
</tr>
<tr>
<td>7</td>
<td><em>M. gallinarum</em></td>
<td>19±1.9</td>
<td>28±2.4</td>
<td>45±1.1</td>
<td>44±0.3</td>
</tr>
<tr>
<td>8</td>
<td><em>M. bovoculi</em></td>
<td>44±0.4</td>
<td>42±8.8</td>
<td>44±7.1</td>
<td>33±2.9</td>
</tr>
<tr>
<td>9</td>
<td><em>M. bovirhinis</em></td>
<td>36±4.8</td>
<td>40±2.1</td>
<td>44±14.9</td>
<td>44±3.2</td>
</tr>
<tr>
<td>10</td>
<td><em>M. axunthum</em></td>
<td>30±4.2</td>
<td>21±4.0</td>
<td>27±1.5</td>
<td>31±2.0</td>
</tr>
<tr>
<td>11</td>
<td><em>M. capricolum</em></td>
<td>45±21.7</td>
<td>48±0.5</td>
<td>45±1.4</td>
<td>57±4.9</td>
</tr>
<tr>
<td>12</td>
<td><em>Mmm</em>LC</td>
<td>30±1.2</td>
<td>27±2.3</td>
<td>30±0.5</td>
<td>34±3.4</td>
</tr>
<tr>
<td>13</td>
<td><em>M. canadense</em></td>
<td>65±10.4</td>
<td>71±7.4</td>
<td>61±1.8</td>
<td>85±2.0</td>
</tr>
<tr>
<td>14</td>
<td><em>M. bovis</em></td>
<td>44±13.7</td>
<td>39±2.5</td>
<td>41±5.6</td>
<td>49±2.3</td>
</tr>
<tr>
<td>15</td>
<td><em>A. laidlawii</em></td>
<td>39±8.3</td>
<td>43±0.2</td>
<td>42±3.5</td>
<td>39±1.7</td>
</tr>
<tr>
<td>16</td>
<td><em>A. modicum</em></td>
<td>32±6.8</td>
<td>26±0.6</td>
<td>32±0.4</td>
<td>51±1.2</td>
</tr>
<tr>
<td>17</td>
<td><em>A. axanthum</em></td>
<td>28±8.6</td>
<td>26±4.1</td>
<td>35±2.3</td>
<td>45±1.8</td>
</tr>
<tr>
<td>18</td>
<td><em>M. bovine group VII</em></td>
<td>46±6.0</td>
<td>27±2.0</td>
<td>33±4.3</td>
<td>45±7.3</td>
</tr>
</tbody>
</table>

All sera were kindly provided by Dr. Heller, FLI, Jena, and used in a 1:2,000 dilution; phage clones were used as solid-phase antigen. Three of the clones (MSC_0029, MSC_0108, and MSC_0636) showed only limited cross-reactivity with rabbit sera directed against 17 other *Mycoplasma* species. One clone (MSC_1062) showed strong reactivity with serum directed against *M. canadense* and was excluded from further analyses.
4.7 Identification of stop codons near selected immunogenic peptide sequences and construction of recombinant GST-fusion proteins

The genes encoding for the immunogenic epitopes identified by the panning process were identified further based on the genome sequence of strain *Mmm*SC PG1. In order to construct fusion proteins the part of the respective gene encoding the immunogenic peptide and located between two TGA-codons (encoding for tryptophan in *Mycoplasma* spp. but serving as stop codon in *E. coli*) was identified. The sequences were amplified by PCR using primers with a unique restriction enzyme site on their respective 5’ and 3’ ends in order to facilitate directional cloning into the expression vector pGEX5x2 resulting in the plasmids pMSC0029-500, pMSC0108-500 and pMSC0636-500 (Fig. 13). The purity of the cultures of *E. coli* transformants was confirmed by immuno-colony blot with positive control serum; all individual colonies reacted thereby implying that pure cultures had been prepared and that spontaneous mutations did not occur at a detectable frequency (Figure 14).
Results

Fig. 13: Schematic for the construction of expression vectors resulting in GST-fusion proteins. (A) The genes corresponding to the respective phage-derived polypeptide clones were selected and TGA codons (stop) as well as the region present in the phage were marked (yellow box). (B) The DNA-regions between two stop codons encoding the immunogenic peptide identified by phage display were amplified by PCR. (C) The PCR fragments were cloned in-frame into the expression vector pGEX5x2 resulting in plasmids pMSC0029-500, pMSC0108-500, and pMSC0636-500. The three plasmids shown resulted in the production of fusion proteins of the expected size in *E. coli* transformants.
Figure 14: Immuno-colony blots for *E. coli* transformants carrying plasmids (A) pMSC0029-500, (B) pMSC0108-500, (C) pMSC0636-500, and (D) pGE5-x2 as negative control. All individual colonies carrying an expression plasmid reacted with the positive control serum (dilution 1:100) thereby showing that pure cultures had been prepared and that spontaneous mutations did not occur at a detectable frequency.
4.8 Characterization of recombinant GST fusion protein

The fusion proteins were prepared as inclusion bodies and analysed by SDS PAGE (Fig. 15A) and by Western blot using an anti-GST antibody (Fig. 15B), as well as with serum from CBPP-infected animals (positive control; Fig. 15C) and serum from uninfected animals (negative control sera; Fig. 15D). The recombinant fusion proteins expressed reacted positive with both the anti-GST and the positive control serum but did not react with the negative control serum. All proteins had a molecular mass corresponding to the calculated masses of 32.5, 32.0, and 45.0 kDa, respectively.
Fig. 15: Characterization of the GST fusion proteins. (A) SDS PAGE showing aggregate preparation of recombinant fusion proteins of *E. coli* transformants containing pMSC0029-500, pMSC0108-500, pMSC0636-500 and the cloning vector pGEX-5x2 (Lines 1 to 4). (B) Western blot using anti-GST antibody (1:2000), (C) Western blot using the positive control serum obtained from CBPP-infected animals (1:100) and (D) Western blot using the negative control serum (1:100). All three clones pMSC0029-500, pMSC0108-500, pMSC0636-500 were found to strongly react with the positive control serum and not with the negative control serum. In addition, GST showed only minor reactivity with the positive control serum.
4.9 GST fusion protein (aggregate) ELISA's

The GST fusion-proteins were used as solid-phase antigens in an ELISA. In a first ELISA using recombinant fusion proteins as solid phase antigen, rabbit hyperimmune serum raised against *Mmm*SC were used in serial two-fold dilution starting from 1:100. A good discrimination between positive and negative serum (32-fold difference in serum concentration leading to an OD of 1) was observed (Fig. 16).

In a second ELISA, the CBPP-positive and -negative control sera were used in serial two-fold dilutions starting from 1:250. The ELISA results revealed poor discrimination between positive and negative control serum (< 2-fold) and a high inter-assay variation (Fig. 17). Further, these fusion proteins were tested against ten individual CBPP infected and six individual uninfected animals sera. In this assay, despite a less than 2-fold difference in OD% values between positive and negative sera no overlap of OD%-values for positive and negative sera was observed (Fig. 18).
Fig. 16: Characterization of the GST fusion proteins by ELISA using rabbit hyperimmune serum raised against MmmSC. All three fusion proteins and their mixture showed good discrimination between rabbit hyperimmune and negative control serum (32-fold difference in serum concentration leading to an OD of 1). Coating with MmmSC whole cell lysate served as positive control.
Fig. 17: Characterization of the GST fusion proteins by ELISA using the positive and negative control sera. Neither the individual proteins nor their mixture showed discrimination between positive and negative control serum. The bars depict the standard deviations from three independent experiments. Coating with *Mmm*SC whole cell lysate served as positive control.
Fig. 18: Characterization of the GST fusion proteins by ELISA using 10 individual positive and six individual negative sera. The columns depict the arithmetic mean of the positive and negative sera, and the bars depict the standard deviation. For two of the fusion proteins (MSC_0029 and MSC_0108) the ELISA-reactivity differentiates between the positive and negative sera. For fusion protein MSC_0636 and for the mixture of all three fusion proteins no differentiation of positive and negative sera can be seen based on the ELISA results. Coating with MmmSC whole cell lysate served as positive control.
5 Discussion

*Mycoplasma mycoides* subsp. *mycoides* small colony type (*MmmSC*), the causative agent of contagious bovine pleuropneumonia (CBPP) belongs to class *Mollicutes*. The identification of immunogenic proteins of *MmmSC* and other bacteria belonging to the *Mollicutes* has been limited by the lack of classical translational systems and inability to express cloned genes in native *Mycoplasma* (DYBVIG and VOELKER 1996; MINION 1998). The low G+C-content of 24% (WESTBERG et al. 2004) and the use of TGA as tryptophane codon (MINION 1998) rather than (serve) as a termination codon (INAMINE et al. 1990) has hampered genetic approaches. The current diagnostic tests namely the complement fixation test (CFT) and the competitive-ELISA (cELISA) are hampered by a limited sensitivity (MAROBELA-RABOROKGWE et al. 2003). The currently used live vaccines (T144, T144 SR) suffer from a limited duration of protection of only one year, and severe side effects (PILO et al. 2007; TULASNE et al. 1996) and also exhibit poor efficacy and stability (RWEYEMAMU et al. 1995; THIAUCOURT et al. 2000). Several publications and expert meetings have reported on the inability of current vaccines to control the disease in Africa (ANON 1999; ANON 2001; MASIGA and DOMENECH 1995; NICHOLAS et al.) and research designed to produce ‘next generation’ vaccines has proceeded, although with little apparent success (ABUSUGRA et al. 1997; WAITE and MARCH 2002).

5.1 Construction of *MmmSC* phage display library, packaging in to Hyperphage and panning

The construction of genomic libraries in phage display vectors could be a crucial tool to identify immunogenic polypeptides, particularly if they cannot be identified by proteome-based methods due to lack of expression in vitro. For *Mycoplasma* species the enrichment of small but continuous ORFs in a phage display library is highly advantageous, because this approach – in contrast to conventional expression libraries is not severely hampered by the use of TGA as tryptophane codon (KÜGLER et al. 2008). So far, there have been few successful studies using genomic
phage display libraries to identify mycoplasmal antigens (BEGHETTO et al. 2006; BEGHETTO et al. 2009) with only Kügler et al., 2008 using an M13-based system. The enrichment of ORFs from genomic phage display libraries is only possible if the corresponding polypeptides are presented on the phage surface. When using an M13-based system the foreign peptide has to be located at the aminoterminal end of the mature pIII protein. Therefore, statistically only one in 18 random DNA-fragments will contain a complete ORF consisting of the signal peptide, the cloned DNA insert, and the pIII protein (HUST et al. 2006). As a result, when a conventional packaging system based on a complete helper phage is used, a subsequent panning would be performed with a phage library containing more than 90% of “junk clones”. Since each phage display selection is based on the ability to distinguish single molecular binding events in a background of $10^8 - 10^{11}$ “unspecific” molecules, a library with more than 90% “junk” sequences would hamper the success of the panning (KÜGLER et al. 2008). This is prevented by using the Hyperphage packaging system which consists of a helperphage not encoding the pIII protein and, therefore, can only package those phagemids where the pIII protein is expressed in-frame with the foreign polypeptide (HUST et al. 2006).

The average size of the cloned insert decreased after Hyperphage packaging. This decrease in insert size could be attributed to a number of reasons, mainly (i) longer gene fragments statistically contain more UGA codons than short gene fragments and are, therefore, lost during the enrichment steps, and (ii) phagemids containing a short insert will be propagated faster in *E. coli* than phagemids with longer inserts. After packaging, the rate of ORF enrichment was analysed by DNA sequencing showing that 90% of the clones contained an ORF consisting of a *MmmSC* genomic DNA fragment in-frame with the *pelB* signal sequence and the pIII protein-encoding ORF.

PCR analyses of individual clones after packaging showed that approximately 70% of the clones contained an insert of a length that was clearly distinguishable from the negative control upon agarose electrophoresis (Fig. 6). The other clones are likely to either have short inserts (< 100 bp) or are part of the pHORF3x library where the pIII protein is expressed even if no insert is present. This ORF-enrichment of the
constructed *MmmSC* genome library is in accordance with recently published results (HUST et al. 2006; KÜGLER et al. 2008).

The restriction to smaller polypeptides and the Sec pathway of *E. coli* used by the phage display system might hamper the representation of the entire spectrum of immunogenic epitopes of *MmmSC* antigens. The loss of complex antigens with structural epitopes as well as of antigens which can not be secreted into the periplasm of *E. coli* via the Sec pathway, like membrane or cytosolic proteins, have to be considered. However, in this study the Hyperphage based ORF-enrichment and phage display system already resulted in the identification of lipoproteins, membrane proteins and intracellular proteins representing different types of proteins (Table 11). Presumably, the display of smaller polypeptides are much more independent of the folding and secretion apparatus in *E. coli* and should be less problematic regarding the Sec pathway required for this phage display system. However, a combination with phagemid vectors facilitating secretion via the twin-arginin-translocation pathway (PASCHKE and HOHNE 2005) could provide the display of a broader spectrum of polypeptide antigens.

Four panning rounds using a pooled serum from infected animals and including stringent washing steps (increase of 10 fold washing in every panning round) were performed. The specifically bound phages were eluted and reamplified by infection of *E. coli*. This is in accordance with the work of other authors showing that for enrichment of specific binders more than two panning rounds can be carried out (HUST et al. 2007). The preincubation of positive serum on Hyperphage before panning and the addition of Hyperphage particles and *E. coli* lysate into the blocking buffer during the panning procedure were performed because unspecific binding of M13 phage has been demonstrated for many sera (ESHAGHI et al. 2005) and is probably caused by previous contact and immunization with these *E. coli* phage. Therefore, this approach was considered to reduce background binding and to allow a specific selection of phage particles displaying *MmmSC*-derived polypeptides. During the screening of individual phage clones the signal to noise ratio was
optimized by using purified phage particles produced in glass tubes as the solid phase antigen instead of culture supernatant. One hundred and forty-seven individual phage clones were identified for which the relative optical density (%OD) with the positive control serum was at least twice as high as that of the negative control serum. The insert DNA of these 147 clones was sequenced resulting in identification of 22 unique clones encoding different peptides (Table 11). Each of the clones was present at least twice among the clones sequenced, thereby showing the success of the panning procedure.

5.2 Function of the immunogenic proteins identified in the phage display library

It is well known that Mycoplasmas lack the classical modulins such as lipopolysaccharides (LPS), lipoteichoic acid, and murein fragments (HENDERSON et al. 1996), but they are still potent activators of macrophages. One of the most important mycoplasmal factors in pathogenicity are lipoproteins or lipopeptides (FENG and LO 1994; MUHLRADT et al. 1998). Lipoproteins are considered to be highly immunogenic due to their surface exposure and amino-terminal lipoylated structure (CHAMBAUD et al. 1999). Four MmmSC immunogenic membrane lipoproteins (Lpps) have been identified previously and their role in MmmSC-specific humoral response has been shown (ABDO et al. 2000; CHENG et al. 1996; PILO et al. 2003; VILEI et al. 2000). Further studies also reported the strong potential of mycoplasmal Lpps to trigger immune responses, with the lipid moiety acting with adjuvant-like proinflammatory activity while epitopes of the protein part evoke the immune response (CHAMBAUD et al. 1999; YOU et al. 2006). The lipoprotein LppQ has been characterized as immunogenic (ABDO et al. 2000), and an indirect ELISA has been established (BRUDERER et al. 2002).

Among the 22 immunogenic peptides identified in this study, two peptides encode epitopes of putative lipoproteins MSC_0079 and MSC_0617. None of these putative lipoproteins has been described to date in MmmSC or in other mycoplasmal species.
Also, they don’t have homologues in other bacterial species and, therefore, their function is currently unknown.

For proteins potentially useful as components in subunit vaccines it is advantageous if they are known virulence factors or are part of metabolic pathways deemed essential for survival in the host. A strong humoral immune response to these proteins is likely to prevent clinical disease and reduce shedding upon infection (KAUFMANN 1996). The in silico investigation performed on the immunogenic proteins identified in this study revealed a number of potential vaccine components.

Thus, pyruvate dehydrogenase appears to be located on the surface and to mediate adhesion in some Mycoplasmas (LAYH-SCHMITT et al. 2000; REGULA et al. 2001). A recent study employing mouse sera and a MmmSC lambda phage library – among other proteins – also identified members of the pyruvate dehydrogenase complex to be highly immunogenic (MARCH et al. 2006). Interestingly, pyruvate dehydrogenase has been described to be immunogenic in other Mycoplasma species like M. hyopneumoniae (PINTO et al. 2007) and M. pneumoniae (DALLO et al. 2002). In our study we also identified pyruvate dehydrogenase (MSC_0266).

Lactate dehydrogenase (LDH) has already been described as immunogenic in M. hyopneumoniae (FREY et al. 1994) and its activity seems to be common to different Mycoplasma species (MANOLUKAS et al. 1988). In this study LDH (MSC_0034) was also identified as being immunogenic.

Mannitol-1-phosphate 5-dehydrogenase (MSC_0017) has also been described as immunodominant 72 kDa lipoprotein of MmmSC (CHENG et al. 1996) and 67-kDa lipoprotein of Mycoplasma sp. bovine group 7 (FREY et al. 1998). In this study Mannitol-1-phosphate 5-dehydrogenase (MSC_0017) was also identified as being immunogenic.

In 1976 it was shown that intravenous injection of the capsule from MmmSC into calves evoked pulmonary edema as in natural lesions of CBPP, indicating that the capsule has a direct toxic effect (BUTTERY et al. 1976). There are also some indications that increased capsular content is associated with reduced phagocytosis by the host cells (MARSHALL et al. 1995). The MmmSC genome contains two clusters involved in the synthesis of the capsule; one is located between position
127,251 and 130,842 (WESTBERG et al. 2004), and the second one is located between position 1,108,435 and 1,133,176 (WESTBERG et al. 2004). Proteins encoded on both clusters were identified in this study with the glycosyl transferase gene MSC_0108 (position 127,251-128,600) in the first and the glycosyl transferase gene (MSC_973, MSC_980, MSC_987 and MSC_993 with 99% approximate identity to MSC_108) and UDP glucose 4-epimerase gene (MSC_0978; position 1,116,217-1,117,221) in the second cluster.

In this study also a number of membrane proteins (MSC_135, MSC_0910 and MSC_1066) were identified representing different types of proteins. Among these, MSC_0135 and MSC_1066 are hypothetical and are currently not characterized. One of the peptides maps to the acyl carrier protein phosphodiesterase (MSC_0029), a cytoplasmic protein having 100% identity with MmmSC type strain PG1 and close identity to the putative membrane protein MCAP_0345 of M. capricolum. In E. coli, ACP is a small anionic protein that functions as an essential component of enzyme systems for the biosynthesis of fatty acids (VAGELOS et al. 1966), membrane phospholipids and lipopolysaccharides (COOPER et al. 1989).

One of the peptides maps to the ATP dependent protease ClpB (MSC_0613). Transcriptional and translational analyses of heat shock in M. pneumoniae indicated that clpB transcription is significantly upregulated, reinforcing its status as a critical responder to heat stress (KANNAN et al. 2008). It is identified as heat shock protein in different bacteria (ERIKSSON and CLARKE 1996; GRANDVALET et al. 1999; INGMER et al. 1999; SQUIRES et al. 1991). The clpB gene is also identified as being involved in cellular invasion in-vitro and virulence in-vivo in Porphyromonas gingivalis (YUAN et al. 2007).

Proteins of the AAA family ATPase (MSC_1062) are found in all organisms and are essential for cell cycle functions, vesicular transport, mitochondrial functions, peroxisome assembly and proteolysis (FROHLICH 2001). Likewise, oligopeptide ABC transporters (MSC_0184), hydrolases (MSC_0614) tRNA synthase (MSC_0336), RNA polymerase (MSC_1009), transcriptional regulators (MSC_0068), cell division proteins (MSC_1062) and hypothetical proteins (MSC_0636, MSC_0656, MSC_0826) are present in all bacterial species with the
first two having been associated with virulence (HOPFE and HENRICH 2008).

5.3 Selection of three proteins as potential diagnostic antigens

Among the 22 immunogenic peptides the eight peptides showing the strongest immunogenic reaction were selected for further investigation. Five of these peptides showed 100% homology solely to *Mmm*SC type strain PG1 (MSC_0108, MSC_0135, MSC_0617, MSC_656 and MSC_1062) whereas all other identified (17) peptides (MSC_0017, MSC_0029, MSC_0034, MSC_0068, MSC_0079, MSC_0184, MSC_0266, MSC_0336, MSC0613, MSC_0614, MSC_0636, MSC_0826, MSC_0908, MSC_978, MSC_1009, MSC_1017 and MSC_1062) were also identical to proteins of *M. capricolum* which has been reported to cause CBPP-like lesions in immunosuppressed cattle (AJUWAPE A.T.P.T et al. 2003). On the basis of discrimination between positive and negative control serum initially six of these eight clones were selected (MSC_0029, MSC_0108, MSC_636, MSC_656, MSC_1009 and MSC_1062; Fig. 11). Among these six clones only four clones showed a consistently strong reaction with 10 individual positive sera and no reaction with six individual negative sera (MSC_0029, MSC_0108, MSC_0636 and MSC_1062; Fig. 12). Among these four clones one clone (MSC_1062; Table 10) showed high cross reactivity with *M. canadense* serum (Table 12) and, therefore, was excluded. The remaining three clones were expressed as GST-fusion proteins. Although there is a His-Tag in the phagemids in order to facilitate purification of soluble proteins this subcloning as a fusion protein was required because the peptides were not large enough for an efficient production and purification.

Fusion proteins were prepared as inclusion bodies and shown to be immunoreactive and specific in Western blot analyses using the bovine positive control serum (Fig.15) and in an ELISA using rabbit hyperimmune serum (Fig. 16). However, an ELISA using the bovine positive and negative control serum showed a strong reactivity also with negative serum as well as a high inter-assay variation and did not allow a significant discrimination between positive and negative serum.
Therefore, the immunogenic antigens identified are potentially valuable for the development of new diagnostic tests and/or vaccines but additional work is required to reduce background signals and to transfer their use into practical application. Higher background is caused by previous contact and immunization of animals with *E. coli*. Therefore, need of either pre-incubation of sera with *E. coli* cell lysate or purification of sera (infected and uninfected animals sera) or aggregate preparation is necessary.

### 5.4 Out Look and future prospective

In the future the immunogenic proteins identified have to be further investigated for their possible application in veterinary diagnostics. Initially this would involve the improvement of the ELISA by purification of the antigen by dialysis to remove small *E. coli*-derived polypeptides which cause a reaction in the ELISA but are absent in the Western Blot. Alternatively, affinity chromatography using a commercial anti-GST-antibody system could be applied. Subsequently, the recognition of recombinant immunogenic proteins should be verified with higher numbers of field serum samples.

In addition to using the immunogenic antigens in diagnostics they might have potential as components of subunit vaccines. Here, lipoproteins and other surface-exposed proteins which have been shown to be involved in virulence might be particularly promising. For this approach it might be advantageous to construct recombinant full-length proteins by removal of the TGA-codons in order to optimize the immune response.

Lastly, most of the proteins identified are not characterized to date and no experimental evidence is available on their function, therefore, the molecular characterization of these proteins could be helpful for future research on the virulence mechanisms of *MmmSC*. 
6 Summary

Identification and molecular characterization of immunogenic antigens of
*Mycoplasma mycoides* subsp. *mycoides* small colony type
Shamoon Naseem

Contagious Bovine Pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp. *mycoides* SC (*MmmSC*) is one of the economically most important diseases of cattle in large parts of sub-saharan Africa, and there is an increasing threat of reintroducing the disease into other parts of the world. The pathogenicity of CBPP is poorly understood, and diagnostic tests available are applicable at the herd level but are of limited use for individual testing. Currently used vaccines do not protect reliably against infection and can cause severe side effects. The identification of previously unknown immunogenic proteins is a prerequisite for the development of novel diagnostic tests and vaccines. In order to achieve this goal, a phage M13-based display method based on the helperphage “Hyperphage” was used. To construct the *MmmSC* expression library genomic DNA was restricted with five different restriction endonucleases resulting in blunt ends. DNA fragments of 200 to 800 bp were isolated and ligated into the vector pHORF3 and two derivatives constructed in this work. The library was packaged into “Hyperphage” and screened by panning using an anti-*MmmSC* serum obtained by pooling the sera of 10 individual CBPP-positive animals. After four panning rounds 147 individual phage clones were sequenced resulting in the identification of 22 different immunogenic *MmmSC* proteins some of which have been described as immunogenic or as being involved in virulence.

In order to identify the clones potentially best suited as antigen in a diagnostic ELISA an ELISA-based selection was performed. Initially the eight clones resulting in the highest ELISA titre with the positive control sera were selected. These were tested for their discriminatory power comparing positive and negative control sera. Six clones resulting in an at least 4-fold difference in titre were tested with 10 individual
positive sera. Four clones encoding epitopes of acyl carrier protein phosphodiesterase (MSC_029), glycosyl transferase (MSC_108), conserved hypothetical protein (MSC_636) and AAA family ATPase (MSC_1062) were highly positive with all sera; they were tested for reactivity with rabbit sera against 17 other mycoplasmal species. One clone (AAA family ATPase MSC_1062) showed strong cross reactivity with the serum directed against *M. canadense*. Three clones (MSC_029, MSC_108 and MSC_636) did not show significant cross reactivity.

In order to facilitate efficient production of these three antigens the immunoreactive epitopes were expressed as GST fusion proteins. The part of the respective gene encoding the immunogenic peptide, located between two TGA-codons, was amplified by PCR using primers with a unique restriction enzyme site on their respective 5′-ends in order to facilitate directional cloning into the expression vector pGEX5x-2. Fusion proteins were prepared as inclusion bodies and shown to be immunoreactive and specific in Western blot analyses using the bovine positive control serum and in an ELISA using rabbit hyperimmune serum. However, an ELISA using the bovine positive and negative control serum showed a strong reactivity also with negative serum as well as a high inter-assay variation and did not allow a significant discrimination between positive and negative serum.

Therefore, the immunogenic antigens identified are potentially valuable for the development of new diagnostic tests and/or vaccines but additional work will be required to transfer their use into practical application.
7 Zusammenfassung

Identifizierung und molekulare Charakterisierung immunogener Antigene von *Mycoplasma mycoides* subspecies *mycoides* small colony type

Shamoon Naseem

Die durch *Mycoplasma mycoides* subspecies *mycoides* small colony type (MmmSC) ausgelöste Lungenseuche ist eine der wirtschaftlich verlustreichsten Rindererkrankung in vielen Teilen von Afrika südlich der Sahara und es besteht eine erhöhte Gefahr der Wiedereinschleppung in andere Teile der Welt. Die Pathogenität der Lungenseuche ist bisher nur unzureichend geklärt; die verfügbaren diagnostischen Tests eignen sich für die Herdendiagnose, sind aber für die Einzeltierdiagnose nur begrenzt aussagekräftig. Die gegenwärtig genutzten Impfstoffe schützen nicht zuverlässig vor einer Infektion und können schwere Impfreaktionen auslösen.

Um eine effiziente Produktion dieser drei Antigene zu erreichen, wurden die immunreaktiven Epitope als GST-Fusionsprotein exprimiert. Der Teil des jeweiligen Gens, der das immunogene Peptid kodiert und zwischen zwei TGA-Kodons lokalisiert ist, wurde mittels PCR amplifiziert; dabei wurden Primer verwendet, die eine singuläre Restriktionenschnittstelle an ihrem jeweiligen 5'-Ende tragen, um so eine gerichtete Klonierung in den Expressionsvektor pGEX5x-2 zu ermöglichen. Die Fusionsproteine wurden als Einschlusskörperchen präpariert und es wurde gezeigt, dass sie spezifisch-immunreaktiv im Western Blot mit dem positiven Kontrollserum reagierten; sie zeigten ebenfalls eine spezifische und positive Reaktion in einem ELISA mit Kaninchenhyperimmunserum. In einem ELISA mit positivem und negativem bovinem Kontrollserum zeigte jedoch auch das negative Kontrollserum eine deutliche Reaktion und Zudem zeigte sich eine hohe „inter-assay“ Variation; Dadurch war keine aussagekräftige Diskriminierung zwischen positivem und negativem Serum möglich.
Zusammenfassung

Somit sind die in dieser Arbeit identifizierten immunogenen Antigene potentiell für die Entwicklung neuer diagnostischer Tests und/oder Impfstoffe geeignet, aber weitere Arbeiten sind erforderlich, um ihre praktische Applikation zu ermöglichen.
8 References

Antigenic and genetic characterization of lipoprotein LppQ from *Mycoplasma mycoides* subsp. *mycoides* SC.

ABUSUGRA, I., G. WOLF, F. THIACOURT and B. MOREIN (1997):
ISCOM vaccine against contagious bovine pleuropneumonia (CBPP). 1. Biochemical and immunological characterization.
Veterinary Immunology and Immunopathology 59, 31-48.

Pathogenicity of *Mycoplasma capricolum* subspecies *capricolum* for cattle immunosuppressed with trypanosoma congolense
Israel Journal of Veterinary medicine vol.59 (4) 2003.

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS and D. J. LIPMAN (1990):
Basic local alignment search tool.
J. Mol. Biol. 215, 403-410

ANON (1999):
Summary of Presentations and Discussions.
FAO, Rome. pp 3-16.
ANON (2001):

Discovery of new Mycoplasma pneumoniae antigens by use of a whole-genome lambda display library.
Microbes Infect. 11, 66-73

Discovery of novel Streptococcus pneumoniae antigens by screening a whole-genome lambda-display library.
FEMS Microbiol. Lett. 262, 14-21

Sensitivity and specificity of serological and bacteriological tests for contagious bovine pleuropneumonia.
Rev. Sci. Tech. 17, 654-659

Cloning of the pelA gene from Bacillus licheniformis 14A and biochemical characterization of recombinant, thermostable, high-alkaline pectate lyase.
Appl. Microbiol. Biotechnol. 64, 560-567
References

BIRNBOIM, H. C. and J. DOLY (1979):
A rapid alkaline extraction procedure for screening recombinant plasmid DNA.
Nucleic Acids Res. 7, 1513-1523

BREITLING, F. and S. DÜBEL (1997):
Rekombinante Antikörper. 1997,
Heidelberg Berlin: Spektrum Akademischer Verlag.

BREITLING, R., K. GASE and D. BEHNKE (1990):
Intracellular expression of hIFN alpha genes in Escherichia coli and Bacillus subtilis
directed by staphylokinase signals.
J. Basic Microbiol. 30, 655-662

Serodiagnosis and monitoring of contagious bovine pleuropneumonia (CBPP) with
an indirect ELISA based on the specific lipoprotein LppQ of Mycoplasma mycoides
subsp. mycoides SC.
Vet. Microbiol. 84, 195-205

BUTTERY, S. H., L. C. LLOYD and D. A. TITCHEN (1976):
Acute respiratory, circulatory and pathological changes in the calf after intravenous
injections of the galactan from Mycoplasma mycoides subsp. mycoides.
J. Med. Microbiol. 9, 379-391

CAMPBELL AND TURNER 1953 Studies on contagious bovine pleuropneumonia of
cattle: An improve complement fixation test.
CHAMBAUD, I., H. WROBLEWSKI and A. BLANCHARD (1999):
Interactions between *mycoplasma* lipoproteins and the host immune system.
Trends Microbiol. 7, 493-499

Characterization of the gene for an immunodominant 72 kDa lipoprotein of *Mycoplasma mycoides* subsp. *mycoides* small colony type.
Microbiology 142, 3515-3524

Identification of natural ligands for SH2 domains from a phage display cDNA library.
J. Mol. Biol. 297, 89-97

2-Acylglycerolphosphoethanolamine acyltransferase/acyl-acyl carrier protein synthetase is a membrane-associated acyl carrier protein binding protein.
J. Biol. Chem. 264, 7384-7389

Tapping allergen repertoires by advanced cloning technologies.
Int. Arch. Allergy Immunol. 124, 43-47

CRAMERI, R., ET. AL. (2003):
Identification of natural protein-protein interactions with cDNA libraries.


FAO (1997):

Induced mouse spleen B-cell proliferation and secretion of immunoglobulin by lipid-associated membrane proteins of Mycoplasma fermentans incognitus and Mycoplasma penetrans.
Infect. Immun. 62, 3916-3921

Genetic and serological analysis of the immunogenic 67-kDa lipoprotein of Mycoplasma sp. bovine group 7.
Res. Microbiol. 149, 55-64

Immune response against the L-lactate dehydrogenase of Mycoplasma hyopneumoniae in enzootic pneumonia of swine.
Microb. Pathog. 17, 313-322

FREY, J. and J. NICOLET (1997):
Molecular identification and epidemiology of animal mycoplasmas.
Wien. Klin. Wochenschr. 109, 600-603

FROHLICH, K. U. (2001):
An AAA family tree.
J. Cell Sci. 114, 1601-1602


OppA, the ecto-ATPase of *Mycoplasma hominis* induces ATP release and cell death in HeLa cells.
BMC Microbiol. 8, 55-59

HSU, T., S. ARTIUSHIN and F. C. MINION (1997):
Cloning and functional analysis of the P97 swine cilium adhesin gene of *Mycoplasma hyopneumoniae*.
J. Bacteriol. 179, 1317-1323

HUDSON, J. R. (1968a):
Contagious bovine pleuropneumonia. Experiments on the susceptibility and protection by vaccination of different types of cattle.

HUDSON, J. R. (1968b):
Contagious bovine pleuropneumonia. The keeping properties of the V5 vaccine used in Australia.

Mating antibody phage display with proteomics.
Trends Biotechnol. 22, 8-14

Phage display vectors for the in vitro generation of human antibody fragments.
Methods Mol. Biol. 295, 71-96
HUST, M., S. DÜBEL and T. SCHIRRMANN (2007):
Selection of recombinant antibodies from antibody gene libraries.
Methods Mol. Biol. 408, 243-255

HUST, M., M. MEYSING, T. SCHIRRMANN, M. SELKE, J. MEENS, G. F. GERLACH
and S. DÜBEL (2006):
Enrichment of open reading frames presented on bacteriophage M13 using
hyperphage.
Biotechniques 41, 335-342

Evidence that UGA is read as a tryptophan codon rather than as a stop codon by
Mycoplasma pneumoniae, Mycoplasma genitalium, and Mycoplasma gallisepticum.
J. Bacteriol. 172, 504-506

Disruption and analysis of the clpB, clpC, and clpE genes in Lactococcus lactis:
ClpE, a new Clp family in gram-positive bacteria.
J. Bacteriol. 181, 2075-2083

JORES, J., I. NKANDO, A. STERNER-KOCK, W. HAIDER, J. POOLE, H. UNGER,
C. MURIUKI, H. WESONGA and E. L. TARACHA (2008):
Assessment of in vitro interferon-gamma responses from peripheral blood
mononuclear cells of cattle infected with Mycoplasma mycoides ssp. mycoides small
colony type.
Vet. Immunol. Immunopathol. 124, 192-197


Contagious bovine pleuropneumonia: immunoenzymatic test and kinetics of antibody formation in experimental infection. Relation of complement fixation, excretion and the study of circulating antigen.

LE GOFF, C. and F. THIAUCOURT (1998):
A competitive ELISA for the specific diagnosis of contagious bovine pleuropneumonia (CBPP).
Vet. Microbiol. 60, 179-191

Presence of anaplerotic reactions and transamination, and the absence of the tricarboxylic acid cycle in mollicutes.
J. Gen. Microbiol. 134, 791-800

Phage library screening for the rapid identification and in vivo testing of candidate genes for a DNA vaccine against *Mycoplasma mycoides* subsp. *mycoides* small colony biotype.
Infect. Immun. 74, 167-174
Comparison of complement fixation test, immunoblotting, indirect ELISA, and competitive ELISA for detecting antibodies to Mycoplasma mycoides subspecies mycoides small colony (SC) in naturally infected cattle from the 1995 outbreak in Botswana.
Onderstepoort J. Vet. Res. 70, 21-27

The phagocytosis of mycoplasmas.
J. Med. Microbiol. 43, 239-250

MASIGA, W. N. and J. DOMENECH (1995):
Overview and epidemiology of contagious bovine pleuropneumonia in Africa.
Rev. Sci. Tech. 14, 611-630

Manifestation and epidemiology of contagious bovine pleuropneumonia in Africa.
Rev. Sci. Tech. 15, 1283-1308

Contagious bovine pleuropneumonia (CBPP) caused by vaccine strain T1/44 of Mycoplasma mycoides subsp. mycoides SC.
Vet. Microbiol. 98, 229-234

MCCAFFERTY, J., A. D. GRIFFITHS, G. WINTER and D. J. CHISWELL (1990):
Phage antibodies: filamentous phage displaying antibody variable domains.
Nature 348, 552-554
Identification and immunological characterization of conserved *Mycoplasma hyopneumoniae* lipoproteins Mhp378 and Mhp651.
Vet. Microbiol. 116, 85-95

Mycoplasma gene expression in *Escherichia coli*.
Methods Mol. Biol. 104, 259-265

Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis*.
Infect. Immun. 66, 4804-4810

Biotechnology 24, 17-27

NEWTON AND NORRIS (2000):
CSIRO Publishing, Collingwood, Australia.


Antigenic and genetic characterisation of lipoprotein lppC from *Mycoplasma mycoides* subsp. *mycoides* SC.
Vet. Res. 34, 761-775

Proteomic survey of the pathogenic *Mycoplasma hyopneumoniae* strain 7448 and identification of novel post-translationally modified and antigenic proteins.
Vet. Microbiol. 121, 83-93

PROVOST, A. (1996):
[Strategies for prevention and eradication of contagious bovine pleuropneumonia with or without vaccination].
Rev. Sci. Tech. 15, 1355-1371

RAZIN, S., D. YOGEV and Y. NAOT (1998):
Molecular biology and pathogenicity of mycoplasmas.

Defining the mycoplasma 'cytoskeleton': the protein composition of the Triton X-100 insoluble fraction of the bacterium *Mycoplasma pneumoniae* determined by 2-D gel electrophoresis and mass spectrometry.
Microbiology 147, 1045-1057
Cloning allergens via phage display.
Methods 32, 212-218

Dealing with animal disease emergencies in Africa: prevention and preparedness.
Rev. Sci. Tech. 18, 59-65

RONDOT, S., J. KOCH, F. BREITLING and S. DÜBEL (2001):
A helper phage to improve single-chain antibody presentation in phage display.
Nat. Biotechnol. 19, 75-78

Sequence of thioredoxin reductase from *Escherichia coli*. Relationship to other flavoprotein disulfide oxidoreductases.
J. Biol. Chem. 263, 9015-9019

Contagious bovine pleuropneumonia vaccines: the need for improvements.
Rev. Sci. Tech. 14, 593-601

SAMBROOK AND RUSSELL (2001):
ISBN: 0-87969-576-5(cloth), 0-87969-577-3 (pbk)
Cold Spring Harbor Laboratory, New York.
ISBN: 0-87969-309-6
Cold Spring Harbor Laboratory, Press 2 New York.

SCHNEIDER, H. P., O. J. B. HÜBSCHLE and J. J. VAN DER LUGT (1994):
Contagious bovine pneupneumonia. In: Infectious Diseases of Livestock.

Exploring protein-protein interactions with phage display.
Chembiochem. 4, 14-25

Phage display for engineering and analyzing protein interaction interfaces.
Curr. Opin. Struct. Biol. 17, 481-487

Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome.
PLoS Genet. 3, e75
SKERRA, A. (2007):
Alternative non-antibody scaffolds for molecular recognition.
Curr. Opin. Biotechnol. 18, 295-304

Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface.
Science 228, 1315-1317

Libraries of peptides and proteins displayed on filamentous phage.
Methods Enzymol. 217, 228-257

On the influence of vector design on antibody phage display.
J. Biotechnol. 127, 626-637

ClpB is the Escherichia coli heat shock protein F84.1.
J. Bacteriol. 173, 4254-4262

Surveillance of contagious bovine pleuropneumonia in Switzerland.
Rev. Sci. Tech. 14, 621-629
Cloning and expression of a species-specific early immunogenic 36-kilodalton protein
of Mycoplasma hyopneumoniae in Escherichia coli.
Infect. Immun. 59, 1217-1222

TAUSSIG, M. J., O. STOEVESANDT, C. A. BORREBAECK, A. R. BRADBURY, D.
CAHILL, C. CAMBILLAU, A. DE DARUVAR, S. DUBEL, J. EICHLER, R. FRANK, T.
J. GIBSON, D. GLORIAM, L. GOLD, F. W. HERBERG, H. HERMJAKOB, J. D.
HOHEISEL, T. O. JOOS, O. KALLIONIEMI, M. KOEGL, Z. KONTHUR, B. KORN, E.
KREMMER, S. KROBITSCH, U. LANDEGREN, M. S. VAN DER, J. MCCAFFERTY,
S. MYULDERMANS, P. A. NYGREN, S. PALCY, A. PLUCKTHUN, B. POLIC, M.
PRZBYLSKI, P. SAVIRANTA, A. SAWYER, D. J. SHERMAN, A. SKERRA, M.
TEMPLIN, M. UEFFING and M. UHLEN (2007):
Proteome Binders: planning a European resource of affinity reagents for analysis of
the human proteome.
Nat. Methods 4, 13-17

THIAUCOURT, F., L. DEDIEU, J. C. MAILLARD, P. BONNET, M. LESNOFF, G.
LAVAL and A. PROVOST (2003):
Contagious bovine pleuropneumonia vaccines, historic highlights, present situation
and hopes.
Dev. Biol. (Basel) 114, 147-160

THIAUCOURT, F., A. YAYA, H. WESONGA, O. J. HUEBSCHLE, J. J. TULASNE and
A. PROVOST (2000):
Contagious bovine pleuropneumonia. A reassessment of the efficacy of vaccines
used in Africa.
Ann. N. Y. Acad. Sci. 916, 71-80


Genomic and antigenic differences between the European and African/Australian clusters of *Mycoplasma mycoides* subsp. *mycoides* SC.
Microbiology 146, 477-486

Effect of HEPES buffer systems upon the pH, growth and survival of *Mycoplasma mycoides* subsp. *mycoides* small colony (*MmmSC*) vaccine cultures.
FEMS Microbiol. Lett. 201, 291-294

Capsular polysaccharide conjugate vaccines against contagious bovine pleuropneumonia: Immune responses and protection in mice.
J. Comp. Pathol. 126, 171-182

The genome sequence of *Mycoplasma mycoides* subsp. *mycoides* SC type strain PG1T, the causative agent of contagious bovine pleuropneumonia (CBPP).
Genome Res. 14, 221-227

WINDSOR, R. S. (2000):
The eradication of contagious bovine pleuropneumonia from south western Africa. A plan for action.
Ann. N. Y. Acad. Sci. 916, 326-332
Aminopeptidase N/CD13 induces angiogenesis through interaction with a pro-
angiogenic protein, galectin-3.

Interactions between mycoplasma lipid-associated membrane proteins and the host
cells.

YUAN, L., P. H. RODRIGUES, M. BELANGER, W. DUNN, JR. and A. PROGULSKE-
FOX (2007):
The Porphyromonas gingivalis clpB gene is involved in cellular invasion in vitro and
virulence in vivo.
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