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Analysis of protein-protein interactions linked to the formation of a bacterial Cytoskeleton in *Mycoplasma pneumoniae*

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Summary

Mycoplasma pneumoniae has a cytoskeleton-like structure. Based on genetic evidence, it was proposed that the 1818 amino acids long protein HMW2 plays a central role in both formation of the cytoskeleton-like structure and adherence to its host cell (cytadherence).
As gene products of the *hmw2* gene (MPN310), two proteins were identified, the full-length protein HMW2 with a molar mass of 216 kDa and a smaller one (HMW2-s) with only 25 kDa. HMW2-s was considered to be the processing product of HMW2, but it could be shown by determining the N-terminus of HMW2-s and by expression studies with an artifical *hmw2-s* gene in *M. pneumoniae* that HMW2-s was synthesized by a new internal start within the *hmw2* gene but in the same raster as HMW2. This internal expression unit also ensures the transcription of the two genes, MPN311 and MPN312, located immediately downstream.
To characterize HMW2, it was expressed in *Escherichia coli* under various condition and with alternative *E. coli* strains, but, it was poorly expressed and degraded rapidly independent of the applied conditions. Therefore, it was impossible to isolate enough soluble full-length protein to do biochemical and structural analyses.
The proposed function of HMW2 requires its interaction with other proteins of *M. pneumoniae*. Pilot experiments with the two-hybrid system suggested several candidates. By applying the "pairwise tests", an internal fragment of HMW2 was found to interact with the C-terminal fragment of HMW1 (MPN447) and with the MPN297 encoded 17-kDa protein. The latter one has not yet been implicated in cytoskeleton formation. In addition, the interaction between the main adhesin P1 with HMW1 and the gene product of MPN297 was established linking indirectly HMW2 to the P1 adhesion complex consisting of at least three proteins: P1, P40 and P90.
Further evidence for the interaction between HMW2 and the P1 adhesin (complex) derived from comparative protein analyses of *M. pneumoniae* WT and the mutant *M. pneumoniae* A3 (*hmw2*$^{-}$). Western blot analyses showed that in *M. pneumoniae* A3 the turnover rate of the proteins of the P1 complex was significantly higher. This was interpreted as a consequence of the missing binding partner, because without HMW2, the P1 complex can not be formed and inserted properly in the membrane, making those proteins useless for the bacterium.
First attempts to isolate protein complexes containing HMW2, to which a TAP tag was fused, were succesful. Twelve genes/ proteins were identified from the purified complexes: MPN015, MPN140 (ORF4 gene product), MPN141 (P1), MPN142 (P90), MPN160, MPN297, MPN392 (PdhB), MPN426 (P115), MPN430 (GAPDH), MPN447 (HMW1), MPN573 (GroEL), MPN665 (EF-Tu). These results confirm the two-hybrid analyses of proteins interacting with HMW2. Eight of them (P1, P90, PdhB, GAPDH, HMW1, GroEL, EF-tu and the gene product of MPN297) were also found in the Triton X-100 insoluble fraction, which contains almost all of the known cytoskeletal proteins including HMW2. Furthermore, the interaction of HMW2 with EF-Tu (elongation factor Tu) and PdhB (pyruvate dehydrogenase E_1-beta subunit), of which a subfraction was reported to be surface exposed, provide evidence, that HMW2 might also have an important function in organizing other proteins than cytoskeletal proteins.
Finally, a new antiserum against the N-terminal part of HMW2 was generated, which improved the immunocytochemistry and allowed to co-localize HMW2 with the rod structure (co-operation with Dr. Hegermann), which is one of the predominant structures seen in thin sections of *M. pneumoniae*.

Zusammenfassung

Mycoplasma pneumoniae besitzt eine Cytoskelett-ähnliche Struktur. Auf der Grundlage der Ergebnisse genetischer Experimente wurde vorgeschlagen, daß das 1818 Aminosäuren große Protein HMW2 eine zentrale Rolle bei der Organisation Cytoskelett-ähnlicher Strukturen und bei der Anheftung an die Wirtszelle (Cytadhärenz) spielen würde. Als Genprodukte des Gens *hmw2* (MPN310) wurden zwei Proteine identifiziert, das vollständige HMW2 Protein mit einer molaren Masse von 216 kDa und ein kleineres Protein mit einer molaren Masse von nur 25 kDa (HMW2-s). Letzteres wurde bisher als Prozessierungsprodukt von HMW2 angesehen, aber in der vorliegende Arbeit konnte durch Bestimmung des N-terminus von HMW2-s und Expressionsstudien mit einem künstlichen Gen *hmw2-s* gezeigt werden, daß HMW2-s von einem internen Start-Codon von *hmw2* neu synthetisiert wird. Zum Zweck der weiteren Charakterizierung wurde HMW2 in *Escherichia coli* unter verschiedene Bedingungen und mit alternativen Stämmen exprimiert. Es war aber nicht möglich, ausreichend lösliches, vollständiges HMW2 zu synthetisieren, um biochemische und strukturelle Analysen durchzuführen, da HMW2 in *E. coli* nur schwach exprimiert und zudem schnell abgebaut worden ist. Die vorgeschlagene Funktion von HMW2 erfordert dessen Wechselwirkung mit anderen Proteinen von *M. pneumoniae*. In Pilotexperimenten mit dem "Two-hybrid system" wurden mehrere Kandidaten gefunden. Durch Anwendung des "Pairwise tests" konnte gezeigt werden, daß ein internes Proteinfragment von HMW2 sowohl mit dem C-terminalen Proteinfragment von HMW1 (MPN447) als auch einem Protein von 17 kDa, dem Genprodukt von MPN297, in Wechselwirkung steht. Letzteres war bisher noch nicht mit der Ausbildung des Cytoskeletts in Verbindung gebracht worden. Außerdem wurde die Wechselwirkung zwischen dem P1 Adhäsionskomplex, bestehend aus den Proteinen P1, P40 und P90, und HMW1 und dem Genprodukt von MPN297 nachgewiesen und damit indirekt eine Wechselbeziehung zwischen HMW2 und P1 hergestellt.

Zusätzliche Hinweise auf eine Wechselwirkung von HMW2 und dem P1 Komplex kamen von vergleichenden Proteomanalysen zwischen *M. pneumoniae* WT und der *hmw2*$^-$ Mutante *M. pneumoniae* A3, die zeigten, daß die Abbaurate der Proteine des P1 Komplexes in der Mutante deutlich höher waren. Das wurde damit erklärt, daß in Abwesenheit des Bindungspartners HMW2 der P1 Komplex nicht gebildet und daher auch nicht in die Membran integriert werden kann und dessen Komponenten aus diesem Grund, als nutzlos angesehen, schneller abgebaut werden. Erste Versuche, Proteinkomplexe zu isolieren, die HMW2 enthielten, welches durch ein Peptid (TAP-tag) modifiziert waren, verliefen erfolgreich. In angereicherten Komplexen konnten zwölf verschiedenen Proteine identifiziert werden: MPN015, MPN140 (ORF4 gene product), MPN141 (P1), MPN142 (P90), MPN160, MPN297, MPN392 (PdhB), MPN426 (P115), MPN430 (GAPDH), MPN447 (HMW1), MPN573 (GroEL), MPN665 (EF-Tu). Damit wurden die Ergebnisse der „Two-hybrid" Analyse bestätigt. Acht der zwölf Proteinen (P1, P90, PdhB, GAPDH, HMW1, GroEL, EF-Tu und das Genprodukt von MPN297) wurden außerdem in der Triton X-100 unlöslichen Fraction von *M. pneumoniae* gefunden. In dieser Fraktion sind alle bisher bekannten Cytoskelett-Proteine, einschließlich HMW2, angereichert.

Die Wechselwirkung zwischen HMW2 und EF-Tu (Elongationsfaktor Tu) und PdhB (E_1-beta Untereinheit der Pyruvatdehydrogenase), die nach Berichten teilweise Oberflächen-exponiert sind, zeigt, daß HMW2 auch bei der Organization solcher Proteine eine Rolle spielen könnte, die nicht an der Bildung des Cytoskeletts beteiligt sind. Schließlich wurde noch ein Antiserum gegen die N-terminale Region von HMW2 synthetisiert. Damit konnte eine verbesserte Immunogold-Elektronenmikroskopie durchgeführt werden, welche es erlaubte, HMW2 mit der „rod" Struktur, einer besonders markanten, in Ultradünnschnitten sichtbaren Struktur, zu co-lokalisieren (Zusammenarbeit mit Dr. Hegermann).

Abbreviations

1-D	one dimensional
2-D	two dimensional
aa	amino acid(s)
A, Ade	adenine
AP	alkaline phosphatase
approx.	approximately
APS	ammonium persulfate
ATP	adenosin triphosphate
α	anti (for antiserum)
BCIP	5-brom-4-chlor-3-indolylphosphate
bp	base pair
BSA	bovine serum albumin
°C	degree Celsius
C	cytosine
Ci	Curie
cpm	counts per minute
DIG	digoxigenin-11-dUTP
Da	Dalton
DNA	deoxyribonucleic acid
DNA-AD	DNA-activation domain
DNA-BD	DNA-binding domain
dNTP	deoxyribonucleic triphosphate
EDTA	ethylendiamintetra acedic acid
e.g.	for instance (exempli gratia)
et al.	and others
EtOH	ethanol
FCS	fetal calf serum
Fig.	figure
FHS	fetal horse serum
g	gravitational constant
G	guanosine
h, hr	hour(s)
HEPES	N-2-hydroxyethylpiperazin-N ethane sulfonic acid

HPLC	high performance liquid chromatography
i.e.	that is (id est)
IPG	immobilized pH gradient
IPTG	isopropyl-beta-D-thiogalactopyranoside
k	kilo
kbp	kilo base pair
kDa	kilo Dalton
L	liter(s)
LiAc	lithium acetate
LiCl	lithium chloride
µ	micro
m	milli
M	molar
mA	milliampere
MALDI-TOF	matrix-assisted laser desorption ionization–time-of-flight
MCS	multiple cloning site
$MgSO_4$	sodium sulfate
min	minute(s)
ml	milliliter(s)
MPN	prefix for ORF numbering used by Dandekar et al., 2000
mr	Molar mass
mRNA	messenger RNA
MS	mass spectrometry
MW	molecular weight
m/z	mass-to-charge ratio
n	nano (10^{-9})
nm	nanometer
NaCl	sodium chloride
NaOH	sodium hydroxide
$NaSO_4$	sodium sulfate
NBT	nitro-blue tetrazolium chloride
NH_4Ac	ammonium acetate
NP-40	nonidet-P40
nr	number

OD	optical density
ORF	open reading frame
p...	plasmid
p.A.	per analysis
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pI	isoelectric point
PPLO	pleuopneumoniae-like organism
PVDF	polyvinylidene fluoride
ref	"literature" reference
RNA	ribonucleic acid
mRNA	messenger RNA
rpm	rounds per minute
RT	room temperature
sec	second
SolA / B / C	solution A / B / C
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamide
tRNA	transfer RNA
U	unit of enzyme activity
UV	ultraviolet
V	volt(s)
vol	volume(s)
v/v	volume/ volume
vs.	versus
W	Watt(s)
WT	wild type
w/v	weight/volume
YT	yeast-trypton medium
Ω	Ohm

1 Introduction

1.1 *Mycoplasma pneumoniae* : general information

The first Mycoplasma to be isolated in culture was the bovine pleuropneumonia agent now known as *Mycoplasma mycoides*, which was described initially by Nocard and Roux in 1896 [Nocard and Roux, 1990].

The organism now known as *Mycoplasma pneumoniae* was first isolated in 1944 by Eaton and coworkers from the tissue culture of the sputum of a patient with primary atypical pneumonia. Thereafter it became known as the Eaton agent [Eaton et.al, 1944], later as PPLO (*pleuropneumonia* like organisms). Chanock and coworkers succeeded in culturing the Eaton agent in a cell-free medium [Chanock et al., 1962] and proposed the taxonomic designation *Mycoplasma pneumoniae* in 1963 [Chanock et al., 1962].

The species *M. pneumoniae* belongs to the genus *Mycoplasma*. Together with the eight following genera: *Ureaplasma, Entomoplasma, Mesoplasma, Phytoplasma, Spiroplasma, Acholeplasma, Asteroplasma* and *Anaeroplasma,* Mycoplasma belongs to the bacterial class named *Mollicutes* [Tully et.al, 1993]. About 200 known species have been detected in humans, vertebrate animals, arthropods, and plants. Several important differences between the *Mollicutes* and other eubacteria are summerized in Table 1.1.

Table 1.1: Molecular properties distinguishing mollicutes from eubacteria (*E. coli)*

Property	Mollicutes	Eubacteria
Cell size	0,3 – 0,8 µm	> 1 µm
Cell wall	absent	present
Requirement of cholesterol in the plasma membrane	most species	no
Genome size	580 – 2220 kbp	*E. coli*: 4639 kbp
G/C content of the DNA (mol%)	23 – 40 *M. pneumoniae*: 40	25 – 75
Number of rRNA Operons	1 - 2	1 - 11
Length of the 5S rRNA	104 - 113 nucleotides	114 nucleotides
Number of genes for tRNA	25 – 35 *M. pneumoniae*: 35	84 (*B. subtilis*), 86 (*E. coli*)
Number of proteins	460 - 1500	*E. coli*: 4288
UGA codon usage	tryptophan encoded by Mycoplasma,Ureaplasma, Spiroplasma, Mesoplasma	stop codon
RNA-polymerase	rifampicin resistent	rifampicin sensitiv
cilia and flagella	absent	present

Members of the class *Mollicutes* are characterized by their small genomes consisting of a single circular chromosome, the complete lack of a cell wall or cell-wall precursors and their commensal or parasitic life style [Razin et al., 1998].

Among the mycoplasmas, M. genitalium is so far the smallest and simplest self-replicating organisms with a minimal genome of only 580 kbp[Razin et al., 1998]. The drastic reduction of their genomes has significant consequences. The mollicutes lack many synthetic and metabolic pathways (with some exceptions in the genus *Acholeplasma*) such as the enzymes of the tricarboxylic acid (TCA) cycle, most enzyms for synthesis of fatty acids- and amino acid, etc. Therefore, many essential molecules have to be imported from the environment of the bacteria. As a consequence, mollicutes appear in nature only as parasites of humans,mammals, reptiles, fish, arthropods, and plants [Razin, 1992]. Furthermore, some species are pathogenic to their host cells. They infect particularly animal and human respiratory- and urogenital tracts, others damage useful plants.

Among the mollicutes, *Mycoplasma pneumoniae* is the only pathogen that fulfills Koch's postulates. Although never found freely living, it can be grown in the laboratory without host cells in a rich medium containing 5-20% serum. *M. pneumoniae* is a prominent pathogen of humans and perhaps the most throughly characterized of the mollicutes [Tylor-Robinson, 1996]. It is the leading cause of an atypical pneumonia in older children and young adults. Symptoms are flu-like but characteristically chronic in onset and recovery. Infection can be treated with antibiotics like erythromycin and tetracyclin. No immunity remains after infection for unknown reasons [Jacobs, 1991; Taylor-Robinson, 1996]. Also important is the observation that, after successful therapy, part of the bacteria still survive in the host, probably by invasion of the host cells. *M. pneumoniae* moves by gliding on surfaces [Bredt et al., 1979]. The damage of tissues of lung epithelial cells begins after colonization of *M. pneumoniae* [Razin and Jacobs, 1992]. It has been proposed that the intimate contact of the wallless mycoplasmas with the host cell membrane may result in local, perhaps transient fusion of the two membranes or exchange of membrane components and hence in direct "injection" of the mycoplasma cytoplasmic content, including hydrolytic enzymes, into the cytoplasm of the host cell. Thus, the potent nucleases of mollicutes combined with superoxide radicals may be responsible for clastogenic effects [Almagor et al., 1983; Razin et al., 1998]. So far, bacterial toxins have not been found to be a cause of *M. pneumoniae* pathogenicity.

M. pneumoniae has a pleiomorphic shape with sometimes long filamentous extensions [Krause, 1996]. Electron micrographs of infected bronchial epithelia reveal that the

mycoplasmas firmly attach to the host cells (= cytadherence), often, although not exclusively, by a differentiated terminal structure (attachtment organelle) which was called a “tip” [Krause, 1996]. The correct assembly of this organelle is a prerequisite for binding of *M. pneumoniae* to specificc receptors of the host cell [Krause, 1996; Razin and Jacobs, 1992].

This attachment organelle is a membrane-bound extention of the mycoplasma cell and is characterized by an electron-dense core orientated laterally within the tip [Boatman, 1979]. *M. pneumoniae* attaches to receptors common to a wide variety of cell types [Razin, 1985]. Two types of receptor moieties have been identified for *M. pneumoniae* binding: glycoprotein having sialic acid linked to the terminal galactose [Baseman et al., 1987; Roberts et al., 1989], and the galactose moiety of sulphatide glycolipides [Krivan et al., 1989].Therefore, *M. pneumoniae* is able to bind to erythrocytes. The molecular basis of the host-pathogen interaction is one of the most interesting scientific questions concerning infection by *M. pneumoniae*.

Among the proteins known to be present in an intact attachment organelle are the proposed adhesin proteins P1 [Hu et al., 1977; Inamine et al., 1988; Krause, 1996; Su et al.,1987] and P30 [Dallo et al., 1990; Romero-Arroyo et al.,1999], and a number of other proteins including P40 and P90, cleavage products derived from the ORF6 gene (MPN142) of the P1 operon [Inamine et al., 1988; Layh-Schmitt and Harkenthal, 1999; Sperker et al., 1991], and HMW3 [Ogle et al., 1991; Stevens and Krause, 1992]. The P1 protein was identified as the main adhesin. It mediates the binding of the bacteria to the host cell [Feldner et at.,1982; Hu et al.,1982]. It is densely clustered at the tip organelle but also appears to be scattered elswhere along the mycoplasma surface [Baseman et al., 1982]. P30, P40 and P90 are responsible for the the correct localization of the adhesin protein P1 in the terminal organelle. By cross-linking experiment, these four proteins were linked to each other in the intact bacterial membrane, indicating that these proteins are located at a maximal distance of 12 Å in the tip structure of *M. pneumoniae* [Layh-Schmitt and Herrmann, 1994] (Fig. 1.3).

The complete genome of *M. pneumoniae* was sequenced and annotated [Himmelreich et al.,1996]. It has 816,394 bp and contains 688 open reading frames, according to the latest re-annotation [Dandekar et al., 2000]. About 65% of these hypothetical proteins are assigned to function based on significant similarities to functionally defined proteins from other organisms and therefor their functional features could be predicted and catagorized.

1.2 Cytoskeleton-like structures in prokaryotes and cytoskeletal elements in *M. pneumoniae*

The cytoskeleton plays a crucial role in the organization of eukaryotic cells from yeast to human. The main components of the cytoskeleton are actin filaments, microtubules (MTs, polymers of tubulin) and intermediate filaments. In eukaryotes, microtubules are involved in various transport processes and in forming the mitotic spindle during chromosome segregation, actin forms a dynamic cytoskeleton involved in cell-shape regulation and mobility as well as the cytokinetic ring during cell division, and intermediate filaments provide mechanical support to the cell and nucleus. [Lewis, 2004]

In contrast to the situation in eukaryotic cells, the occurrence of a well defined complex cytoskeleton or cytoskeletal elements in prokaryotes remained unknown for decades even after introduction of the electron microscopy [Mayer, 2003; Nanninga, 2001]. A first hint for the existence of a kind of true bacterial cytoskeleton was obtained in the early 1990s, when the FtsZ system and its functional role was discovered and further investigated [Lutkenhaus, 1990]. The current knowledge of the bacterial cytoskeleton are summerized in this chapter.

Cytoskeleton in prokaryotes

Tubulin-like filaments: FtsZ

FtsZ is the key player in bacterial cell division and forms a filamentous ring around midcell that constricts during division [Lutkenhaus, 1990]. *In vitro* experiments demonstrated the ability to polymerize in a GTP-dependent manner (like tubulin), into straight filaments, flat sheets or tiny rings [Lu et al. 2000; Lutkenhaus and Addinall, 1997]. Later, the structure of FtsZ and tubulin proved to be very similar [Lowe and Amos, 1998; Nogales et al., 1998].

Actin-like filaments: MreB, Mbl and ParB

MreB has been known for a long time to affect cell shape [Wachi et.al, 1987]. Its known relationship to actin [Bork et.al, 1992] inspired an immunofluorescence study of MreB and Mbl (MreB-like) in *Bacillus subtilis* showing that these proteins form dynamic, helical filaments on the inside of the cytosolic membrane [Jones et.al, 2001; van den Ent et al., 2001]. The MreB homologue in *Escherichia coli* (Mbl and ParB) was also found to assemble on the cytoplasmic face of the innered membrane [Carballido-Lopez and Errington, 2003]. Mutations

in these proteins cause bacteria to grow as abnormal spheroids or misshaped rods, indicating these proteins regulate cell shape [Abhayawardhane and Stewart, 1995; Jones et al., 2001].
Mbl is responsible for guiding the machinery for cylindrical growth, and Mbl filaments have been shown to be dynamic with no apparent polarity and with a half-life of 8 min [Carballido-Lopez and Errington, 2003].
The protein ParM forms highly dynamic actin-like filaments in the bacterium *E. coli* and uses a self-assembly mechanism for force production and transport [Mollor-Jensen et al., 2002]. The crystal structure of the ParM monomer has a clear homology to those of actin and MreB [van den Ent et al., 2002].

Other filament systems: EF-Tu, fibril protein and cytoskeletal elements

It has been speculated that the bacterial elongation factor (EF)-Tu has a dual role, acting as a "cytoskeletal web" as well as aiding in translation [Mayer and Gottschalk, 2003]. This is mostly based on the the fact that overexpression of EF-Tu mutants induces *E. coli* to lose shape and lyse [Mayer, 2003], its high intracellular concentration of up to 5–10% of the total fraction of soluble proteins, and its apparent association with the membrane [Lowe et al., 2004]. Many years ago this protein has been shown to be able to form filaments and bundles thereof *in vitro* under certain favorable conditions [Beck, 1979; Cremers et.al, 1981; Helms and Jameson, 1995].
Williamson [Williamson, 1974] reported the isolation of cytoskeleton-like structure in *Spiroplasma* by cell lysis and detergent extraction. In *Spiroplasma citri*, the subunit of the cytoskeletal fibrils is 515 amino acids long (molecular weight of 59,000), as deduced by sequencing the cell's *fib* gene [Williamson et.al., 1991].
It is interesting that the pathogenic bacterium *M. pneumoniae*, although plylogenetically closely related to *B. subtilis* does not encode an *mreB* or related gene [Jones et al., 2001], exhibit a very differentiated cytoskeleton that, according to electron microscopic observations, might funtion in shape preservation [Hegermann et al., 2002]. Most of the proteins forming this cytoskeleton-like structures are not defined. A selection of the most important currently known cytoskeletal proteins in prokaryotes are shown in Fig. 1.1.

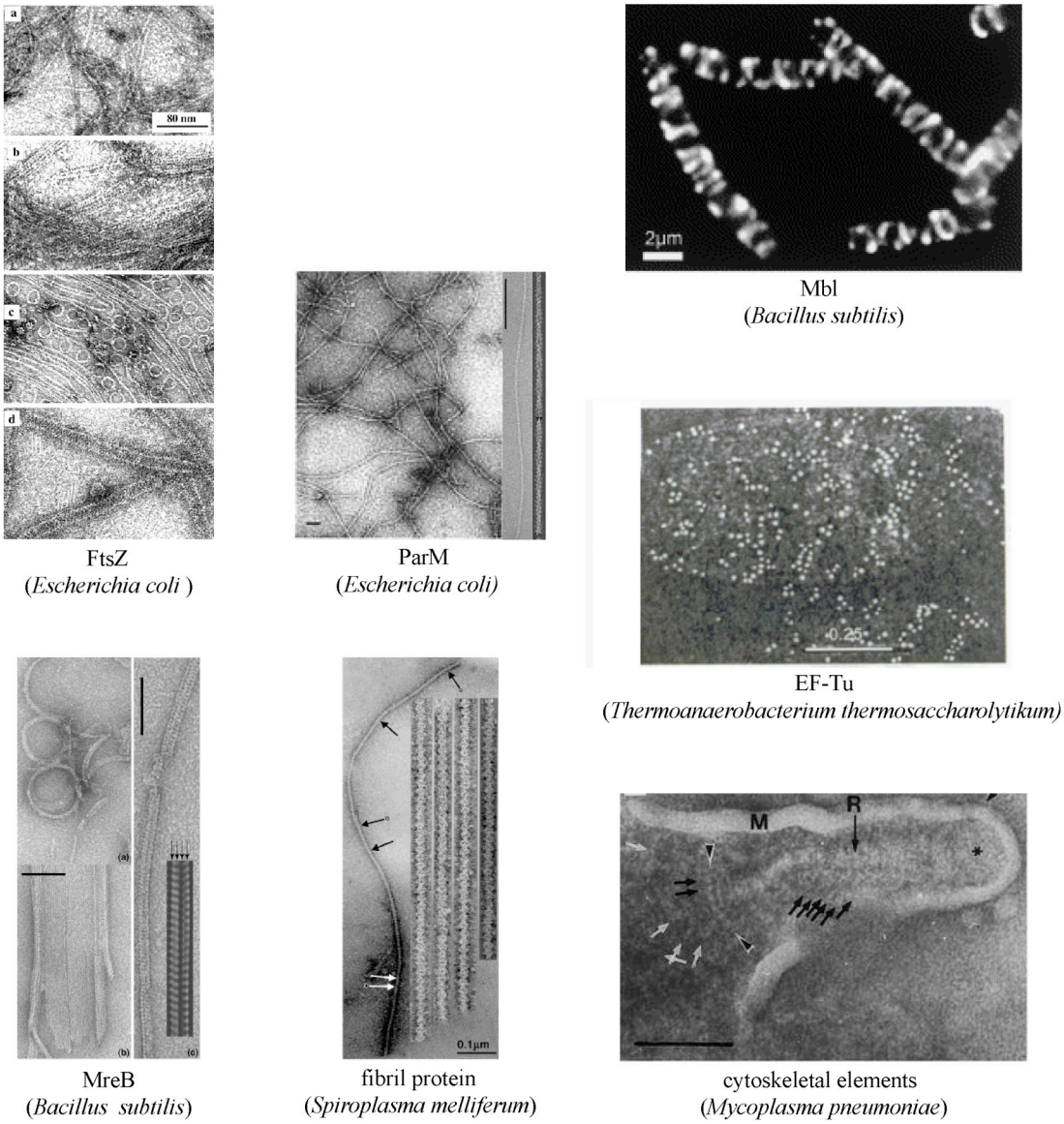

Fig. 1.1: Currently known cytoskeletal proteins in prokaryotes: FtsZ [Lu et al., 2000]; MreB [van den Ent et al., 2001]; Mbl [Jones et al., 2001]; ParM [van den Ent et al., 2002]; EF-Tu [Mayer and Gottschalk, 2003]; fibril protein [Tractenberg and Gilad, 2001] and cytoskeletal elements [Hegermann et al., 2002].

Cytoskeletal elements in *Mycoplasma pneumoniae*

Over the past 20 years, evidence has accumulated that *M. pneumoniae* possesses a cytoskeleton-like structure, probably as a substitute for the missing cell wall [Biberfeld and Biberfeld, 1970; Gobel et al., 1981; Krause,1996; Meng and Pfister, 1980; Wilson and Collier, 1976] . By analogy with eukaryotic cells, such a cytoskeleton could provide the necessary framework for maintaining and stabilizing the shape of *M. pneumoniae*, for motility [Radestock and Bredt, 1977] and for the formation of an asymmetric cell. Furthermore, a cytoskeleton might be involved in cell division, chromosome separation and cytadherence [Krause, 1996].

The first experimental indication for a cytoskeleton-like structure in *M. pneumoniae* was provided in 1980 by Meng and Pfister, who detected by electron microscopy rod-like condensed structures localized in the attachment organelle and thin fibrous structures extending into the cell body. These observations were confirmed by other researchers [Gobel et al., 1981]. In these experiments, *M. pneumoniae* cells grown on cover slips were treated with 1%Triton X-100, a non-ionic detergent which removes membranes and cytoplasm. The remaining proteins were stained with uranyl acetate for electron microscopy. In most pictures, a rod-like condensation, with a terminal button and a basal node was visible [Gobel et al., 1981; Meng and Pfister, 1980]. This structure is sometimes still surrounded by membrane fragments with some filamentous extensions attached [Gobel et al., 1981; Meng and Pfister, 1980]. These experiments and studies on the architecture and composition of eukaryotic cytoskeletons from many different cells [Herrmann and Wiche, 1983; Starger and Goldman, 1977; Steinert et al., 1978] that have been treated with the detergent Triton X-100, suggested that a cytoskeleton-like structure could also be concentrated in a Triton X-100 insoluble fraction of *M. pneumoniae*. A typical Triton X-100 insoluble structure of *M. pneumoniae* is shown in Fig. 1.2.

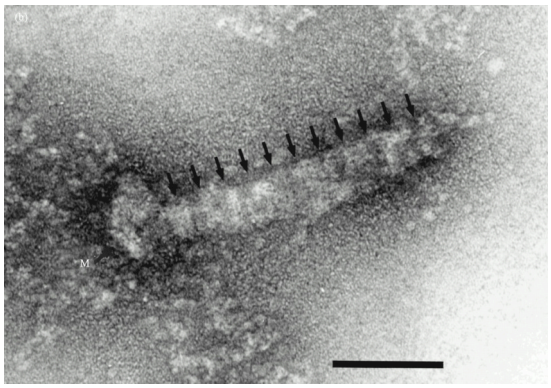

Fig. 1.2: Negatively stained sample of Triton X-100 resistant structure of *M. pneumoniae.* Repeating feature are indicated by arrows. Bar, 100 nm. [Regula et al., 2001].

Some proteins of the Triton X-100 insoluble fraction of *M. pneumoniae* were already identified: HMW1 (high molecular weight), HMW2, HMW3, P65 [Proft and Herrmann, 1994; Proft et al., 1995] and P200 [Proft and Herrmann, 1994; Proft et al., 1996]. These proteins were candidates for structural proteins based upon their predicted features. But none of these proteins, or any other of the predicted 688 ORFs [Dandekar et al., 2000], has a significant sequence similarity to a known cytoskeleton protein from other organisms except FtsZ. This is not surprising, as structural components may not be conserved at the sequence level even though the proteins have similar structural features.

Based on the complete sequence of the genome of *M. pneumoniae* [Himmelreich et al., 1996], the protein composition of the Triton X-100 insoluble fraction of *M. pneumoniae* was determined using two-dimensional (2-D) gel electrophoresis combined with mass spectrometry (MS) to characterize this fraction and to identify new candidates for structural components of the cytoskeleton-like structure [Regula et al., 2001]. 41 Proteins were identified as components of the Triton-X100 insoluble fraction. Most of the proteins known to be involved in cytadherence (HMW1, HMW2, HMW3, P65, P40, P90, P1, P200) were shown to be components of the Triton X-100 insoluble fraction. In addition, several other proteins were identified, which might be candidates based on their predicted structural features.

The correlation between the correct organization of the attachment organelle and cytadherence was shown in several studies [Krause et al., 1997; Romero-Arroyo et al., 1999] by isolating spontaneous mutants with a cytadherence-negative phenotype that possessed the adhesins P1 and P30 but had lost the high-molecular mass proteins HMW1, HMW2 and HMW3 [Krause et al., 1982; Layh-Schmitt et al., 1995]. Cytadherence positive revertants had regained the HMW proteins [Krause et al., 1997], reinforcing their correlation with cytadherence. This raised the hypothesis that in adherence-negative strains, the putative cytoskeleton-like structure is lacking, malfunctioning or absent [Krause, 1996].

The high molecular weight protein: HMW1, HMW2, HMW3 and structural related proteins

The HMW proteins are elements of a cytoskeleton-like network in *M. pneumoniae*. Their functions are consistent with a possible scaffolding role [Krause, 1996].

HMW1 [Dirksen et al., 1996] and HMW3 [Ogle et al.,1991] are structurally similar, with an internal domain that is dominated by repeating patterns rich in proline and acidic amino acids [Dirksen et al., 1996; Ogle et al., 1992]. They are members of a family of mycoplasma proteins that include P65 and P200, which also have such internal proline-rich motifs. HMW3, which is thought to play a critical role in stabilizing the attachment organelle [Willby and Krause, 2002], is found exclusively at the terminal structure of the attachment organelle [Stevens and Krause 1992]. HMW1 is found on the mycoplasma surface at the attachment organelle [Seto et al.,2001], as well as the trailing filament at the opposite pole [Stevens and Krause, 1991], and is essential for normal cell morphology [Hahn et al., 1998].

HMW2 is a large phosphoprotein (1818 residues, 216 kDa). Its structure is predicted to take the form of periodically broken coiled coils, and its presence is required for proper assembly of a functional attachment organelle [Dirksen et al., 1994; Krause et al., 1997; Fisseha et al.,

1999]. Comparison with protein databases revealed 18–25% identities over much of the length of HMW2 with proteins characterized by their potential to form coiled-coil structures, including the tail region of myosin II heavy chain [Krause et al., 1997], suggesting that HMW2 is capable of forming higher order structures.

HMW2 is required for cytadherence and stability of HMW1, HMW3 and P65. In mutants lacking HMW2, these proteins were synthesized at normal levels, but accelerated turnover results in reduced steady-state levels [Jordan et al, 2001; Popham et al., 1997], suggesting that HMW2 is required early in the process of attachment organelle assembly [Jordan et al., 2001; Krause and Balish, 2001; Popham et al., 1997].

The large number of proteins present at reduced levels in *hmw2* mutants therefor suggests a key role for HMW2 in promoting the stabilization of those proteins, indicating an important role for HMW2 in promoting protein-protein interactions.

Fluorescence microscopy showed that HMW2 is present at the attachment organelle [Balish et al., 2003]. However, a defined correlation between the structures identified in the attachment organelle and HMW2 has not been established by immunoelectron microscopy.

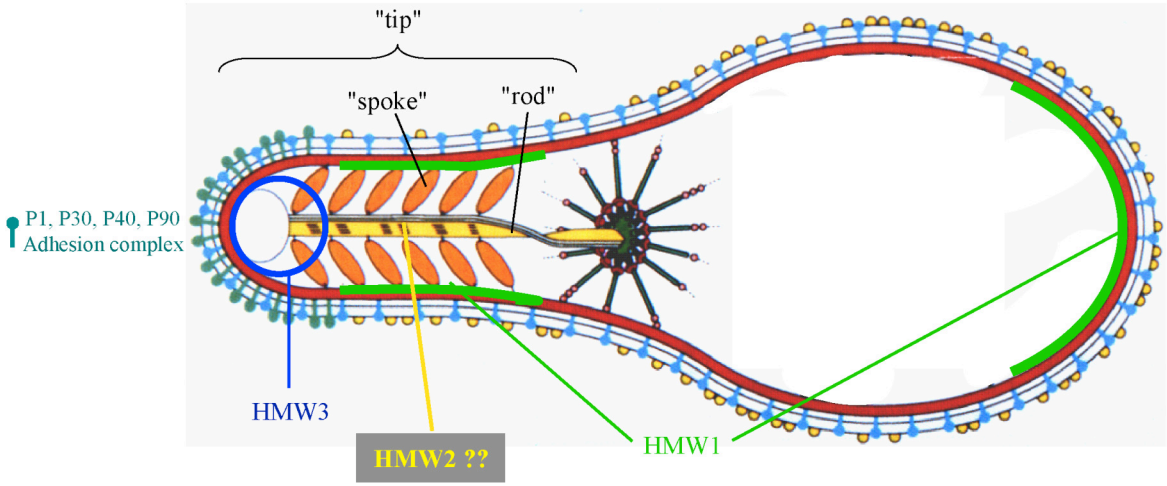

Fig. 1.3: Diagrammatic view of a longitudinal section through a cell of *M. pneumoniae*. In the elongated part of the cell ("tip"), a flat blade ("rod") is shown. It is indicated that this blade can be twisted or bent. The edges of the blade are connected to the cell periphery by "spokes". The proximal end of the blade is in contact with a wheel-like complex that is connected to the cell periphery by fibrils. The distal end of the blade is attached to an undefined mass. Fibrils crossing the cytoplasm are also drown. Subcellular localization of adhesion complex (P1, P30, P40 and P90), HMW1 and HMW3 are indicated [modified from Hegermann et al., 2002].

1.3 Methods for detection and analysis of protein-protein interactions

It is evident that protein-protein interactions are much more widespread than once suspected. Many fundamental cellular processes involve interactions among proteins and other biomolecules. To properly understand their significance in the cell, one needs to identify the different interactions, understand the extent to which they take place in the cell, and to determine the consequences of the interaction [Phizicky and Fields 1995].
Comprehensively identifying these interactions is an important step toward systematically defining protein functions [Eisenberg et al., 2000; Lan et al., 2003]. Function of an unknown protein can therefor be inferred from the identification of annotated interaction partners [Drewes and Bouwmeester, 2003]. A number of physical, biochemical, molecular biological and genetic approaches have been used to detect protein-protein interactions. An overview of such methods is presented in the following chapter.

Physical/ chemical methods to select and detect proteins that bind another protein

Protein Affinity Chromatography

A protein can be covalently coupled to a matrix such as Sepharose under controlled conditions and used to select ligand proteins that bind and are retained from an extract of soluble protein [Cuatrecasas and Wilchek, 1968].

Affinity Blotting

In a procedure analogous to the use of affinity columns, proteins can be fractionated by PAGE transferred to a nitrocellulose membrane, and identified by their ability to bind a protein, peptide, or other ligand. This method is similar to immunoblotting (Western blotting), which uses an antibody as the probe. Complex mixtures of proteins, such as total-cell lysates, can be analyzed without any purification [Schaltmann and Pongs, 1980].

Immunoprecipitation

Coimmunoprecipitation is a classical method of detecting protein-protein interactions and has been used in literally thousands of experiments [Phizicky and Fields, 1995]. The basic experiment is simple. Cell lysates are generated, antibody is added, the antigen/ antibody complex precipitates and coprecipitated proteins are eluted and analyzed.

Cross-Linking

In cross-linking experiments, chemical cross-linkers are used to covalently link proteins which are in close association, e.g. enzyme complexes, ribosomes, etc [Phizicky and Fields, 1995].

Typical chemical cross-linker reagents are *N*-hydroxysuccinimide (NHS) esters, imido esters, carbodiimides, maleimides, thiol-disulfide exchange reagents and aryl azides. A most usefulness feature of this method is that some of the covalent bounds are reversible. The stabilized protein complexes can be isolated and their subsunit compositions determined by several methods, mainly by mass spectrometry.

Library-based methods

Protein Probing

A labeled protein can be used as a probe to screen an expression library to identify genes encoding proteins that interact with this probe [Phizicky and Fields, 1995]. Interactions occur on nitrocellulose filters between an immobilized protein (generally expressed in *E. coli* from a genomic DNA/cDNA library) and the labeled protein probe. The method is very general and therefor widely applicable. Proteins as diverse as transcription factors and growth factor receptors have been used succesfully as probes.

Phage display

It was first demonstrated in 1985 by Smith [Smith, 1985] that an *E. coli* filamentous phage can express a fusion protein bearing a foreign peptide on its surface. These foreign peptides were accessible to antibodies, such that the 'fusion phage' could be enriched over unmodified phages by immunoaffinity purification. In the past few years, there have been numerous developments in this technology to make it applicable to a variety of protein-protein interactions [Gupta et al., 2003].

Two-Hybrid System

The two-hybrid system [Chien et al., 1991; Fields and Song, 1989; Fields and Sternglanz, 1994] is a genetic method that uses transcriptional activation as a measure of protein-protein interaction. This method was chosen for studying protein-protein interactions in *M. pneumoniae* and will be described in more detail in the next chapter.

Genetic methods

Extragenic Suppressors

Suppressor mutations are mutations that partially or fully revert the phenotype caused by an original mutation [Hartman, 1973]. A mutation of protein Y to Y* compensates for the defect X* to restore activity to the XY dimer.

Synthetic Lethal Effects

Mutations in two genes can cause death (or another observable defect) while mutation in either one does not. This phenomenon is called a synthetic effect and can be caused by the physical interactions between two proteins required for the same essential function. For instance, the formation of the dimer XY is required for function and loss of this function results in a detectable phenotype [Dobzhansky, 1946].

Overproduction Phenotypes

The overproduction of a wild type protein (X) can suppress phenotypes caused by mutations in genes encoding another protein (Y) [Rine, 1991]. In other cases, overproduction of a protein (X) can cause a phenotype that is altered by overproduction of an interacting protein (Y) [Meeks-Wagner and Hartwell, 1996]. The overproduction of the mutant protein is rather used to investigate interacting domains/ subunits within proteins which are functional as dimers or multimers. Overproduction of a nonfuntional version of a subunit [Herskowitz, 1987] can result in a mutant phenotype due to disruption of the activity of the wild type protein by competitive binding to wild type subunit to form an inactive dimer/multimer of the protein. An analysis of the sites of mutations is a standard methods to define the domains involved in the organization and function of a protein.

Unlinked Noncomplementation

Individuals, heterozygous for two different recessive mutations, sometimes display a mutant phenotype. This unlinked noncomplementation is often interpreted as being due to mutation in two genes that encode interacting products.

Others experimental techniques

Fluorescence resonance energy transfer (FRET)

One of the few techniques that are capable of detecting molecular interactions with subcellular resolution relies on fluorescence resonance energy transfer (FRET) from one fluorophore (the donor) to another fluorophore or non-fluorescent dye (the acceptor) [Phizicky et al., 2003]. Energy is transferred through the resonant coupling of the dipole moments of donor and acceptor. The Observation of a significant transfer efficiency between donor and acceptor strongly suggests actual physical interaction [Zal and Gascoigne, 2004].

Protein microrarrays (Protein Chips)

Protein chips allow the detection of different kinds of binary interactions *in vitro*, such as protein-protein, protein-lipid or antibody-antigen interactions. Proteins are covalently attached to a solid support and screened with fluorescently labelled probes (e.g. proteins or lipids).

Mass spectrometry analysis of purified protein complexes

General approaches for protein-complex purification use targeted proteins fused to a standard affinity tag as bait or entry point, which are in turn captured by antibodies or affinity resins. The tandem affinity purification (TAP) procedure developed by Seraphin and colleagues [Rigaut et al., 1999; Puig et al., 2001] has been used to purify protein complexes under native conditions. This method was also chosen for studying protein-protein interactions in *M. pneumoniae* and will be described in more details in the next chapter.

Databases for Biomolecular Interactions

Many databases have been created to store the tremendous amount of data required for and contained in these networks; some of which are summarized in Table 1.2. Some databases are more comprehensive than others; for instance, MIPS contains not only protein-protein physical interaction data but also information on genetic interaction as well [Mewes et al., 2002].

Table 1.2: Summary of the databases for biomolecular interactions

Name	URL	Type of networks	Reference
Database of Interacting Proteins (DIP)	http://dip.doe-mbi.ucla.edu/	Physical	Xenarios et al., 2002
Biomolecular Interaction Network Database (BIND)	http://www.bind.ca/	Physical	Bader et al., 2003
Human Protein Reference Database (HPRD)	http://www.hprd.org/	Physical	Peri et al., 2003
Munich Information Center for Protein Sequences (MIPS)	http://mips.gsf.de/	Physical & genetic	Mewes et al., 2002
The Yeast Proteome Database (YPD)	http://www.incyte.com/sequence/proteome/index.shtml	Physical, genetic & regulatory	Csank et al., 2002
The Transcription Factor Database (TRANSFAC)	http://transfac.gbf.de/TRANSFAC/	Regulatory	Wingender et al., 2001
Regulon Data Base (RegulonDB)	http://www.cifn.unam.mx/Computational_Genomics/regulondb/	Regulatory	Salgado et al., 2001
Kyoto Encyclopedia of Genes and Genomes (KEGG)	http://www.kegg.com/	Metabolic	Kanehisa and Goto, 2000
Encyclopedia of Metabolic Pathways (MetaCyc)	http://metacyc.org	Metabolic	Karp et al., 2002
Alliance for Cellular Signaling (AfCS)	http://www.cellularsignaling.org/	Signaling	Gilman et al., 2002

1.4 "The Yeast Two Hybrid System"

In the few years since its introduction [Fields and Song, 1989; Chien et al., 1991], the two-hybrid system has been widely used for identifying physically interacting proteins.

The conceptual basis for the two-hybrid system was established in studies on the regulation of eukaryotic transcriptional factors [Allen et al., 1995]. Certain transcriptional factors, such as the yeast GAL4 proteins [Chien et al., 1991; Harper et al., 1993], were shown to consist of two distinct and separable domains: a site-specific DNA-binding domain (DNA-BD), and an acidic region that is required for transcriptional activation (activation domains; DNA-AD) [Hope and Struhl, 1986; Keegan et al., 1986] (Fig. 1.4).

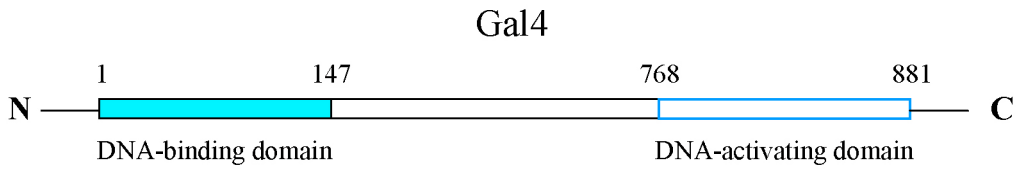

Fig. 1.4: Shematic illustration of the yeast Gal4 protein. The given number referred to corresponding amino acids. The protein contains 881 amino acids. The DNA-binding domain is 147 amino acids long and the DNA-activaing domain is 114 amino acids long.

A GAL4-DNA-binding domain binds to a specific *upstream*-activator sequence (UAS) [UAS; Heslot and Gaillardin, 1992] of the DNA [Keegan et al., 1986]. A DNA- activation domain binds to a DNA-binding domain, thereby takes a contact with the transcriptional apparatus and activates the transcription of the gene downstream of the UAS [Hope and Struhl, 1986; Keegan et al., 1986; Ma and Ptaschne, 1987]. Both domains are necessary for the gene activation. In case of the native yeast GAL4 protein, both domains are part of the same protein. When they are physically separated by moleculargenetic technique and expressed together again in the same cell, they could not interact directly with each other and therefor could not activate transcription of a specific gene [Ma and Ptashne, 1988; Brent and Ptaschne, 1985]. However, when both domains are physically brought together near a promotor region, where the DNA-binding domain can bind to a DNA-binding sequence, then transcription will

be activated. Based on the described features of the two domains, the two-hybrid technique was developed to provide a method for identifying proteins that physically interact *in vivo* (normally in yeast cells) [Frederickson, 1998].

Typically, the two-hybrid system utilizes two plasmid-borne gene fusions that are co-transformed into a host yeast strain containing inducible reporter genes. The protein of interest (X), or 'bait', is encoded as a gene fusion with a DNA binding domain (BD) of a well-characterized transcription factor (e.g. GAL4). A second protein (Y) is encoded as a gene fusion with a transcription activation domain (AD). Interaction between the two proteins in the nucleus of a yeast host strain results in the binding of the transcription activation domain to the DNA of the host strain, activating transcription of the adjacent reporter genes and generating a phenotypic signal, such as the expression of nutritional markers (e.g. *HIS3, ADE2*), which provides a scorable phenotype.

An important useful application of the two-hybrid system was extended to the isolation of unknown interacting partners of protein of interest from a genomic DNA gene bank. This has typically been done by screening a protein of interest against cells expressing a random plasmid library of genomic DNA and applying a genetic selection for monitoring interaction. Plasmid DNA is recovered from cells expressing interacting proteins and their identities are determined by DNA sequencing. This application makes the two-hybrid system to be the most widely used genetic assay to detect protein-protein interactions [Guo et al., 2004; Mendelsohn and Brent, 1994]. Fig. 1.5 illustrates the principle of the two-hybrid system.

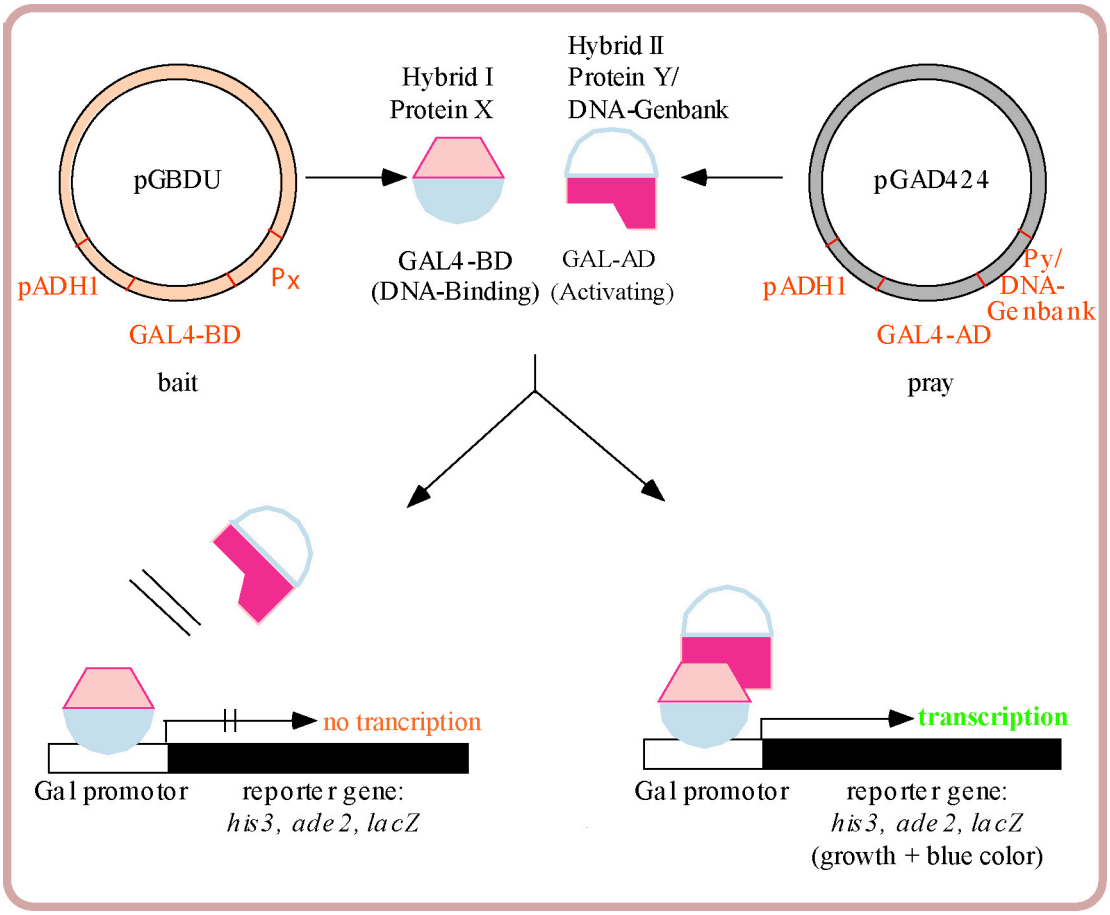

Fig. 1.5: Schematic illustration of the two-hybrid-based protein-protein interactions in the yeast nucleus (strain PJ49-4; James et.al, 1996). Informations of the reporter genes are described on the next page.

The two-hybrid host strain used in this work is PJ69-4A/α, constructed by James and coworkers [James et.al., 1996]. The terms A and α correspond the two different mating types of the haploid yeast cell. Yeast strains previously used in two-hybrid system employ only a single promoter (gal1 UAS or lexA operator) for all their reporter genes. The yeast strain PJ69-4A/α contains three different reporter genes, which utilize different inducible promoters: The *GALI**, *GAL2,* and *GAL7* promoter (Fig. 1.6). This was thought to eliminate many false positives and allow an extremely efficient two-hybrid selections.

The first reporter gene is the gene *HIS3*,* which is essentiel for the biosynthesis of the amino acid histidine. This reporter gene allows only yeast cells carrying interacting proteins to grow on selective media lacking histidine. This reporter gene is under control of the *GAL1* promotor. A

* these nomenclatures are corresponding to the article James *et al.,* 1996, by which the yeast strain PJ69-4 was described

GAL1-HIS3 region was introduced downstream of the *LYS2* gene. This construct exhibits more stringent regulation than other *GAL1*driven *HIS3* alleles, reducing or eliminating the need for the drug 3-aminotriazole [James et al., 1996], a competitive inhibitor of the *HIS3* gene product which helps to reduce the activity of the *HIS3* gene and eliminate weak positive signals.

The *ade2-101* allele of the yeast strain was replaced by a wild-type *ADE2* gene under the control of the *GAL2* promoter. *ADE2* was chosen as a reporter because *ade2* mutants form red colonies, while wild-type cells form white colonies. Intermediate expression of *ADE2* results in colonies in various shades of pink; thus, the colony color can be used as an first indication of the strength of an interaction or of a hint for loss of the plasmids [James et al., 1996]. The *GAL2-ADE2* reporter is especially useful, because it is very sensitive to weak interactions and eliminates nearly all false positives in the selection

The third reporter gene is the *lacZ* gene, which encodes the enzyme beta-galactosidase. This enzyme initiates the conversion of the colorless indicator X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranosid) to a blue product. Yeast cells with an activated *lacZ* reporter gene can be easily identified in a medium containg X-gal by their color. A *lacZ* gene under the control of the *GAL7* promoter was inserted into the coding region of the *MET2* gene to provide a quantitative measure of interaction strength [James et al., 1996].

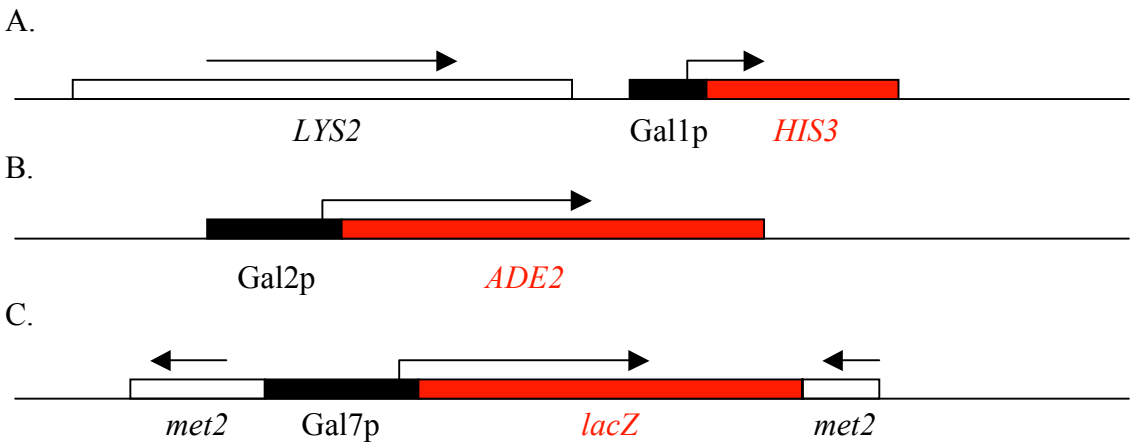

Fig. 1.6: Genomic structure at the reporter gene loci in PJ69-4A/α. Gal4-induced promoters are indicated by ■; reporter genes by ■; and adjacent coding regions by □
(A) Structure of the *LYSZ::GALI-HIS3* reporter locus.
(B) Structure of the *GAL2-ADE2* reporter locus.
(C) Structure of the *met2::GAL7-lacZ* reporter locus.

1.5 "The Tandem Affinity Purification (TAP) Method": A general procedure of protein complex purification

The TAP method was developed by Séraphin's group at the European Molecular Biology Laboratory (EMBL, Heidelberg) [Puig et.al, 2001]. They descirbed the fusion of the TAP (Tandem Affinity Purification) tag to the target protein and the introduction of the construct into the host cell or organism. The fusion protein as well as associated partners are recovered from total protein cell extracts by two specific affinity purification/elution steps. The material recovered will be analyzed in several ways. For protein complex characterization, proteins are concentrated, fractionated on a denaturing gel by SDS-PAGE, and the individual proteins were identified by mass spectrometry (Alternatively, Edman degradtion or Western blot may be used.) Because the various TAP purification steps are done in a gentle native manner, purified complexes may also be tested for their biological activities or used for structural analysis [Gavin et al., 2002].

The TAP tag consists of two IgG binding domains of *Staphylococcus aureus* protein A (ProtA) and a calmodulin binding peptide (CBP) separated by a TEV protease cleavage site. Originally, a C-terminal TAP tag was described, but an N-terminal TAP tag was also generated (Fig. 1.7), by which the relative order of the modules of the TAP tag are inversed in the two tags because the ProtA module needs to be located at the extreme N or C terminus of the fusion protein.

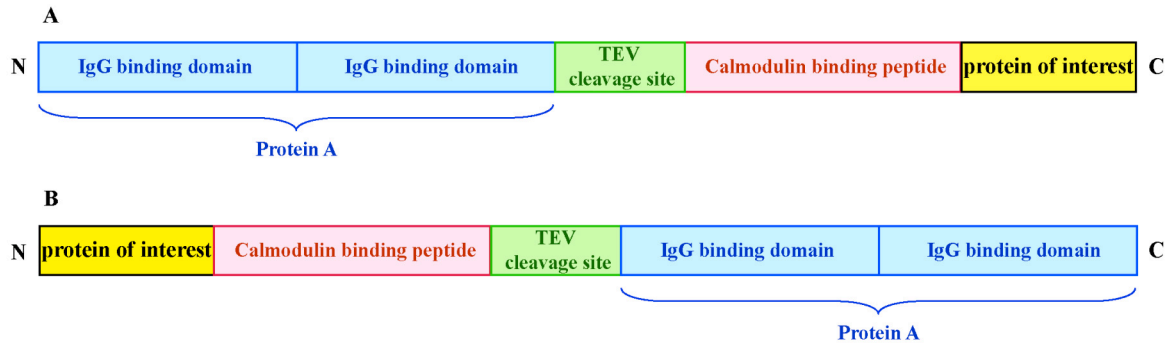

Fig. 1.7: Structure of the TAP tag. The various domains constituting the TAP-tag are indicated. A = the N-terminal construct, B = the C-terminal construct.

Both affinity tags have been selected for highly efficient recoveries of proteins present at low concentration. ProtA binds tightly on an IgG matrix, requiring the cleavage by the TEV protease to elute material under native conditions (Fig.1.8). The eluate of this first affinity purification step is then incubated with calmodulin-coated beads in the presence of calcium. After a washing step, which removes contaminants and the TEV protease remaining after the first affinity selection, the bound material is released under mild conditions with EGTA. Optimized conditions have been developed for the general use of the TAP strategy.

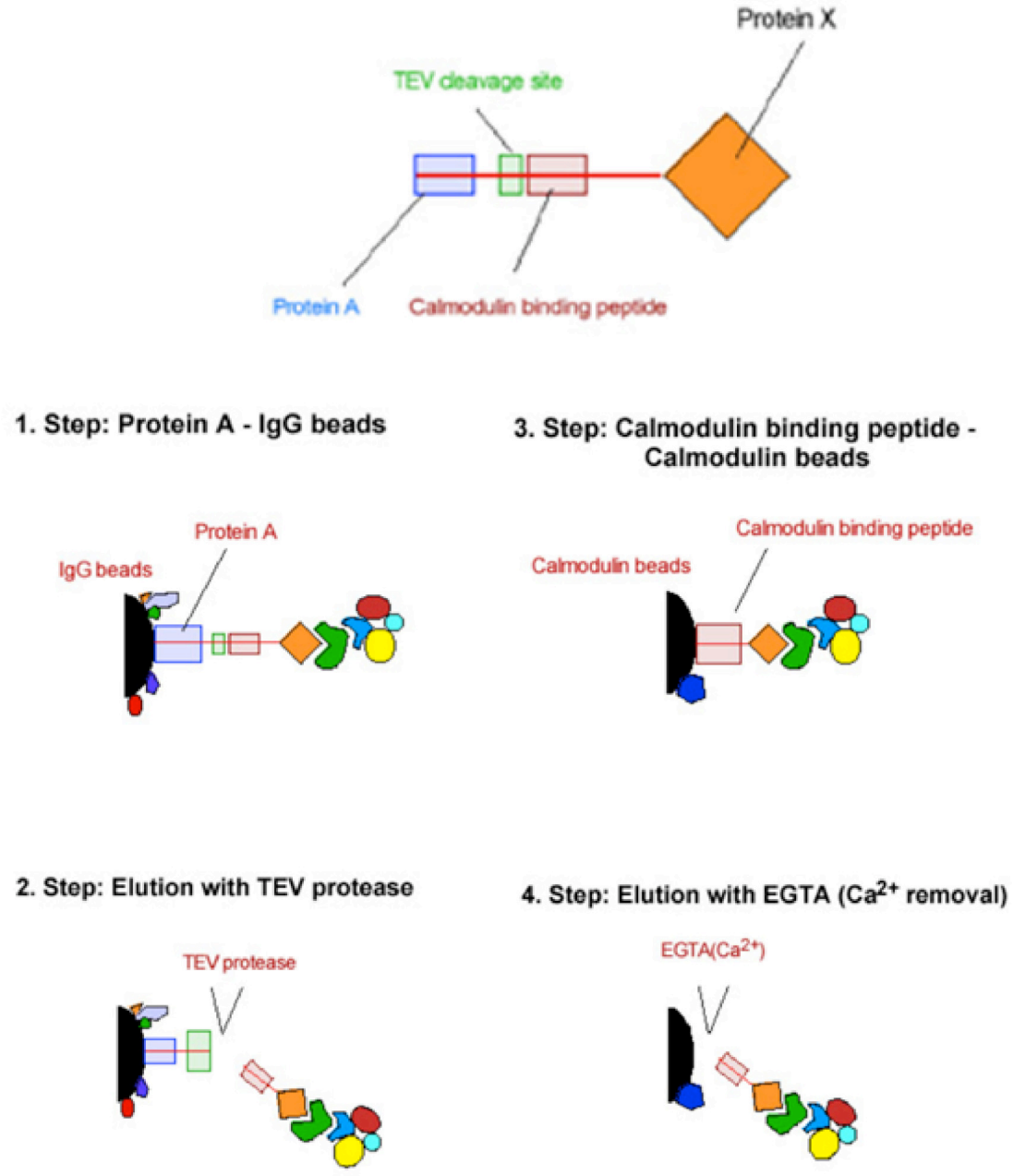

Fig. 1.8: Flow chart of the TAP tag purification.

1.6 The outline for the experimental approach

The aim of my thesis was to contribute to the understanding of the formation of cytoskeleton-like structures in *Mycoplasma pneumoniae*. In particular, the following experimental approaches were taken.

1) Re-analyses of the gene products of the *hmw2* gene (MPN310) with emphasis on a second small protein which is believed to be the processing product of the full-lemgth HMW2 protein.

2) Expression of the HMW2 protein in *Escherichia coli* or in other hosts, to isolate sufficient material for doing structural and biochemical studies.

3) Identification of direct or indirect binding partners of HMW2 applying the "two-hybrid" analysis and supportive methods.

4) Localization of the HMW2 protein within *M. pneumoniae* by cytochemistry (in cooperation with Dr. Hegermann). Improving the hitherto unsuccessful attempts by generating a new HMW2 specific antiserum and adding "new" structural features to HMW2 with a high binding specificity.

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2 Materials

2.1 Bacteria strains

Escherichia coli

TOP10 (Invitrogen). Genotype: F– *mcrA* Δ*(mrr-hsdRMS-mcrBC)* Δ*80lacZ*Δ*M15* Δ*lacX74 recA1 ara*Δ*139* Δ *(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG*

TOP10F' (Invitrogen). Genotype: F'*{lacI^q,* Tn*10(Tet^R)} mcrA (mrr-hsdRMS-mcrBC) 80lacZM15 lacX74 recA1 araD139 (ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG*

XL1-Blue (Stratagene). Genotype: *recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [*F*', proAB, lacI^q, DM15* Tn*10 (Tet^r)];* [Bullock et al., 1987]

M15 [pRep4] (Qiagen). It is derived from *E. coli* K12 and habouring pREP4 plasmid carrying *lacI* gene encoding for lac-repressor. Phenotype NaI^S, Str^S, Rif^S, Thi$^–$, Lac$^–$, Ara$^+$, Gal$^+$, Mtl$^–$, F–, RecA$^+$, Uvr$^+$, Lon$^+$.

TG1 (Maxim Biotech) Genotype: F' traD36 lacI^q Δ(lacZ) M15 proA$^+$B$^+$ /supE Δ(hsdM-mcrB)5 (r_k - m_k - McrB$^-$) thi Δ(lac-proAB)

JM109 (NEB) Genotype: F' *traD36 proA$^+$B$^+$ lacI^q* Δ*(lacZ)M15/* Δ*(lac-proAB) glnV44e14$^-$ gyrA96 recA1 relA1 endA1 thi hsdR17*

Mycoplasma pneumoniae

M129-B18 (ATCC 29342) is considered as the wild type strain of *Mycoplasma pneumoniae* throughout this work. The genome of this strain was completely sequenced [Himmelreich et al., 1996]. All experiments, unless described differently, were done done with this strain of the 30th passage. *M. pneumoniae* M129 represents subtype I.

M129 mutant A3 was a kind donation of D. Krause, University of Georgia, Goergia/USA. It was generated by transposon mutagenesis [Krause et al., 1997]. It is cytadherence-negative and lacks HMW2.

2.2 Yeast strains

Saccharomyces cerevisiae PJ69-4 was a kind donation of Dr. M. Kögl, RZPD, Heidelberg. Mating typA and α, genotype: *Trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3GAL2-ADE2 met2::GAL7-lacZ*;. [James et al.,1996].

2.3 Culture media

2.3.1 Supplementary ingredients and culture media

Bacto Agar, Difco Laboratories, Augsburg

Bacto Peptone, Bio101 Inc., USA

Complete Supplement Mixture minus variable aminoacids (CSM), Bio101 Inc., USA

Difco™ PPLO Broth (Beckton, Dickinson and Company; MD, USA)

Drop Out Base for Synthetic Defrod (DOB), Bio101 Inc., USA

Yeast extract, Difco Laboratories, Augsburg

2.3.2 Antibiotics

Ampicillin (Serva; Heidelberg, Germany)

Carbenicillin (Sigma-Aldrich; Freiburg, Germany)

Chloramphenicol (Serva; Heidelberg, Germany)

Gentamicin (Serva; Heidelberg, Germany)

Kanamycin (Serva; Heidelberg, Germany)

Penicillin (Serva; Heidelberg, Germany)

Streptomycin (Serva; Heidelberg, Germany)

Tetracyclin (Serva; Heidelberg, Germany)

2.3.3 Culture medium for *Escherichia coli*

• *LB medium (Luria broth)*

1 % (w/v) tryptone

0.5 % (w/v) yeast extract

1 % (w/v) NaCl

• *Standard I medium*

2.5 % (w/v) Standard I Medium

• *YT medium*

0.8 % (w/v) tryptone

0.5 % (w/v) yeast extract

0.25 % (w/v) NaCl

adjust to pH 7.0, add 40 % sterile glucose to a final concentration of 1 %

2.3.4 Agar plates for *E. coli*

400 ml Standard I broth (25 g/ L) were supplemented with 6 g Bacto Agar. The suspension was autoclaved.

Agar plates contained antibiotics at the following concentrations

Antibiotic	*concentration*	*stock solution*
Ampicillin	100 µg/ ml	100 mg/ ml in H_2O
Carbenicillin	100 µg/ ml	50 mg/ ml in H_2O in 70 % ethanol
Chloramphenicol	25 µg/ ml	25 mg/ ml in 70 % ethanol
Kanamycin	25 µg/ ml	25 mg/ ml in H_2O
Streptomycin	10 µg/ ml	10 mg/ ml in H_2O
Tetracyclin	10 µg/ ml	10 mg/ ml in 70% ethanol

2.3.5 Culture medium for *M. pneumoniae*

M. pneumoniae M129 was grown at 37°C in cell culture flasks (500 cm^2) containing 100 ml of modified Hayflick medium [Hayflick, 1965] supplemented with 20% horse serum. The full medium is composed of two components: component A and component B.

Component A

7,3 g PPLO broth w/o CV

2 ml phenol red (5 g/ L)

11,9 g HEPES

set pH with 10 M NaOH (~ 2,5 ml) to pH 7.6

ad. 400 ml ddH_2O

Component B

100 ml fetal horse serum (inactivated)
10 ml glucose (500 g/ L), autoclaved
5 ml penicillin G (100.000 U / ml), steril filtered

Component A was autoclaved and stored at room temperature. Component B was stored at -20°C. The full medium can be stored at 4°C

Phenol red solution

One gramme phenol red was crushed in a mortar while 30 ml 0.1 M NaOH is slowly added. The solution was then filled up to 200 ml with H_2O and stored over night at 4°C. The next day, the solution was filtered, the pH is adjusted to pH 7.5 and autoclaved. The solution could be stored for several months at 4 °C.

2.3.6 Agar plates for *M. pneumoniae*

400 ml component A were supplemented with 5 g Agar Bacteriological. The suspension was autoclaved. After cooling to 56 °C, 115 ml component B was added. Ten to twelve milliliters agar were plated in Ø 9 cm Petri dishes. Gentamicin agar plates contained 80 µg/ ml gentamicin.

2.3.7 Culture medium and agar plates for *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is normally grown in YPDA-Medium.

• *YPDA (Yeast-Peptone-Dextose-Adenin)*

21 g Bacto Peptone
10.5 g yeast extract
2% (w/v) Glucose
2 ml adenine (10 mg/ml)

• *SD –LU (Synthetic Defined Dropout Medium, leucine-uracil free)*

13.5 g DOB
0.37 g CSM-Leu,-Ura
2 ml adenine (10 mg/ml)
ad 500 ml deionized H_2O

• *SD –LUH (Synthetic Defined Dropout Medium, leucine-uracil-histidine free)*

13.5 g DOB
0.33 g CSM-Leu,-Ura,-His
2 ml adenine (10 mg/ml)
ad 500 ml deionized H_2O

• *SD –LUHA (Synthetic Defined Dropout Medium, leucine-uracil-histidine-adenine free)*

13.5 g DOB
0.285 g CSM-Leu,-Ura, -His,-Ade
ad 500 ml deionized H_2O

Solutions could be stored for several months at 4 °C after autoclave.
For agar plates, 400 ml Medium was supplemented with 8 g Bacto Agar and autoclaved.

2.4 Nucleic acids

2.4.1 Cosmids

All Cosmids used for the polymerase chain reaction were from the the cosmid bank of the genome of *Mycoplasma pneumoniae* [Wenzel and Herrmann, 1989; Himmelreich et al., 1996].

2.4.2 Plasmids

- pBC sk+: for blue/white screening, Stratagene, Heidelberg
- pBS1761: TAP tagged containing plasmid [Puig et al., 1998], Dr. Jansen, ZMBH, Heidelberg
- pCT461: cloning vector for gene expression in *M. pneumoniae* [Pirkl et al., unpublished].
- pGAD424: for expression of GAL4-AD fusion proteins, Dr. Koegl, RZPD, Heidelberg.
- pGBDU-C3: for expression of GAL4-BD fusion proteins [James et al., 1996], Dr. Jansen, ZMBH, Heidelberg.
- pKV74, pKV104: for gene expression in *M. pneumoniae* [Hahn et al., 1996, 1999], Prof. Krause, University of Goergia, USA
- pMT-Red: cloning vector for gene expression in *M. pneumoniae* [Zimmermann, 2005].
- pQE9, pQE60 for expression of His-tagged proteins in *E. coli* (Qiagen, Hilden)

2.4.3 Oligonucleotides

Primers used for cloning were synthesized by metabion (http://www.metabion.com); they were HPLC purified. Primers used for sequencing were synthesized either by Thermohybaid (http://www.interactiva.de) or by the lab of Dr. Frank in the ZMBH; Thermohybaid oligonucleotiedes were HPLC purified.

Oligonucleotides for sequencing:

Nr.	Name	Sequence
7135	5' A2	AAC GCG TTT GGA ATC ACT ACA GGG ATG TTT
7136	3' A2	TGC ACA GTT GAA GTG AAC TTG CGG GGT TTT
7171	5' U	GTG CGA CAT CAT CAT CGG AAG AGA G
7172	3' U	CGC CCG GAA TTA GCT TGG CTG CAG G
7221	9050	GAT AAA GTC CGT ATA AT
7297	pQE60_vor_Ecoforw	CAT AAA AAA TTT ATT TGC
	oPQE60/forw/Eco	GAA ATC ATA AAA AAT TTA TTT GC
8008	pQE60_rev	CTA GCT TGG ATT CTC ACC
7403	hmw2_Forw2	GCA GCT CAA CGA ACA G
7404	hmw2_Forw3	GGA AAA AGA ACG TTT G
7405	hmw2_Forw4	GCC GCG AAT TGG AGA AG
7406	hmw2_Forw5	GAA AAG CAA AAG GAA ATG
7407	hmw2_Forw6	GCA GAG CTA GAT AAG
7408	hmw2_Forw7	GAA TTG AGG CAA AAT G
7409	hmw2_Forw8	GTT GCA ACG CGA AGC
7410	hmw2_Forw9	GAA ACC AAA AAG AAA C
7411	hmw2_Forw10	GCA ATC TAT CAT TTC
7412	hmw2_Forw11	GAA TTA ACT GAG TTG C
8050	*S.aureaus* Tn*4001* Transposase	GAT AAA GTC CGT ATA ATT GTG T

Standard sequencing primers T3 and T7 were provided from the firm GATC (Konstanz) for sequencing inserts in the vector pBC sk+.

Oligonucleotides for cloning:

Nr.	Name	Sequence (**bold** = restriction site)
5137	HMW2_Start	ATG AAT GAT ACT GAC AAG AAG
5143	HMW2_Start_2AS	AAT GAT ACT GAC AAG AAG TTC
6504	HMW2/BamHI_3'	CG**G GAT CC**T TTA GCT GCT TTT TGG GCA ATT AG
7174	HMH2_BamHI_start	C**GG GAT CC**A TGA ATG ATA CTG ACA AGA AG
7175	ClpB_BamHI_forw	CG**G GAT CC**C GGA AAT TAT GCC TCA AAC A
7176	ClpB_BamHI_forw_neu	GCG C**GG ATC C**GC GAA ATT ATG CCT CAA ACA C
7291	hmw2_start_ATG	ATG AAT GAT ACT GAC AAG AAG TTC CCC TTG
7292	HMW2_Start_ATG_24nt	ATG AAT GAT ACT GAC AAG AAG TTC
7293	6 × his_BamHI_3	C**GG GAT CC**G TGA TGG TGA TGG TGA TG
7294	clpB_EcoRI_Forw	G**GA ATT C**CG AAA TTA TGC CTC AAA CAC AGC
7491	hmw2_FW_25kDa	TTA TTT CCA CCG TTA TTT AAA ATT AAC
7492	hmw2_FW_25kDa+32AS	GCG GTA GAA CAG CAA TAC CAA G
7493	hmw2_FW_25kDa-10AS	CCT TAC CCA TAT CCT TGA TTT TAT C
7519	hmw2_CTerm_2.AS_FW	AAC TAT CCT TAC CCA TAT CCT TGA TTT TAT CC
7520	hmw2_RV_9His_stop	CTA GTG GTG GTG GTG ATG GTG ATG GTG ATG AGA TCT TTT AG
7541	1his_stop_Bam_RV	CGC **GGA TCC** GCG CTA GTG G
7567	hmw2C_300FW_Bam	CGC **GGA TCC** AAC AAA CAA CGC GAA GAG
7568	hmw2_ohne_TAA_RV	TTT AGC TGC TTT TTG GGC AAT
	o9his_hmw2/5'	ATG CAC CAC CAC CAT CAT CAT CAC CAC CAC AAT GAT ACT GAC AAG AAG TTC
	ohmw2/rev/3×his_573AS	TGG TGA TGA TCC TCT GCA ATC TTT TGT G
	ohmw2/rev/ganz_9his_Bam	GCG **GGA TCC** TTA GTG GTG GTG GTG ATG GTG ATG GTG ATG TTT AGC TGC TTT TTG
	ohmw2/rev/ganz_Bgl_ 9his_Bam	GCG C**GG ATC C**GC GCT AGT GGT GGT GGT GAT GGT GAT GGT GAT G**AG ATC T**TT TAG C
	ProtA_5'	ATG GCA GGC CTT GCG CAA C
	CBP_EK_3'	CTT ATC GTC ATC ATC AAG TGC CCC G
	ohmw2/stop/Bam3'	CGC **GGA TCC** GCG TTA TTT AGC TGC TTT TTG GG
	ohmw2/Cterm/2.AS	AAC TAT CCT TAC CCA TAT C
	ohmw2/for/Bam_2ndAA	GCG C**GG ATC C**AA TGA TAC TGA CAA GAA G

Oligonucleotides for cloning in the Two-hybrid system:

Nr.	Name	Sequence (**bold** = restriction site)
7578	H10_orf119/FW/Eco/ATG	CCG **GAA TTC** ATG ATT CTA GGC ATA GGG
7579	H10_orf119/RV/TAA/Bam	CGC **GGA TCC** TTA ATT TTT TCA TGG TGT TTG
7580	H10_orf149FW/Eco/ATG	CCG **GAA TTC** ATG GAT AAC GAT AAA AAG
7581	H10_orf149/RV/TAA/Bam	CGC **GGA TCC** TTA AAA GAA ATC ATC ATC
7582	P115/FW/Eco/ATG	CCG **GAA TTC** ATG GTT TTT CTC AAA CGG
7583	P115/RV/TAA/Bam	CGC **GGA TCC** TTA GTT CTG CTT ATC TTT AC
	ohmw2-4a/Eco/1414	CCG **GAA TTC** AAT GAA GCT ACG GCA CAC
	ohmw2-4b/Bam	CGC **GGA TCC** TTA TTT AGC TGC TTT TTG G
	OP1/3'Bam/2HS	CGC **GGA TCC** GCG TTA CGG CAA CAC GTA ATC AGG CCA CTG GTT AAA CGG ACT AAA CAA GGT TTG GGG

Oligonucleotides for mutagenesis:

Nr.	Name	Sequence (**bold** = point mutation)
7539	hmw2-CTerm_mutATG_RV	GGG TAA GGA TAG TT**A** ATA TCA TTA CC
7540	hmw2-CTerm_mutATG_FW	GGT AAT GAT AT**T** AAC TAT CCT TAC CC

Oligonucleotides for other approaches:

Nr.	Name	Sequence
7330	CAT/for/412	GCT TTT TCT CTT TCT ATT ATT C
7331	CAT/rev/203	GTC GCT ATT GTA ACC AGT TC
7343	CAT/stop/3'	TTA TAA AAG CCA GTC ATT AG

2.4.4 DNA Ladders and markers for DNA gel electrophoresis

- GeneRuler™ DNA Ladder Mix, MBI Fermentas
- GeneRuler™ 100bp DNA Ladder, MBI Fermentas
- *M. pneumoniae* cosmids, digested with *Eco*RI [Wenzel et al., 1992; Pirkl and Herrmann, unpublished].

2.5 Proteins

2.5.1 Restriction endonucleases

Enzyme	*Manufacturer*	*U/ ml*
*Bam*HI	New England Biolabs	2,000
*Bcl*I	New England Biolabs	15,000
*Bgl*II	New England Biolabs	10,000
*Eco*RI	New England Biolabs	12,000
*Hind*III	New England Biolabs	20,000
*Nde*I	New England Biolabs	20,000
*Pst*I	New England Biolabs	20,000
*Sma*I	New England Biolabs	20,000
*Xho*I	MBI fermentas	10,000

2.5.2 Polymerases

Enzyme	*Manufacturer*	*U/ ml*
AccuPrime™ *Pfx* DNA Polymerase	Invitrogen	2,500
Platinum® *Pfx* DNA Polymerase	Invitrogen	2,500
DeepVent® DNA Polymerase	New England Biolabs	2,000
Phusion ™ DNA Polymerase	Finnzymes	2,000
Taq DNA Polymerase	Promega	5,000

2.5.3 DNA modifying enzymes and other enzymes

Enzyme	*Manufacturer*	*U/ ml*
Desoxyribonuklease (*DNase*I)	Roche, Mannheim	10,000
Lysozyme from chicken egg white	Sigma	57,500/ mg
Proteinase K	Fluka	290
T4-Polynukleotidkinase	New England Biolabs	10,000
T4-DNA Ligase	New England Biolabs	400,000
TEV protease	Invitrogen	100,000

2.5.4 Antibodies

primary antibodies:

A collection of approximately 50 monospecific antibodies against fusionproteins and synthetic peptides were supplied by the lab.

Name of antiserum	*Antiserum number*	*dilution*
anti-EF-G	66708	1:3000
anti-EF-Tu	AIR8	1:5000
anti-H10_orf 149	MX-95	1:5000
anti-HMW2_N573aa	MX-94	1:5000
anti-HMW2-Cterm	88256	1:1000
anti-HMW2-Nterm	28050	1:2000
anti-HMW1	84267	1:5000
anti-HMW3	65376	1:5000
anti-P1	45790	1:1000
anti-P24	85952	1:250
anti-P40	42326	1:1000
anti-P41	86630	1:500
anti-P65	66578	1:3000
anti-P90	43558	1:1000
anti-P115	35	1:1000
anti-His tag	Sigma, Prod. No. H1029	1:3000
anti-Triton X-100 insoluble Fraction	60631	1:5000

secondary antibodies:

- Alkaline phosphatase (AP) conjugated goat-anti-rabbit antibody IgG (H&L) (Dianova GmbH, Hamburg; Code No.111-055-144)
- Alkaline phosphatase (AP) conjugated goat-anti-mouse antibody IgG (Fcγ fragment specific) (Dianova GmbH, Hamburg; Code No.115-055-071)

2.5.5 Protein ladders and markers for SDS-PAGE

- PageRuler™ Protein Ladder (MBI Fermentas, #SM0661), a mixture of 14 recombinant, purified, unstained proteins with melecular weight from 10 to 200 kDa
- PageRuler™ Prestained Protein Ladder (MBI Fermentas, # SM0671), a mixture of 10 recombinant, purified colored proteins with the apparent molecular weights from 10 to 170 kDa.
- Precision Plus Protein unstained standards (BioRad Laboratories, catalog #161-0363), a mixture of 10 recombinant, purified unstained proteins with the apparent molecular weights from 10 to 250 kDa.

2.5.6 Other standard protein

- Albumin Standard (BSA) 2 mg/ ml in ampoules (Pierce, Rockford, USA).

2.5.7 Peptides

Peptide from P115 protein for immunization of rabbit was made by Peptide Specialty Laboratories GmbH, Heidelberg.
amino acid sequence: CGGKAEQYISKDKQN (bold = amino acid of P115)
amino acid position: 971-982 (the last 12 amino acid at the C-terminus of P115)

2.6 Buffers and solutions

2.6.1 Reaction solutions for enzymes

For all enzyme reactions, delivered buffers from the manufacturers were used.

2.6.2 Solutions for plasmid isolation

SolA	*SolB*	*SolC*
50 mM glucose	0.2 N NaOH	3 M sodium acetate
25 mM Tris-HCl pH 8.0	1 % SDS	2 M acetic acid
10 mM EDTA		

2.6.3 Solutions for Colony transfer

Solution A	*Solution B*	*Solution C*
10% SDS (w/v)	500 mM NaOH	500 mM Tris pH8
	1.5 M NaOH	1.5 M NaCl

2.6.4 Solution for Southern blot transfer

Denaturation solution

0.5 M NaOH

1.5 M NaCl

Neutralizing solution

1 M NH_4Ac

20 mM NaOH

2.6.5 Buffers for hybridization

- *Buffer for hybridization of Colony blots with radioactive labeled oligonucleotides*

Prehybridizing and hybridizing buffers

5 × SSC

1 % SDS (w/v)

Wash buffer

5 × SSC

0.05 % SDS (w/v)

- *Buffer for hybridization of Colony blots or Southern blots with DIG- labeled probes*

Prehybridizing and hybridizing buffers (50 ml)

5 × SSC

3 % milk powder

0.1 % N-lauryl sarcosine (w/v)

0.02 % SDS (w/v)

Wash buffer 1

2 × SSC

0.1 % SDS (w/v)

Wash buffer 2

0.5 × SSC

0.1 % SDS (w/v)

2.6.6 Buffers for DIG-detection

DIG 1

0.1 M Maleic acid

0.15 M NaCl

pH 7.5 (the pH was adjusted by addition of NaOH pellets to the 10 × solution)

DIG 2

DIG 1

3 % milk powder

DIG 3

0.1 M Tris /HCl pH 9.5

0.1 M NaCl

DIG-wash buffer

DIG 1 + 0.3 % Tween 20

2.6.7 Buffer for DNA gel electrophoresis

E-buffer for DNA agarose gels

2 mM EDTA
20 mM sodium acetate
40 mM Tris-HCl
adjust to pH 8.3 with acetic acid

sample dye

50 mM	EDTA
1 mg/ml	Bromphenol blue
2 mg/ml	Orange G
2 mg/ml	Xylenxyanol
500 mg/ml	Sucrose
2 %	SDS

2.6.8 Buffers for protein gel electrophoresis

Protein extraction buffer

10 mM Tris/ HCl, pH 7.5
150 mM NaCl
complete-proteinase inhibitor (1 tablet / 50 ml)

Protein Sample buffer (2x)

125 mM Tris/ HCl pH 6.8
4 % SDS
10 % beta-Mercaptoethanol
10 % glycerol
0.02 % bromophenol blue

Protein running buffer

Concentration and weight for 1 L 10 × buffer

Glycine	192 mM	144 g
Tris	25 mM	30.28 g
SDS	1%	10 g

pH should be 8.8. Do not add acid or base to adjust the pH.

Stacking gel buffer

0.5 M Tris/ HCl, pH 6.8
0.4 % SDS (w/v)

Resolving gel buffer

1.5 M Tris/ HCl, pH 8.8
0.4 % SDS (w/v)

2.6.9 Buffers for 2-D gel electrophoresis

All chemicals with p.A. quality should be used only.

Lysis buffer

	Concentration	For 50 ml solution
Urea	7 M	21 g
thiourea	2 M	7.16 g
CHAPS	4 %	2 g
Tris	40 mM	0.242 g
DTT	1 %	0.5 g
complete protease inhibitor		1 tablet
Pharmalyte, pH 3-10	0.5 %	0.25 ml

Rehydration buffer

	Concentration	For 50 ml solution
Urea	6 M	18 g
thiourea	2 M	7.16 g
CHAPS	2 %	1 g
DTT	0.3 %	0.15 g
Pharmalyte, pH 3-10	0.5 %	0.25 ml
bromophenol blue		traces

Equilibration buffer

	Concentration	For 100 ml solution
Tris/ HCl, pH 6.8	50 mM	10 ml 0.5 M Tris/ HCl pH 6.8
Urea	6 M	36 g
glycerol	30 %	30 g
SDS	2 %	20 ml 10 % SDS

2.6.10 Western blot transfer buffer

Schafer-Nielson buffer

48 mM Tris / HCl
39 mM glycine
20 % methanol (v/v)
0.037 % SDS (w/v)
pH should be 9.2. Do not add acid or base to adjust the pH.

CAPS buffer for electrotransfer of proteins onto PVDF for N-terminal sequencing

10mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), pH 11.0
10 % methanol

2.6.11 Buffers and solutions for Immunoblot analysis

Incubation buffer (TBST)

10 mM Tris/ HCl, pH 8
150 mM NaCl
0.05 % Tween 20

Incubation buffer (TBSTT) for ECF Western blot

10 mM Tris/ HCl, pH 8
150 mM NaCl
0.1 % Tween 20

PBS buffer for anti-His tag antiserum
140 mM NaCl
8 mM $Na_2HPO4 \times 2\ H_2O$
2 mM $NaH_2PO4 \times H_2O$

TBS buffer for ECF substrate
10 mM Tris/ HCl, pH 8
150 mM NaCl

Alkaline phosphatase (AP) buffer
100 mM Tris/ HCl, pH 9.5
100 mM NaCl
5 mM $MgCl_2$

Alkaline phosphatase (AP)-stop solution
20 mM EDTA

Substrate solutions for the alkaline phosphatase
NBT 50µg/ml in 70 % dimethylformamide
BCIP 50µg/ml in 100 % dimethylformamide

2.6.12 Staining solution for polyacrylamide protein gels and protein blots

Coomassie Blue stain solution
50 % methanol (v/v)
10 % acetic acid (v/v)
0.06 % Coomassie Brilliant Blue G-250 (w/v)

Coomassie Blue destain solution
5 % methanol (v/v)
7.5 % acetic acid (v/v)

Colloidal Coomassie stain solution

Component A : Coomassie Brilliant Blue stock (CBB)
5 % Coomassie Brilliant Blue G-250 (w/v)

Component B : Colloidal Coomassie dye stock
50 g ammonium sulfate
6 ml 85 % phosphate acid
ad to 490 ml with H_2O
+ 10 ml component A (CBB)

Colloidal Coomassie stain solution:
200 ml of component B
50 ml methanol

Ponceau-stain solution
5 % acetic acid (v/v)
0.1 % Ponceau S (w/v)

Amido Black solution
45 % methanol (v/v)
10 % acecid acid (v/v)
0.1 % Amido Black (w/v)

2.6.13 Buffers for His-tagged protein purification

By ÄKTAexplorer™ 10

All buffer and solutions are filtered through a 0.2 μm filter.

	Buffer A	*Buffer B*	*Buffer C*	*Buffer D*
Guanidium hydrochloride	6 M	–	–	–
Urea	–	8 M	8 M	8 M
Tris	10 mM	10 mM	10 mM	10 mM
Na_3PO_4	100 mM	100 mM	100 mM	100 mM
Imidazol	–	–	–	1 M
pH (with HCl)	8.0	8.0	6.3	6.3

By gravity-flow chromatography using sepharose column

	Buffer A	*Buffer B*	*Buffer C*	*Buffer D*	*Buffer E*	*Buffer F*
Guanidium hydrochloride	6 M	–	–	–	–	–
Urea	–	8 M	8 M	8 M	8 M	–
Tris	–	10 mM	10 mM	10 mM	10 mM	–
Na_3PO_4	100 mM	100 mM	100 mM	100 mM	100 mM	–
Acetic acid	–	–	–	–	–	0.2 M
pH (with HCl)	8.0	8.0	6.3	5.9	4.0	2.7

By paramagnetic precharged nickel particles

For all purification steps, delivered buffers from the manufacturers were used, with addition of guanidium hydrochloride to a final concentration of 6 M by purification under denaturing condition.

2.6.14 Buffers for TAP-tagged protein purification

IPP150

10 mM Tris/ HCl, pH 8.0

150 mM NaCl

(NaCl can be varied from 150 – 300 mM)

IPP150 was used as basic buffer for further purification steps. By adding chemicals and detergents to following final concentrations, individual buffers for each step could be prepared.

	IgG binding buffer	TEV cleavage buffer	Calmodulin binding buffer	Calmodulin elution buffer
NP-40	< 1 %	–	0.1 %	0.1 %
SDS	< 0.1 %	–	–	–
EDTA	–	0.5 mM	–	–
DTT	–	1 mM	–	–
2-mercapthoethanol	–	–	10 mM	10 mM
Magnesium acetate	–	–	1 mM	1 mM
Imidazol	–	–	1 mM	1 mM
$CaCl_2$	–	–	2 mM	–
EGTA	–	–	–	2 mM

2.6.15 Buffer for *M. pneumoniae* transformation

Hepes / D(+) Saccharose buffer

272 mM D(+) saccharose

8 mM HEPES

2.6.16 Solutions for *S. cerevisiae* transformation

Lithium acetate (LiAc) solution

1 M Lithium acetate

100 mM Tris/ HCl, pH 7.8

10 mM EDTA

For 100 mM LiAc solution, dilute 1:100 with distilled H_2O

SS-DNA solution

distilled H_2O (1 ml) was added to 2 mg Deoxyribonucleic acid, Herring Sperma (ICN, cat.#152274) and stirred overnight at 4°C in a beaker.

2.6.17 Other buffers and solutions

TE (Tris-EDTA)
10 mM Tris/ HCl pH 8.0
0,1 mM EDTA

Enzyme-stop solution
25 mM Tris/ HCl, pH 8.0
25 mM EDTA
300 mM NaCl
1/10 vol Phenol
in TE-buffer

15 × SSC
150 mM NaCl
15 mM Sodium citrate

2.7 Chemical compounds

Standard chemicals in p.A. grade were used. All special chemicals and items which were not nearly described were provided from following companies:

- Amersham Biosciences, Freiburg
- Biomol GmbH, Hamburg
- Biozym, Hess. Oldendorf
- Carl Roth GmbH and Co., Darmstadt
- Roche, Mannheim
- Merck KGaA, Darmstadt
- Qbiogene, Heidelberg
- Serva Feinbiochemika GmbH, Heidelberg
- Sigma Aldrich Chemikalien GmbH, Deisenhofen

2.7.1 Special chemical compounds

- Acetic acid 100% (Merck, Darmstadt)
- Aceton (Merck, Darmstadt)
- Acetonitrile, HPLC grade (Merck, Darmstadt)
- Agar Bacteriological (Oxoid)
- Agarose, Gel Electrophoresis Grade (Biozym, Hess. Oldendorf)
- Amido Black 10 B extra (Serva, Heidelberg)
- Amonium acetate (AppleChem; Darmstadt)

- APS (Ammonium peroxodisulfate) (Carl-Roth GmbH and Co., Karlsruhe)
- β-Mercaptoethanol (Roth, Darmstadt)
- Bacto TM Agar (BD, LePont de Claix; France)
- BCIP (5-Bromo-4-chloro-3-indolyl-phosphate) (Roth, Darmstadt)
- Boric acid (Mallincrodt Baker B.V.; Deventer, Holland)
- Bovine Serum Albumin FractionV (Biomol, Hamburg)
- Bromophenol Blue (Serva, Heidelberg)
- Calcium chloride 2-hydrate (J.T.Baker, Deventer, Holland)
- Calmodulin Affinity Resin (Stratagene, La Jolla, CA, USA)
- CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate Hydrate) (Sigma, Deisenhofen)
- Citric acid monohydrate (Sigma, Deisenhofen)
- Complete EDTA-free (Roche, Mannheim)
- Coomassie Brilliant Blue G250 pure (Serva, Heidelberg)
- D-Glucose-Monohydrate (Merck, Darmstadt)
- Deoxynucleotide triphosphate (Peqlab, Erlangen)
- Digoxigenin-11-dUTP (Roche, Mannheim)
- DTT (Dithiotreitol) (Sigma, Deisenhofen)
- EDTA (Ethylenediaminetetraacetic acid), disodium salt dihydrate (Carl-Roth, Karlsruhe)
- Ethidium bromide (10 mg/ml) (Carl-Roth GmbH and Co., Karlsruhe)
- Formaldehyde (37%) (Merck, Darmstadt)
- Formamide (Merck, Darmstadt)
- Freund's adjuvant (complete and incomplete) (Sigma, Deisenhofen)
- Gel 30 Acrylamide (Carl-Roth GmbH and Co., Karlsruhe)
- Glutardialdehyd 25% for electron microscopy (Merck, Darmstadt)
- Glycerol 99,5% (Gerbu, Gaiberg)
- Glycine (AppliChem, Darmstadt)
- HEPES (N-2-hydroxyethyl piperazine-n-ethanesulfonic acid) (Serva, Heidelberg)
- Horse Serum (Gibco, Invitrogen,
- IgG Sepharose™ 6 Fast Flow (Amersham Biosciences, Freiburg)
- Iodoacetamid (Sigma, Deisenhofen)
- IPTG (Isopropyl-beta-D-thiogalactopyranoside) (Roche, Mannheim)
- Lithium acetate (Sigma, Deisenhofen)
- Magnesium chloride × 6 H_2O (Gruessing, Filsum)

- Maleic acid (Fluka, Buchs)
- Methanol (Merck, Darmstadt)
- NBT (p-Nitro-blue tetrazolium chloride) (Roth, Darmstadt)
- PEG (polyethylene glycol) 6000 (Serva, Heidelberg)
- Penicillin (Serva, Heidelberg)
- Pharmalyte pH 3-10 (Amersham Biosciences, Freiburg)
- Phenol (Carl-Roth GmbH and Co.,Karlsruhe)
- Phenol red (Merck,Darmstadt)
- PMSF (Phenylmethylsulfonyl fluoride) (Roth, Karlsruhe)
- Polyacrylamide solution 30 % (19:1 Acrylamide:Bisacrylamide) (Carl-Roth, Karlsruhe)
- Ponceau S (Sigma, Deisenhofen)
- Potassium acetate (Gerbu; Gaiberg, Germany)
- PPLO broth (Difco Laboratories, Augsburg)
- Quicksafe A Szintillation solution (Zinsser, Frankfurt)
- SDS (Sodium dodecyl sulphate) (AppliChem, Darmstadt)
- Silver nitrate (Sigma, Deisenhofen)
- Sodium acetate (J.T. Baker; Deventer, Holland)
- Sodium chloride (J.T. Baker; Deventer, Holland)
- Sodium hydroxide (Gruessing; Filsum)
- Sodium sulfate (Agros Organics, Geel, Belgium)
- Sodium thiosulfate (Agros Organics, Geel, Belgium)
- Sucrose (J.T. Baker, Deventer, Holland)
- Standard I Medium (Merck, Darmstadt)
- TCA (Trichloroacetic acid) (Riedel-de Haen, Seelze)
- TEMED (N,N,N',N'- tetramethylethylenediamine) (Carl-Roth GmbH, Karlsruhe)
- Trisodium citrate dihydrate, Roth, Karlsruhe
- TRIS PUFFERAN® (Roth, Karlsruhe)
- Triton X-100, SigmaUltra (Sigma, Deisenhofen)
- Tween (Polyoxyethylene sorbitan monolaurate) (Sigma, Deisenhofen)
- Urea (Carl-Roth GmbH and Co., Karlsruhe)
- X-Gal (5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (AppliChem, Darmstadt)

2.7.2 Reagents for biochemical and molecular biological methods

- BCA Protein Assay (Pierce; Rockford/IL, USA)
- BioRad Protein Assay (BioRad Laboratories, Munich)
- DIG-DNA Labeling Kit (Roche, Mannheim)
- ECF™ Substrate for Western Blotting (Amersham Biosciences, Freiburg)
- Novex Colloidal Blue™ Stain Kit (Invitrogen, Karlsruhe)
- High Pure PCR Product Purification Kit (Roche, Mannheim)
- MagneHis™ Protein Purification System (Promega GmbH, Mannheim)
- Nucleobond PC100 Midiprep Kit (Machery and Nagel)
- Plasmid Miniprep Kit Quantum Prep® (BioRad Laboratories, Munich)
- QIAquick® Gel Extraction Kit (Qiagen, Hilden)
- Quantum Prep™, Plasmid Miniprep Kit (BioRad Laboratories GmbH, Munich)

2.7.3 Isotopes

- [γ-^{32}P] ATP (3000 Ci/ mmol) (Amersham Biosciences, Freiburg)
- [α-^{33}P] dATP (3000 Ci/ mmol) (Amersham Biosciences, Freiburg)

2.8 Laboratory articles/ consumer items

- Cell culture flask (TPP), 500cm², 150cm² and 50cm², polystyrol (Renner GmbH, Darmstadt)
- Cell scraper (Corning Inc.; Corning, NY, USA)
- Cellophane foil (Deti GmbH, Meckesheim)
- Cover Fluid (Amersham Biosciences, Freiburg)
- Immobilized pH-gradient (IPG) DryStrips (Amersham Biosciences, Freiburg)
- Immobilon P PVDF-Membran, Millipore, Bedford (USA)
- Milk powder Glücksklee, nonfat (Glücksklee GmbH, Munich)
- Milk powder Naturaflor, nonfat (Töpfer GmbH, Dietmannsried)
- Microfuge ultracentrifuge tubes, polyallomer 9.5×38 mm (Beckman-Coulter; Fullteron , CA, USA)
- Nitrocellulose membranes (Sartorius, Göttingen)
- Nitrocellulose membranes (Schleicher & Schuell, Dassel)
- Nylon membranes (Amersham Biosciences, Freiburg)
- Sephadex G-25 and G-100 (Amersham Bioscience, Freiburg)
- Ultracentrifuge tubes, polyclear 11×60 and 15×95 mm, Science Services GmbH, Munich)
- Vivaspin 0.5 ml Concentrator, Vivascience
- Xar X-ray film, Kodak
- ZipTips C-18 (Millipore; Bedford, MA, USA)

2.9 Apparatuses

2.9.1 Gel electrophoresis

- IPGphor™ and IPGphor™ strip holder (13 cm) (Amersham Biosciences, Freiburg)
- Hoefer Mighty Small-SE250- chamber mit gel pouring apparatus for 10 × 8 and 10 × 11 cm glass plates (Amersham Biosciences, Freiburg)
- Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad Laboratories, Munich)

2.9.2 Chromatography

- Äktaexplorer™ 10 System, Hi-Trap™ Chelating column, Superloop 10 ml and 150 ml (Amersham Biosciences, Freiburg)

2.9.3 Other apparatuses

- Biofuge *pico* (Heraeus Instruments, Osterode)
- Biofuge *fresco* (Heraeus Instruments, Osterode)
- Beckman Tl 100 Ultracentrifuge, TLA 45-Rotor (Beckman Coulter GmbH, Krefeld)
- Branson Sonifier with Micro-Tip or Cuphorn (Branson, Danbury; CT, USA)
- DuPont Sorvall Centrifuge RC5C with SS-34, GSA and GS-3 rotors (Kendro Laboratory Products GmbH, Langenselbold)
- Eppendorf thermocycler (Mastercycler® Gradient) (Eppendorf GmbH, Hamburg)
- Eppendorf Centrifuge 5402 (refrigerated) (Eppendorf, Hamburg)
- Gene Pulse and Pulse Controller (BioRad, Munich)
- Mini Hybridization Oven (Oncor-Appligene; Illkirch, France)
- Speed-Vac-Concentrator (Bachofer Laboratoriumsgeraete; Reutlingen, Germany)
- UV/ Visible Specrophotometer Ultrospec 3000 (Amersham Biosciences, Freiburg)
- Microscopes: LEICA DMIRB, LEICA DMRXA
- PhosphorImager (Amersham Biosciences, Freiburg)
- PhosphorImager FLA 3000; (FUJIFILM)
- Sharp JX-330 Scanner and Film Scanning Unit (Amersham Biosciences, Freiburg)
- Stratalinker 1800 (Stratagene; CA, USA)
- Szintillation Counter Tri-Carb 1500 (Packard Instrument; Frankfurt)
- Thermomixer Comfort (Eppendorf, Hamburg)

2.10 Computer Software

• *Lasergene Program (DNA STAR, Inc. WI, USA)*
- Sequence Editing & Analysis: for creating DNA or amino acid sequences; converts DNAsequences to amino acid sequences
- Restriction Analysis & Mapping: reveals restriction sites in a DNA sequence as list or in other forms; restriction sites can be viewed in vector map or ORF map forms
- Sequence Project Management: for aligning and evaluating sequenced DNA

• *Microsoft Office 97; Microsoft Corp.*
- Microsoft® Excel 97: used for creating spredsheets and calculation of signal data
- Microsoft® Word 97: used for creating text

• *Photo Editing and imaging software:*
- Adobe Photoshop® 5.0: a bitmap program for editing digital images (Adobe Systems Incorporated, USA)
- Adobe Illustrator® 10: a vector based imaging tool for creating illustrations (Adobe SystemsIncorporated, USA)

• *Internet browsers:*
- Netscape Communicator™ 4.76; Netscape® Communications Corp.
- Microsoft® Internet Explorer 5.0; Microsoft Corp.
- Website of Medline Database: http://www.ncbi.nlm.nih.gov/entrez
- Website of *M. pneumoniae* database: ORFs, gene map, annotations, primer list: http://www.uni-heidelberg.de/~pneumo

• *Signal detection software tools:*
- Image Reader V1.8E; FUJIFILM: reads Ips and fluorescence signals and converts the image to a digital file
- Image Gauge V3.45; FUJIFILM: converts the digital image created by Image Reader to a *.tif file
- VisualGrid; used for evaluating signal from Western Blots and 2-D gels images; "VisualGrid(R), Implemented by Markus Kietzmann with contributions from David Bancroft and Igor Ivanov. Copyrighted and Licensed by GPC Biotech AG 1998 - 2000"

• *Other softwares*
- BLAST program for database searching [Altschul, 1990]
- 373A Software Paket with SepEd v1.0.3 for editing DNA sequences (Applied Biosystem)
- Oligo 4.0: program for selection of sequencing and cloning oligonucleotides (National Biosciences Inc.)

3 Methods

All methods which are not described in more detailed were done after the laboratory manual: Molecular Cloning [Sambrok et al., 2001].

3.1 Microbiological Methods

3.1.1 Standard cultivation of *E. coli*

E. coli strains were either inoculated in liquid standard I medium or streaked on standard I agar (2% agar) plates and allowed to grow 14-16 h at 37 °C. Liquid cultures were grown 14-16 h with agitation (100-130 rpm) at 37 °C. If required, antibiotics were added in concentrations as described above. A fresh overnight culture contained approx. 1-2 × 10^9 cells/ml. For further cultivation, a fresh overnight culture was diluted 1:100 with medium. The bacterial titer was determined by counting bacteria cells in a Neubauer chamber or by using a photometer to measure optical density of cell suspension (appoximate value: 1 OD_{600} = 8 ×10^8 bacteria/ml).

3.1.2 Standard cultivation of *M. pneumoniae*

M. pneumoniae strain M129 (Wild type)

M. pneumoniae cells were grown adherently in cell culture flasks. Precultured cells, either freshly prepared or frozen stocks were diluted 1:10 with serum-complemented, modified Hayflick medium. A 100 ml culture was incubated in a 500 cm^2 cell culture flasks for 96 h at 37 °C. The medium for the transformants contained either gentamicin (80 μg/ml) or chloramphenicol (25 μg/ml). The cells were harvested when the medium changed the color from red to orange.

M. pneumoniae mutant A3 (hmw2¯)

M. pneumoniae mutant A3 cells were grown non-adherently in 500 ml glass flasks. Precultured cells, either freshly prepared or frozen stocks were diluted 1:10 with serum-complemented, modified Hayflick medium containing 20 μg/ml gentamicin. A 500 ml culture was incubated in 1000 cm^2 glass flasks for 96 h with gentle agitation at 37 °C. The cells were harvested by centrifugation for 15 min at 8,000*g* at 4 °C. The medium for growing the transformants contained 20 μg/ml gentamicin and 25-50 μg/ml chloramphenicol.

3.1.3 Standard cultivation of *Saccharomyces cerevisiae*

YPD-medium (5 ml) was inoculated with a large colony (diameter ca. 2-3 mm) of *S. cerevisiae* strains picked from a fresh plate (less than 2 months) and incubated for 16-18 h with agitation (230-270 rpm) at 30 °C. By these conditions, the yeast culture will reach the stationary phase (OD_{600} > 1.5). For further cultivation, a fresh overnight culture was diluted and adjusted to OD_{600} = 0.2 – 0.3.

3.1.4 Storage of *E. coli*

For long-term storage, glycerol is added to a liquid overnight culture to 20% final concentration (v/v) and stored at -80° in aliquots.

3.1.5 Storage of *Saccharomyces cerevisiae*

A single colony was picked from the agar plate and resuspended very well in 200-500 µl YPD medium (or appropriate selection medium). Glycerol (50 %) was added to this cell suspension (final concentration 25%), mixed and stored at -80 °C. Instead of a single colony, a fresh overnight culture could also be used.

3.1.6 Storage of *M. pneumoniae*

M. pneumoniae strain M129 (Wild type)
After standard incubation, the medium was drained off and the adhering cells were washed once with fresh medium (containing appropiate antibiotics) and then scraped in 1-2 ml fresh medium and frozen at -80 °C. Such cultures could be stored at -80 °C for several years.

M. pneumoniae mutant A3 (hmw2)
After standard incubation conditions, each 50 ml of the bacteria culture is aliquoted and frozen at -80 °C. Such cultures can be stored at -80 °C for several years.

3.1.7 Harvest of *M. pneumoniae*

M. pneumoniae strain M129 (Wild type)
After standard incubation, the medium was drained off and the adhering cells were washed three times with cold PBS, scraped and resuspended in 1-2 ml PBS. After centrifugation, PBS was drained off and the cell pellet was stored at -80 °C. The yield was about 70 mg cells (wet weight) or about 6 mg total protein from 100 ml bacteria culture.

M. pneumoniae mutant A3 (hmw2)¯

After standard incubation, the cells were harvested by centrifugation for 15 min at 8,000*g* at 4 °C. The cell pellet was washed by resuspending first with 400 ml cold PBS and centrifuged for 15 min at 8,000*g* at 4 °C. This was repeated twice with each 150 ml cold PBS. The final cell pellet was resuspended in 1-2 ml PBS and centrifuged for 5 min at 8,500*g* at 4 °C. PBS was drained off and the cell was stored at -80 °C. The yield was about 40 mg cells (wet weight) or about 3 mg total protein from 500 ml bacteria culture.

3.1.8 Transformation of *E. coli*

Preparation of competent E. coli (calcium chloride method)

Standard I medium (3 ml) (containing appropiate antibiotics) was inoculated with a single *E. coli* colony from a fresh overnight plate and incubated for 14-16 h with agitation at 37 °C. From this culture, 5 ml was further inoculated in 20 ml Standard I medium (containing appropiate antibiotics) and incubated fro 14-16 h with agitation at 37 °C. From this culture again a 1:100 dilution was prepared in 50 ml Standard I medium and incubated with strong agitation (159 rpm) at 37 °C until the OD_{600} of the culture reached 0.7-0.8. After chilling the culture on ice for 15 min, the cells were harvested by centrifugation for 15 min at 5,000*g* at 4 °C. The cell pellet was resuspended in 25 ml of ice-cold 0.1 M $CaCl_2$ solution and further incubated for 30 min. Thereafter, the cells were harvestd by centrifugation 15 min at 5,000*g* at 4 °C. The cell pellet was then resuspended in 2.5 ml of an ice-cold 0.1 M $CaCl_2$ containg 10% (v/v) glycerol solution and divided in aliquots of 200 µl, shock-freezed by liquid nitrogen and stored immediately at -80 ºC.

Transformation

Frozen competent *E. coli* cells (200 µl) were thawed for 5-10 min on ice. After adding 0.1 to 0.5 µg Plasmid-DNA (dissolved in 5 µl TE) or 4 µl of ligation product, the cell suspension was chilled on ice for 20 min, followed by a 30-sec incubation at 42 °C and immediatly chilled on ice for 1-2 min. Thereafter, 0.8 ml of prewarmed Standard I medium was given to the cells, incubated for 30 min with agitation at 37 °C, then 10 µl, 100 µl, 200 µl and the rest (~ 600 µl) of the cells were plated on Standard I agar plates containing the appropriate antibiotic and incubated for 12-16 h (sometimes upto 36 h) at 37 °C.

3.1.9 Transformation by electroporation of *E. coli*

Preparation of electroporation-competent E. coli

A fresh *E.coli* overnight culture (10 ml) was diluted 1:100 with 500 ml LB medium (containing appropiate antibiotic) and incubated with agitation at 37 °C until the OD_{600} of the culture reached 0.5 to 1.0 (corresponding to approx. 2-3 × 10^8 cells). After chilling the culture on ice for 15-30 min, the cells were harvested by centrifugation for 15 min at 4,000*g* and washed twice with ice-cold sterile water. Thereafter, the cells were washed twice with ice-cold 10% (v/v) glycerol and finally resuspended in 2 ml ice-cold 10% (v/v) glycerol. The cell suspension containing 1-3 × 10^{10} cells/ml was divied in aliquots of 50 µl, shock-freezed by liquid nitrogen and stored immediately at -80 ºC.

electroporation

Frozen competent *E. coli* cells (50 µl) were thawed for 5-10 minutes on ice. About 100 ng of plasmid DNA or 4 µl of ligation product was mixed with 50 µl of competent cells and incubated on ice for 1 minute. Electroporation was done in 1 mm cuvettes with the Biorad GenePulser under the following conditions: field strength 1.25kV/cm, electric capacity 25 µF and resistance 200 W. Thereafter, 1 ml cold Standard I medium was added immediately, the cell suspension was incubated for 1 h with agitation at 37°C and spreaded onto Standard I agar plates containing the appropriate antibiotic and incubated overnight at 37 °C.

3.1.10 Blue/white selection

By using cloning vectors like pBCSK + /KS, there is a possibility provided to recognize recombinant bacteria clones by color of their colonies. *E. coli* strain TOP10 used for general cloning in the lab contains deletions in *lacZ* gene in the α-peptide region, both chromosomal and episomal (F-factor), which leads to loss of ß-galaktosidase activity. This deficiency can be complemented by transforming this bacteria with pBC SK/KS plasmids, which encode an intact α-peptide.

Whenever possible vectors (pBCSK + /KS, pMT 85 etc.) and host cells (*E. coli* TOP10) were used, which permitted the blue/white selection. Bacteria cells were plated on Standard I agar plates with X-Gal (5-bromo-4-chloro-3-indolyl-βD-galactoside). The ß-galactosidase splits the lactose derivative X-Gal, by which 5'-bromo-4-chloro-indolyl is produced, of which the oxidation is promoted by atmospheric oxygen to the blue 5,5'-dibromo-4,4'-dichloro-indigo. The successful insertion of a DNA fragment into the cloning site of the plasmids leads to the destruction of the α-peptide region. Bacteria carrying plasmids containing DNA insertions will not turn to blue color on agar plate containg 2% X-Gal (v/v in dimethyl formamid).

3.1.11 Transformation of *M. pneumoniae* M129 (WT)

M. pneumoniae cells were incubated in 100 ml under standard growth conditions. Cells were washed three times with each 100 ml ice-cold HEPES/ Sucrose buffer, then scraped in 2 ml of the same buffer and centrifuged for 5 min at 8,500*g* at 4 °C. The cell pellet was re-suspended in 150 µl Hepes /Sucrose buffer.

For a standard transformation, 50µl cell suspension was mixed with 10 µg plasmid (in TE) and 1 µl 500 µM tRNA. The suspension was filled up to a final volume of 80 µl with Hepes /Sucrose buffer and incubated for 15 min on ice prior to electroporation.

Electroporation was done in 1 mm cuvettes with the Biorad GenePulser under the following conditions: field strength 1.25kV/cm, electric capacity 25 µF and resistance 100 W. Thereafter, the cells were incubated on ice for an additional 15 minutes, and then suspended in 10 ml modified Hayflick medium without addition of antibiotic. After incubation for 2 h at 37°C, 1 ml of the transformation mix was diluted from 10^{-1} to 10^{-3} in 10 ml modified Hayflick medium containing 80 µg /ml gentamicin or 25-50 µg /ml chloramphenicol and incubated for at least 4 days at 37 °C.

3.1.12 Transformation of *M. pneumoniae* (mutant A3)

The bacteria were grown as described above. Cells from a 500 ml culture are harvested by centrifugation for 15 min at 8,000*g* at 4 °C and washed by resuspending first with 400 ml ice-cold HEPES/ Sucrose buffer and centrifuged for 15 min at 8000*g* at 4 °C. This was repeated twice with each 150 ml ice-cold HEPES/ Sucrose buffer. The final cell pellet was resuspended in 1-2 ml of the same buffer and centrifuged for 5 min at 8,500*g* at 4 °C. The buffer was drained off and the cell was resuspended in 150 µl Hepes/ Sucrose buffer.

For a standard transformation, 50µl cell suspension was used and mixed with 10 µg plasmid (in TE) and 1 µl 500 µM tRNA. The suspension was filled up to a final volume of 80 µl with Hepes /Sucrose buffer and incubated for 15 min on ice prior to electroporation.

Electroporation is done in 1 mm cuvettes with the Biorad GenePulser under the following conditions: field strength 1.25kV/cm, electric capacity 25 µF and resistance 100 W. Thereafter, the cells are incubated on ice for an additional 15 minutes, and then suspended in 10 ml modified Hayflick medium without addition of antibiotic. After incubation for 2 h at 37°C, 1 ml of the transformation mix is diluted from 10^{-1} to 10^{-3} in 10 ml modified Hayflick medium containing antibiotic containg 20 µg /ml gentamicin and 25-50 µg /ml chloramphenicol and incubated for at least 7 days at 37 °C.

3.1.13 Isolation of transformants

After several days (between 4 to 7 days for WT or 7-14 days for the mutant A3) of incubation of the transformed mixture of *M. pneumoniae* cells at 37°C, colonies develope at the surface of the culture flask. The cells were scraped in the 10 ml growth medium, sucked into a syringe with a 0.9 mm needle and filtrated. The following three filters were used: 0.45 µm, 0.2 µm and 0.1 µm. From both, the 0.2 µm and 0.1 µm filtration, dilutions were made in 2 ml medium from 10^{-1} to 10^{-3}, and from each dilution, 200 µl were plated on mycoplasma agar containing antibiotic (10 ml agar in Ø 9 cm petri dishes or 15 ml agar in Ø 13 cm petri dishes). The plated cells were incubated at 37 °C until colonies developed (between 1 to 2 weeks). Colonies were picked with a sterile, wooden toothpick and transferred into 1 ml medium in a reaction tube and incubated for further several days at 37 °C. When the medium changes color from red to orange (usually within one week), the 1 ml was then transferred into 10 ml and incubated at 37 °C until ready for passaging.

3.1.14 Transformation of *S. cerevisiae* by the Lithium acetate (LiAc) method

Preparation of LiAc-competent yeast cells and transformation

Two-three single colonies of the yeast strain were inoculated into 5 ml of YPDA liquid medium and incubated for 16-18 h on a rotary shaker (200 rpm) at 30°C. The titer of the yeast culture was determined by pipetting 0.1 ml of cells into 0.9 ml of water in a spectrophotometer cuvette and measuring the OD at 600 nm. For many yeast strains a suspension containing 1×10^6 cells/ml will give an OD_{600} of 0.1. The OD_{600} of the overnight culture should be between 3 and 6. The overnight culture was diluted with 50 ml prewarmed YPDA to a final OD_{600} at 0.2-0.3 in a 250-ml flask and incubated on a rotary shaker (200 rpm) at 30°C. This culture will give sufficient cells for 10 transformations. When the cell titer is at least 2×10^7 cells/ml or $OD_{600} = 1$, which should take about 4 h, the cells are harvest by centrifugation for 10 min at 3,000*g*. The cells were then wash in 25 ml of sterile water, resuspend in 1 ml of 100 mM LiAc and transferred to a 1.5-ml microfuge tube. The cell suspension was centrifuged for 15 sec at 13,000*g* and LiAc was removed with a micropipette. The cell pellet was resuspended in a final volume of 500 µl (about 2×10^9 cells/ ml) and kept on ice.

The cell suspension was vortexed and divided in aliquots of 50 µl, centrifuged for 15 sec at 13,000*g* and the supernatant was remove with a micropipette. The transformation mixture was made as followed and had to be kept in ice/water:

240 µl PEG (50% w/v)

36 µl 1.0 M LiAc

25 µl SS-DNA (carrier DNA, Herring sperma DNA, 2.0 mg/ ml)*

50 µl water and plasmid DNA (0.1-10 µg)

(* Carrier DNA (1 ml) was boiled for 5 minutes and quickly chilled in ice/water prior to use).

The order is important, the PEG should be pipetted first, which shields the cells from the detrimental effects of the high concentration of LiAc. The transformation mixture was resuspended by vortexing vigorously for 1 min. The tubes were incubated in a 30 °C water bath for 30 min and the cells exposed to a heat shock for 20-25 min at 42 °C. The cell suspenion was centrifuged at 6-8,000*g* for 15 sec and the transformation mix is removed with a micropipette. One ml sterile water is pipetted into each tube, the pellet is resuspended by gently pipetting up and down. Twenty µl, 200 µl and the rest (about 750 µl) of the transformation mix was plated on onto SD selective agar plates (-L, -U or –LU by cotransformation). The plates were incubated for 3 to 4 days at 30°C.

3.1.15 Yeast drop test (applied for the pairwise test)

S. cerevisiae strain PJ69-4α was cotransformed with a pair of bait and pray vector as described in 9.1.14. After cells were grown, 2-3 single colonies of this yeast strain were inoculated into 5 ml of -LU liquid medium and incubated for 16-18 h on a rotary shaker at 200 rpm at 30°C. The titer of the yeast culture was determined by pipetting 0.1 ml of cells into 0.9 ml of water in a spectrophotometer cuvette and measuring the OD at 600 nm. The OD_{600} of the overnight culture should be between 3 and 6. The overnight culture is diluted with 1 ml sterile water to a final OD_{600} at 1.0. The cell suspension (100 µl) was further diluted from 10^{-1} to 10^{-4} in 1 ml sterile water. Each dilution (5 µl) was gently dropped on selective agar plates (-LU, -LUH and –LUHA) by pipetting. The plates were incubated for 3 to 4 days at 30°C and the growth of the yeast cells was monitored.

3.2 Molecular biological methods

3.2.1 Restriction of DNA

DNA restrictions were done according to the standard protocols [Sambrock et al., 2001]. The reaction conditions were corresponded to the recommendations of the enzyme manufacturers. In general, the enzyme concentrations was calculated for a reaction time of 1 h. The completeness of the restriction was controlled on analytical agarose gels. The following methods were used to inactivate the enzymes:

- Isolation of DNA with the PCR purification kit from Roche.
- Phenol/chloroform extraction.

3.2.2 Ligation of DNA

For ligase reaction, only purified DNA fragments were used, either from gel extraction kit, PCR purification kit or phenol/chloroform extraction. The ligation of double stranded DNA molecules was done by T4 DNA ligase, applying 0.1-0.4 pmol DNA and 200 U T4 DNA ligase (400 U/ µl, NEB) in a reaction volume of 20 µl. DNA with sticky ends was ligated for 2 h at RT and DNA with blunt ends was ligated for 14-16 h at 16 °C

3.2.3 Converting sticky ends DNA to blunt ends

Sticky end at 5'-overhang could be filled-in or sticky end 3'-overhang could be removed with T4 DNA polymerase under following standard conditions;

3'-overhang removal:

0,1 to 1 µg DNA with 3'-overhang ends
2 µl 10 × T4 DNA polymerase reaction buffer
2 µl 10 × BSA
1 µl (3 U) T4 DNA polymerase (NEB)
20 µl final volume (H_2O bidest)

5'-overhang fill-in:

0,1 to 1 µg DNA with 5'-overhang ends
2 µl 10 × T4 DNA polymerase reaction buffer
2 µl dGTP/ dCTP/ dTTP/ dATP (10 mM each)
1 µl (3 U) T4 DNA polymerase
20 µl final volume (H_2O bidest)

After an incubation period of 15 min at 12 °C, the restricted fragments were purified by a PCR purification kit or by phenol/choloroform extraction.

3.2.4 Phosphorylation of 5'-end of DNA

Up to 300 pmol 5'-ends of DNA fragments can be phosphorylated in a 50-µl reaction containing 1× T4 DNA ligase buffer (1mM ATP, NEB) and 10 U T4 polynukleotide kinase (10 U/ml) by incubation for 30 min at 37°C. For blunt-end DNA fragments, preheating of the sample for 5 min at 70 °C prior to the start of the reaction are recommended (NEB, T4 polynukleotide kinase manual).

3.2.5 Radioactive 5'-end labeling of oligonucleotides

Oligonucleotides (5 pmol) were mixed in a final volume of 20 µl with T4 polynucleotide kinase buffer, ddH_2O, 30 pmol [γ-^{32}P] ATP and 10 U T4 Polynucleotide Kinase. This mix was incubated for 30 minutes at 37°C prior to addition of 100 µl Enzyme-Stop mixture. Labeled oligonucleotides were fractionated with a G-25 Sephadex-column.

Standard protocol:

1 µl oligonucleotides (5 pmol/ µl)
2 µl 10 × T4 polynucleotide kinase buffer
1 µl T4 polynucleotide kinase (= 10 U)
3 µl (= 30 µCi [γ-^{32}P] ATP)
13 µl H_2O bidest

3.2.6 Polymerase chain reaction (PCR)

For amplification of DNA fragments, polymerase chain reactions were done in an Eppendorff Thermocycler (Mastercycler® Gradient). Gene constructs for cloning experiments were amplified with the following proofreading polymerases: *DeepVent* DNA polymerase (NEB), *Platinum Pfx* DNA polymerase (Invitrogen), *AccuPrime Pfx* DNA polymerase (Invitrogen) or *Phusion* DNA polymerase (Finnzymes). A protocol for amplifying DNA is given below, some details were modified corresponding to the recommendations of the enzyme manufacturers.

Reaction mixture	*50 µl*
Template DNA	0.01-0.1 µg
10 × polymerasebpuffer/ 5 × polymerase buffer	5 µl/ 10 µl
Primer 1	20-40 pmol
Primer 2	20-40 pmol
dNTP mixture (10 mM each)	1-1.5 µl (200-300 µM)
DNA polymerase	1-2.5 U
bidest. H_2O	ad 50 µl

PCR protocol:

Cycle step	Tempetature	Time	Cycle number
preheating	94-98 °C	hold (prior to start)	
Initial denaturation	94-98 °C	30 sec-2 min	1
Denaturation	94-98 °C	5-30 sec	
Annealing	45-72 °C	10-30 sec	25-35
Extension	68-72 °C	15-30 sec/ 1 kb (Phusion) 1 min/ 1 kb (Accuprime Pfx, Platinum Pfx, Taq) 2 min/ 1 kb (DeepVent)	
Final extension	68-72 °C	5-10 min	1
	4 °C	hold	

3.2.7 PCR mediated site-directed mutagenesis

The aim of this experiment was to replace the UGA codon (tryptophan) by the UGG codon (stop codon in *E. coli*) and replace the ATG internal start codon of HMW2-s by ATT. The method, which was applied to exchange a single nucleotide was based on a PCR mediated site-directed mutagenesis [Zoller and smith, 1987; Barettino et al, 1994], by which the desired mutation was incorporated in the primers used for the PCR program. These primers are generally 25-35 bases long and carry the nucleotide which had to be changed in the middle of their sequences. The 'mutation' was finally incorporated into both DNA strands during PCR-cycles, by which the whole vector was amplified. The number of cycles for the PCR-mutagenesis was reduced to only 16 cycles. The parental template DNA and the linear, mutagenesis-primer incorporating newly synthesized DNA were treated with *Dpn*I (10 U) as follow:

17 µl purified PCR sample
2 µl 10 × NEB4 buffer
1 µl *Dpn*I (10 U/ µl, NEB)

incubated for 1.5 h at 37 °C

*Dpn*I digested only the methylated parental template and hybrid DNA. The treated DNA sample (3-5 µl) was used to transformed competent *E. coli*.

3.2.8 DIG (Digoxigenin-dUTP) -labeling of DNA by PCR

DNA sequences used for Southern blot experiments were DIG-labeled during the PCR with the *Taq* DNA polymerase. The PCR program for this PCR was set up according to chapter 3.2.6. The reaction mixture was however modified:
Premixture was prepared in 3-fold volumn and divided in 2 PCR reaction tubes. One of them was used for a control reaction with the standard dNTPs and another one for a reaction, where dTTP was replaced by DIG-dUTP. The replacement of dTTP by DIG-dUTP during the PCR caused an increase of the molecular weight of the PCR-product. Therefore, the success of the DIG-labeling reaction could be monitored by agarose gel electrophoresis, where the DIG-labeled DNA migrates slower than the control DNA.

Premixture	*45 µl*
Template DNA	0.05 µg
10 × *Taq* polymerase buffer (with $MgCl_2$)	5 µl
Primer 1	40 pmol
Primer 2	40 pmol
Taq DNA polymerase	0.5 µl (1µl if PCR product larger than 1 kb)
H_2O (bidest)	ad 45 µl

Reaction 1: control PCR

45 µl PCR premixture
2 µl dNTP mixture (10 mM each)
3 µl bidest. H_2O

Reaction 2: DIG-labeling

45 µl PCR premixture
5 µl PCR DIG probe synthesis mix (#158550, Roche)

3.2.9 Colony hybridization (radioactive)

This procedure facilitated the search for bacterial colonies containing recombinant plasmides by applying DNA hybridization techniques. For this propose, bacteria colonies were transferred on nitrocellulose filters and their DNA fixed to the filters. The prepared filters could be then hybridized with labeled probes.

Colony transfer

A nitrocellulose membrane was marked, placed on an agar plate containing grown bacteria colonies. After one minute the filter was peeled off carefully. For a lysis of the cells, the membran was then briefly placed for 3 min on a 3 MM paper (on transparent foil) soaked with solution A. To denature the DNA, the filters is further placed for 5 min on 3 mm paper soaked with solution B, after that the nitrocellulose is neutralized by placing for 5 min on 3 MM paper soaked with solution C, before it was air dried, then 0.05 μg positive control (plasmid DNA, PCR products or oligonucleotides containing sequence corresponding to the labeled probe) is mixed 1:1 to 0.8 M NaCl and dropped on one edge of the membrane. DNA was immobilized by automatic UV crosslinking (Stratalinker; 120 mJ).

Colony hybridization

The colony filters are prehybridized twice for each 2 h in hybridizing buffer (5 × SSC, 1% SDS, 10 ml/ 1-2 filters) at hybridizing temperature (5-10 °C under the melting temperature of the oligonucleotids). Hybridization was followed with radioactive-labeled probes (≅ 10^6 cpm) in 10 ml hybridization buffer (per 1-2 filters) for 14-16 h at the same temperature. The membranes were washed shortly with 10 ml wash buffer (0.05% SDS, 5 × SSC), then for 10 min at RT with 40 ml wash solution. The detection of the signal was facilitated by Phosphor Imager Screens (see 3.5.6).

3.2.10 Colony hybridization (DIG)

Colony transfer

A nitrocellulose membrane was marked, placed on an agar plate containing grown bacteria colonies. After one minute the filter was peeled off carefully. The membran was briefly placed for 15 min on a 3 MM paper (on transparent foil) soaked with solution B, for 15 min on 3 MM paper soaked with solution C, and finally for 10 min on 3 MM paper soaked with 2 × SSC, before it was air dried, then 0.05 μg positive control (plasmid DNA, PCR products or oligonucleotides containing sequence corresponding to the labeled probe) is mixed 1:1 to 0.8 M NaCl and dropped on one edge of the membrane. DNA was immobilized by automatic UV crosslinking (Stratalinker; 120 mJ).

Additional step before hybridizing with DIG-labeled probes, the colony filters had to be treated with proteinase K to digest all cellular debris which would cause high background by detection. For this perpose, the colony filters were placed on plastic foil, 1 ml of 2 mg/ ml (in 2 × SSC) proteinase K per one membrane was spreaded over the membrane, then incubated for 1 at 37 °C h. The filters were placed between 2 Whatman paper (3MM) soaked with sterile water, and rolled with a heavy rolling pin to remove all cell debris.

Colony hybridization

The colony filters were prehybridized for 1 h in 50 ml DIG-hybridization buffer (per 1-2 filters) at 60 °C. Hybridization was followed with 0.1-0.2 µg DIG-labeled probe in 5 ml DIG-hybridization buffer (per 1-2 filters) for 14-16 h at 60 °C. The Dig-labeled probe was denatured for 5 min at 95 °C and chilled on ice prior to addition to the hybridization buffer. The membranes were washed twice for 5 min with wash buffer 1 and twice for 15 min in wash buffer 2 at 60°C. The DIG-detection is described in 3.2.11.

3.2.11 DIG-detection

The following steps were done at room temperature. Membranes were incubated for 1-2 min in DIG-wash buffer, then blocked in 40-50 ml DIG 2 for 30 min-3 h. Anti-DIG antibody (1 µl) was suspended in 10 ml DIG 2 and membranes were incubated in this solution for 45 min. Membranes were then washed twice in DIG-wash buffer for 15 min and incubated in DIG 3 for 2-3 min. Probes were stained with NBT/BCIP in AP-buffer (10 ml AP-buffer plus 66 µl NBT and 33 µl BCIP) (usually from 5-15 min, or even up to 16 h). To stop the staining reaction, the AP-buffer was discarded, the membranes were washed with TE buffer and air dried.

3.2.12 Colony Western blot

The Colyny Western blot was used for a rapid identification of overexpressed fusion proteins in a polyclonal cell culture. For this perpose, LB agar plate containing bacteria colonies grown under selection pressure was chilled for 30 min at 4 °C. A nitrocellulose round filter was marked, placed on an agar plate. After one minute the filter was peeled off carefully, placed on a fresh LB agar plate containg 10 mM IPTG (colonies upward) and incubated for 2-4 hr at 37 °C. The induced bacteria are fumigated with chloroform for 15 min and incubated with lysis buffer (6ml/ filter) for 14-16 h at RT. After washing with TBST, the filters were treated as described in chapter 3.3.9 (Immunoblot analysis).

3.2.13 Southern blotting

Standard protocol:

Genomic DNA was diluted with loading buffer and separated in a 1 % agarose gel in E-buffer at 150 mA or 250 mA depending on gel size. DNA is visualized by staining with ethidium bromide (3 µg/ml).

Prior to blotting the DNA onto nylon membranes, gels are incubated as follows:

2 × 15 min in 0.125 M HCl

30 min in denaturing solution (0.5 M NaOH ; 1.5 M NaCl)

30 min in neutralizing solution (1 M NH_4Acetate ; 20 mM NaOH)

The gel was blotted overnight by capillary transfer to a nylon membrane. Two gel blotting papers (GB 005; Schleicher & Schuell) were soaked with neutralizing solution and placed on a flat surface. The gel was laid on the wet gel blotting papers. A nylon membrane was placed on top of the gel and covered with two soaked gel blotting papers. A stack of dry paper towels is laid on top to draw the liquid and transfer the DNA onto the nylon membrane. On the following day, the membrane was dried and the DNA was crosslinked to the nylon by UV light exposure. Hybridization was done with DIG-labeled probes.

Hybridization protocol (DIG)

Southern blots were pre-hybridized once in 10 ml DIG-hybridization solution (per 100 cm^2) for 4 h at 60 °C. Hybridization was followed with 0.1-0.2 µg DIG-labeled probe in 3.5 ml DIG-hybridization solution (per 100 cm^2) for 14-16 h at 60 °C. The Dig-labeled probe was denatured for 5 min at 95 °C and chilled on ice prior to addition to the hybridization solution. The membranes were washed twice with wash solution 1 for 5 min and twice in wash solution 2 for 15 min at 60°C. The DIG-detection is described in 3.2.11.

3.3 Biochemical and physical methods

3.3.1 Preparation/ isolation of plasmids from *E. coli*

Protocol:

A bacteria culture (1.5 ml) was transferred into a 1.5 ml reaction tube and centrifuged for 1 min at 13,000*g*. The cell pellet was resuspended in 100 µl SolA and incubated for 5 min at RT. SolB (200 µl) was then added to the cell suspension and inverted several times until the lysate was homogenous and clear. Then 150 µl SolC was added, the tube wss inverted five times and placed on ice for 10 min. The precipitate is collected by centrifugation for 20 min at 13,000*g* at 4 °C. The supernatant was transferred into a new reaction tube and precipitated by ethanol.

Plasmid isolations from 100 - 200 ml cell suspensions were done with the "Nucleobond PC 100 Midiprep Kit" from Macherey and Nagel. The isolation protocol was used as described in the manufacturer's manual.

3.3.2 Preparation/ isolation of genomic DNA from *M. pneumoniae*

M. pneumoniae cell were cultured in a 100 ml modified Hayflick medium. After 96 h, cells were washed twice with 1 × PBS and centrifuged at 8,500*g* at 4 °C. The cell pellet was resuspended in 750 µl Tris/EDTA buffer (50 mM Tris pH 8.0, 25 mM EDTA) and 19 µl of an RnaseH solution (1 mg/ ml, final conc. 25 µg/ml) was added and incubated for 15 min at 37 °C. A 50 µl of proteinase K (25 mg/ml) and 100 µl of a 10 % SDS solution were added to lyse cells. The lysate was incubated for 1-2 h at 50°C or for 14-16 h at RT, then 300 µl 5 M NaCl was added and mixed well by inverting the tube several times until a white precipitate had developed. The lysate was incubated on ice for 20 min-1 h. The precipitate was collected by centrifugation for 25 min at 13,000*g* at 4 °C. The supernatant was moved into a new reaction tube, 1 ml ice-cold isopropanol (100 %) was added and the DNA was precipitated by mild inversion of the tube. The precipitated DNA was centrifuged for 15 min at 13,000*g* and washed twice with 70 % ethanol. DNA was air dried for at least 2 h, 300 µl TE was added to the DNA pellet without pipetting up and down (to avoid shearing genomic DNA) and allowed to stand for 1-2 h at RT. During this time, the DNA will be slowly dissolved in the buffer.
For Southern blot analysis, 20 µl genomic DNA was digested by a restriction endonuclease in a final volume of 100 µl and samples of 10 to 20 µl was loaded onto agarose gels for further analysis.

3.3.3 Phenol/ chloroform extraction

Phenol was added to the liquid solution in a 1:1 ratio (at least 100 µl of DNA sample). This suspension was vortexed and centrifuged for 5 min at 13,000*g*. The water phase was transferred into a new reaction tube and equal volume TE was added to the phenol phase. The suspension was treated like before and the water phase was added to a new tube. An equal volume chloroform was added to the water solution and treated twice as described above. Again the water phase was transferred into a new tube for ethanol precipitation.

3.3.4 Ethanol precipitation

To a given DNA solution 0.1 volume of a 3 M NaAc solution and 2.5 volumes ice-cold 100 % ethanol were added. The solutions were mixed by vortexing and incubated for 20-30 min at -20°C or -80°C (by small DNA or small amount incubate longer (1 hr or overnight) before centrifugation at 13,000*g*. After centrifugation, the supernatant was discarded and the pellet was washed three times with 70 % ethanol. The precipitated DNA was dried in a vacuum centrifuge.

3.3.5 Quantification of nucleic acids

The extinction was measured in a final volume of 100 µl in a quartz cuvette at 260 nm. 1 OD_{260} is equivalent to 50 µg/ml dsDNA, 40 µg/ml ssRNA, 33 µg/ml oligonucleotides. Measurements were carried out in UV/ Visible Spectrophotometer Ultrospec 3000.

3.3.6 Purification of PCR products, Isolation of DNA fragment from agarose gel/ restriction reactions

For the separation of full length DNA fragments from primers, buffer, nucleotides and enzymes, PCR reactions were purified with the High Pure PCR Product Purification Kit (Roche, Mannheim). This kit was also used to seperate DNA fragment from restriction enzymes and other addtives in restriction probes. For purification of DNA fragments from agarose gels, the Qiagen Gel Purification Kit was used.

3.3.7 Immunization of rabbits

For the immunization, 300 µl of the coupled peptides were mixed well with 300 µl Freund's Adjuvant (complete for the first, incomplete for the subsequent immunizations) and injected into a rabbit. About 0.25 to 0.6 mg proteins were used for each immunization. The immunization was repeated 3 times in intervals of 3 weeks, sample was taken a week after each immunization and the antibody reaction was monitired by Western blotting. If the reaction was satisfying, the rabbit was sacrified and blood of that rabbit was collected (~ 70 ml). The blood was incubated for 1 h at 37 °C and then at least 12 h at 4 °C. After centrifugation for 30 min at 5,000*g* at 4 °C, the clear supernatant of the serum was be taken, divided in aliquots and kept at -80 °C for a long-term storage or -20 °C prior to use. Dilutions of the serum for Western blotting were experimentally determined.

3.3.8 Western-transfer

Proteins were separated by SDS-PAGE (see 3.4.3). and transferred on nitrocellulose or PVDF membran by a BIO RAD blotting chamber (Model No.Trans-Blot SD) at 25 volts for 1 hr (or 1.5 hr for protein larger than 200 kDa). Three Whatman papers (GB 005; Schleicher & Schuell) are soaked with Schafer-Nielson buffer. A nitrocellulose membrane was soaked shortly with Schafer-Nielson buffer and then laid on top of the wet papers. By using PVDF membrane, it had to be briefly soaked with 100% methanol before blotting. The resolving gel was placed on the membrane and covered with 3 wet Whatman paper.

3.3.9 Immunoblot analysis

After Western transfer, the proteins were now immobilized on a nitrocellulose membrane and could be detected by specific antisera. Immunoblots with antisera were carried out in blocking buffer (5 % milk powder in 1 × TBST). After staining the proteins with Ponceau, membranes were blocked in blocking buffer for 1 hr. The first antibody was diluted in blocking buffer and incubated with the membrane for 1 hr. Membranes were then washed three times (each 5-10 min) in 1 × TBST, followed by incubation with the second antibody diluted in blocking buffer for 1 hr. The washing procedure was repeated. For staining of the protein with NTB/BCIP, membranes were incubated in AP-buffer for 1 min prior to addition of the staining solution (10 ml AP-buffer plus 66 µl NBT and 33 µl BCIP). Thereby, alkaline phosphatase catalyzes the oxidation of NBT to indigo, by which a violett-blue-colored precipitate developed. The reaction was stopped by incubating the membranes in 20 mM EDTA.

Immunoblots with anti-polyHis antiserum (#H1029, Sigma) were carried out in BSA. After staining the protein with Ponceau, membranes were blocked in BSA-blocking buffer (1 % BSA in 1 × PBS) for 1 hr. The anti-His antiserum was diluted 1:3000 in BSA-blocking buffer and incubated with the membrane for 2 hr. Membranes were then washed three times in PBS/ Tween wash buffer (1 × PBS, 0.05 % Tween20), followed by incubation with the anti-Goat-mouse diluted in PBS/ Tween wash buffer for 1 hr. The washing procedure was repeated. For staining of the protein with NTB/BCIP, membranes were incubated in AP-buffer for 1 min prior to addition of the staining solution for Western blots.

3.3.10 Purification of His-tagged proteins

Purification of fusion proteins with His tag is done by an immobilized metal affinity chromatography (IMAC), where matrices like Sephadex CL-6B or Superflow™ coated with Nitrilotriacetic acid (NTA) are generally used. NTA binds Ni^{2+} ions [Hochuli, 1987, Qiagen manual], it has a tetradentate chelating group that occupies four of six sites in the nickel coordination sphere. The principle of this affinity chromatography is based on a selective connection of neighbouring Histidine with two free binding site of the Ni^{2+} ions, which are coupled with NTA.

By automated IMAC

Automated purification of His-tagged proteins are carried out on an ÄKTAexplorer™ 10 (Amersham Biosciences). All buffers and running soltions are filtered through 0.2 µm filter. The purification under denaturing condition was done as follows; the sediment of an *E. coli* culture (500 ml) was lysed in 50 ml buffer A and mixed overnight at 4 °C with a magnet agitator. The bacterial suspension was then ultracentrifuged for 1 hr at 20,000*g* at 4 °C and the supernatant was filtered through a filter with a 0.2 µm pore size. This cleared cell lysate was the loaded onto the column loaded with Ni^{2+} ions (5 ml HiTrap Chelating or 5 to 10 ml Ni-NTA superflow in a XK16/20 column). This column was preincubated with buffer A and the lysate in buffer A was loaded with a superloop. The column was washed with 5 cv (column volume) buffer B. By further washing with buffer C (5 cv), the pH value was lowered to 6.3. The elution was done either by a stepwise gradient with 3 steps (10, 50 and 100% buffers D, 5 cv each) or a linear gradient (0-100% buffer D in 20 cv). Fractions were collected with 0.5 to 1 cv.

Gravity-flow chromatography using sepharose column

Lysates were prepared from 100 ml *E. coli* culture. *E. coli* cells were centrifuged for 10 min at 8000*g* at 4 °C, and the resulting pellet was lysed in 20 ml buffer A. The lysates were stirred over night at 4 °C in a glass cup. The lysate was centrifuged for 20 min at 13,000*g* at 4 °C. To remove unlysed cells. The supernatant was collected and loaded onto a Nickel-bound sepharose column.

Purification protocol:

2 ml Chelating Sepharose™ Flast Flow (Amersham Biosciences) were loaded into an empty column. The sepharose was equilibrated with 10 ml 0.2 M acetic acid, then the following solutions were loaded in the order as mentioned:

10 ml (10 mM nickel sulfate in 0.2 M acetic acid)
10 ml 0.2 M Acidic acid
10 ml buffer A
the lysate
10 ml buffer A
20 ml buffer B
20 ml buffer C
15 ml buffer D
10 ml buffer E (five fractions were collected, each in 2 ml reaction flasks)
10 ml buffer F (five fractions were collected, each in 2 ml reaction flasks)

The purified proteins were normally in the first two to three fractions of buffer F.

Paramagnetic precharged nickel particles

The paramagnetic precharged nickel particles were supplied with the MagneHis™ Protein Purification System (#V8500, Promega) containing all buffers required for purification under native conditions. For a purification under denaturing conditions, all buffers were adjusted with guanidium hydrochloride or urea to a final concentration at 6 M or 4-8 M, respectively. If the tagged protein contained disulfide bridges, up to 10mM DTT were used in all buffers. Lysates were prepared from either 5 × 100 ml *M. pneumoniae* transformants or from 500 ml *E. coli* culture. The cell pellets were lysed in binding/wash buffer for denaturing conditions for 10–60 min (1ml of cultures should be resuspended in 200–600µl binding/wash buffer), then incubated with shaking for 10–20 min at RT on a rotary mixer or shaking platform. The magnetice particles were vortexed to a uniform suspension prior to use.

MagneHis™ Ni-Particles (30 µl) were added to 1 ml of cell lysate, then pipetted up and down and mixed by pipetting approx. 10 times and incubated for 2 min at RT. The tube was placed in the appropriate magnetic stand for approx. 30 sec to capture the magnetic particles with the magnet. The supernatant was carefully removed with a pipette and the tube was removed from the magnetic stand. About 150 µl of binding/wash buffer were added to the magnetic particles mixed well by pipetting. The tube was placed again in the magnetic stand for approx. 30 sec, then the supernatant was carefully removed. The wash step was repeated twice for a total of 3 washes. For the elution of the His-tagged proteins, 100 µl of elution buffer was added to the washed magnetic particles, and mixed and incubated for 1–2 min at RT. Thereafter, the tube was placed in a magnetic stand to capture the magnetic particles with the magnet. The supernatant that now contained the purified protein was removed by pipetting and the samples could be analyzed by SDS-PAGE or Western blotting.

3.3.11 Purification of TAP-tagged HMW2

Extracts were prepared from 30 × 150 ml *M. pneumoniae* transformants, grown at standard growth conditions. The collected cell pellets were resuspended in 7.5 ml IPP150 buffers containing protease inhibitor Cocktails (Complete tablets, Roche), and sonified (Sonifier B-15, Cuphorn, Branson) for 8 × 15 sec with 30 sec intervals at 4 °C. The extract was centrifuged at 25,000*g* for 30 min and the supernatant was transferred into a new tube and centrifuged at 100,000*g* for 1 h. After this centrifugation step three phases were visible in the tube: a lipidic phase floating on top, a pellet of cellular debris on the bottom, and a middle phase containing the extract. This last phase was recovered and used for the next in purification step or kept at -80 °C.

All the binding and elution steps were done in a 0.8 × 4-cm Poly-Prep columns (Bio-Rad). One hundred microliters of IgG Sepharose beads (Amersham Biosciences) corresponding to 200 µl of bead suspensions, was transferred into the column. The beads were washed with 10 ml IPP150. Then the extract was transferred into the column containing the washed beads and rotated for 2 h at 4 °C. Elution is done by gravity flow and the beads were washed 3 times with each 10 ml IgG binding buffer and once with 10 ml of TEV cleavage buffer. Cleavage is done in the same column by adding 1 ml of TEV cleavage buffer and 100 units of TEV protease (Gibco). The beads were rotated for 2 h at 16 °C and the eluate was recovered by gravity flow.

One hundred microliters of calmodulin beads (Stratagene), corresponding to 200 µl of bead suspension, was transferred into a column and washed with 10 ml of IPP150 calmodulin binding buffer. Calmodulin binding buffer (3 ml) and 3 µl of 1 M $CaCl_2$ were added to the 1 ml of eluate recovered after TEV cleavage. This solution was then transferred to the column containing washed calmodulin beads and rotated for 1 h at 4 °C. After the beads were washed with 30 ml of calmodulin binding buffer, the bound proteins were elute with 1 ml of calmodulin elution buffer. Five elution fractions of 200 µl each were collected. The proteins were usually found in fractions 2 and 3.

3.3.12 Determination of protein concentration

BCA method

Protein concentration is determined by the bicinchoninic acid (BCA) Protein Assay Kit (Pierce). This method is based on the reaction of biuret with Cu^{2+} ions in alkaline solution, where a blue-violet copper complex develops (biuret reaction) [Smith, 1985].

Reagent A (containing Na_2CO_3, $NaHCO_3$, bicinchoninic acid and sodium tartrate in 0.1 M NaOH) was mixed in the ratio 50:1 with reagent B (4% $CuSO_4$). One milliliter of the mixture was pipetted to 50 µl of the protein sample and mixed well. After incubation of 30 min at 60 °C, the absorbance was measured at 562 nm. Standards containing a range of 50 to 400 µg/ml protein (50, 100, 200, 300, 400 µg/ml, bovine serum albumin) were used for generating a standard curve.

Bradford method

Assay materials including color reagent, protein standard, and instruction booklet were available from Bio-Rad Corporation. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from

465 nm to 595 nm when binding to protein occurs [Bradford, 1976]. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. The Bradford assay is very fast and is insensitive against various chemicals (eg. urea, SDS). Protein samples (50 µl) were added to 1 ml of the 1:5 diluted dye reagent and incubated for 5 min. The absorbance was measured at 590 nm. Standards containing a range of 50 to 400 µg/ml protein (50, 100, 200, 300, 400 µg/ml, bovine serum albumin) were used for generating a standard curve.

3.3.13 Protein precipitation

TCA precipitation

Precipitation of proteins with trichloroacetic acid (TCA) is a fast method, however proteins are strongly denatured. This makes it sometimes difficult to resuspend the complete pellet even in protein sample buffer. To precipitate proteins, 0.25 volumes of a 50% TCA solution were added to the protein sample and the proteins were allowed to precipitate for 1 h on ice. The precipitated proteins were harvested by centrifugation for 20 min at 13,000*g* at 4 °C. After washing once with 5 volumes of ice-cold 80% acetone, the tubes were set on their sides for about 10 min at RT to let acetone evaporated. The dried pellets were then resuspended in sample buffer and used for further analysis.

Acetone precipitation

It is a very mild method for protein precipitation and suitable for recovering proteins from most aqueous solvents and from SDS containing buffers. It is not recommended for proteins dissolved in urea or guanidine or for peptides. Acetone (3.3 volumes, precooled at -20 °C) were added to the protein or protein sample and mixed by vortexing and incubated for at least 1 h or overnight at -20 °C. The precipitated protein will be visible, usually as a flocculent, white particulate material. The proteins were collected by centrifugation for 20min at 13,000*g* at 4 °C. The pellets were allowed to dry completely, until the acetone was completely removed. Each pellet was dissolved in sample buffer.

Methanol/ chloroform precipitation

This procedure for precipitating proteins from solution, including detergent solutions, was introduced by Wessel and Flügge [Wessel and Flügge,1984] to remove salt and detergents. Methanol (400 µl) were added to 100 µl sample solutions, mixed well by converting tubes several times and centrifuged for 10 sec at 9,000*g*, then 100 µl chloroform were added,

mixed well by converting tubes several times and centrifuged for 10 sec at 9,000*g*. For phase separation, 300 µl water were added, mixed vigorously by vortexing and centrifuged for 1 min at 9,000*g*. Three phases will be observed, a H_2O/methanol phase, an interphase containing the proteins, and a chloroform phase. The H_2O/methanol phase was removed carefully with a pipette without touching the interphase. Methanol (300 µl) were added to the interphase and the chloroform phase, mixed well by converting tubes several times and centrifuged for 2 min at 9,000*g*. The supernatant was removed as carefully as possible without disturbing pellet which containes the protein(s). The pellet was allowed to dry completely and was then dissolved in sample buffer.

3.4 Gel systems

3.4.1 Agarose gels

To analyze plasmid DNA and DNA fragments, a horizontal Gelsystems with an agarose concentration between 0.8 to 2 % (standard DNA gel: 0.8 %) were used. DNA gels were prepared with 1 × E-buffer. To control DNA migration, the samples were mixed with sample buffer (20%). The gels were run in E-buffer with a constant current (160 mA for 30 ml gels, or 320 mA for 120 ml gels).

3.4.2 SDS polyacrylamide gel electrophoresis (PAGE)

Vertical tris-glycin gel systems composed of stacking- (4.5 %) and resolving gels (between 7.5 % and 15 %) were used. Recipes for both gels are given below:

Stacking gel:	4.5 %
30% PAA	3 ml
Stacking gel buffer	5 ml
H_2O	12 ml
TEMED	20 µl
10 % APS	200 µl
final volume	20 ml

Resolving gel:	7.5%	10 %	12,5 %	15 %
30% PAA	15 ml	20 ml	25 ml	30 ml
Resolving gel buffer	15 ml	15 ml	15 ml	15 ml
H_2O	30 ml	25 ml	20 ml	15 ml
TEMED	60 µl	60 µl	60 µl	60 µl
10 % APS	600 µl	600 µl	600 µl	600 µl
final volume	60 ml			

Solutions for the resolving gels were pipetted together as described above, but without addition of APS. The casting cartridge was assembled as described by the manufacturer. The required amount of APS was added to the solutions just before pouring the gel. The resolving gel solution was overlaid with isopropanol is and allowed to polymerize. The polymerization should be finished within 15-30 min. After that the stacking gels solution was overlaid and the combs were placed between the glass plates. The stacking gel should polymerize within 30-45 min. Vertical 2-dimensional gels were prepared without the stacking gel. After polymerization, the combs were removed carefully and the wells are rinsed with water to remove any unpolymerized acrylamide.
To get preliminary information of the analysed proteins, a protein marker is routinely loaded onto the gel. The gel apparatus is connected to the power supply and run at constant current. Cooling was required by running at higher current to prevent the 'smiling' effect (curvature of the migratory band). The gels were normally stopped when bromophenol blue tracking dye had reached the bottom of the gel.

3.4.3 One-dimensional (1-D) gel electrophoresis

M. pneumoniae cells (ca. 70 mg wet weights) were resuspended in 500 µl extraction buffer for SDS-PAGE. For measuring the protein concentration, the protein solution had to be diluted (1:20 – 1:50). The final sample was adjusted to concentration of 1 mg/ml with extraction buffer and 5 × SDS sample buffer. After heating for 5 min at 95 °C, the cells are broken and 5-20 µl (per lane) of clear cell suspension was used for 1-D SDS gel electrophoresis. Procedures for preparing and running gels are described in 3.4.2.

3.4.4 Two-dimensional (2-D) gel electrophoresis

The two-dimensional (2-D) gel electrophoresis with immobilized pH gradient was done according to a manual from Görg [Görg et al., 1988, 1995,1999 and 2000]. The isoelectric focussing was done in the IPGphor™ chamber (Amersham Biosciences). The 18 cm long strips with an immobilized pH gradient from 3-10 (Amersham Biosciences) were used.

Preparation of protein extract for 2-D gel electrophoresis
Extracts were prepared from 100 ml *M. pneumoniae* cells grown at standard growth conditions. Cell pellets (about 70 mg wet weight) were resuspended in 500 lysis buffer and sonified (Sonifier B-15, cuphorn, Branson) for 8 × 15 sec at 4 °C. To solubilize the proteins as completely as possible, the cell suspension was incubated for 1 h with agitation at RT. Insoluble components were separated by ultracentrifugation for 45 min at 55,000*g* at 20 °C. The clear supernatant was stored in aliquots at -80 °C and should not be thawed more than twice. The protein concentration was measured by the Bradford method and adjusted to a concentration from 2 and 5 mg/ml.

First dimension: Isoelectric focussing (IEF) with IPGphor™

IPG-IEF for 2-D electrophoresis can be simplified by the use of an integrated instrument, the IPGphor™ (Amersham Biosciences). The IPGphor™ includes a Peltier element for temperature control (between 18°C and 25°C) and a programmable power supply (8000V, 1.5 mA). The central part of this instrument are so-called strip holders made from an aluminium oxide ceramic, in which IPG (immobilized pH gradient) strip rehydration with sample solution and IEF are done after the strip is placed into the strip holder. The IPGphor™ can handle up to 12 strip holders (length 7, 11, 13 or 18 cm). The strip holder platform regulates temperature and serves as the electrical connector for the strip holders. Besides easier handling, a second advantage of the IPGphor is the reduced time for focussing, because IEF can be done at rather high voltage (up to 8000 V). The IPGphor™ is programmable and can store nine different programs. A delayed start is also possible, which allows the user to load the strip holders with sample dissolved in rehydration buffer in the afternoon, then automatically start IEF during the night so that IEF is finished the next morning.

The extract containg the appropiate amount of proteins was diluted with rehydration solution to a final volumn of 350 µl for 18 cm long IPG strips (Amersham Biosciences). The required number of strip holders were put onto the cooling plate/electrode contact area of the IPGphor™. The sample containing the rehydration solution was pipetted into the strip holder base. The protective cover sheets from the IPG strip was peeled off and the IPG strip was placed slowly (gel side-down) onto the rehydration solution, avoiding trap to air-bubbles. The IPG strips were covered with 1 ml of silicone oil, then the plastic cover was applied. Pressure blocks on the underside of the cover assure that the IPG strip keeps in good contact with the electrodes as the gel swells. The instrument was programmed (desired rehydration time, voltage gradient, temperature). After the IPG gel strips had been rehydrated (which requires six hours at least), IEF started according to the following programmed parameters. After IEF, those IPG gel strips, which were not used immediately for the run in the second dimension were kept for further use, were stored between two sheets of plastic film at -80 °C up to several months.

IPGphor™ running conditions for IPGs 3-10:

Temperatur: 20°C, 0.05 mA/ strip

	analytical:	preparative:
V / h	18 cm	18 cm
30 V	6 h	6 h
60 V	6 h	6 h
200 V	1 h	2 h
500 V	1 h	2 h
1000 V	1 h	2 h
Gradient 1-8 kV	1 h	2 h
8000 V	4 h	10 h

Equilibration of the IPG gel strips

The IPG gel strips were equilibrated twice, each time for 15 min in 2 × 10 ml equilibration buffer. The equilibration buffer contained 6 M urea and 30% (v/v) glycerol to diminish electroendosmotic effects [Görg et al. 1988] which are thought to be responsible for reduced protein transfer from the first to the second dimension. DTT (100-200 mg) were dissolved in 10 ml of equilibration buffer (= equilibration buffer I). The focused IPG gel strips were taken out from the freezer and placed into individual test tubes. Bromophenol Blue solution were added to 10 ml Equilibration buffer I. The test tubes were sealed with parafilm and incubated for 15 min on a shaker, then the equilibration buffer I was discarded. Iodoacetamide (IAA) (460 mg) were dissolved in 10 ml of equilibration buffer (= equilibration buffer II, 260 mM iodoacetamide). Bromophenol Blue solution were added to the equilibration buffer II. The IPG gel strips were incubated with the equilibration buffer II for another 15 min on a shaker. Iodoacetamide removes excess DTT, which is responsible for the 'point streaking' in silver stained patterns [Görg et al. 1988]. The equilibrated IPG gel strips were slightly rinsed with deionized water for a second, then placed on a piece of filter paper at one edge for a few minutes to drain off excess equilibration buffer. The strip could be now applied onto the second dimension SDS gel.

Second dimension: SDS-PAGE)

The second dimension can be run on horizontal or vertical systems [Görg et al. 1988, 1995]. In this work, a vertical gel system was used. The electrophoresis chamber was filled with protein running buffer and the cooling (15°C) was turned on. The SDS gel cassettes (gel

size 20 × 20 cm) were supported in a vertical position to facilitate the application of the first dimension IPG strips. After equilibration, The IPG gel strip was immersed in protein running buffer for a few seconds, placed on top of of a vertical SDS gel and overlaid with 2 ml of hot agarose solution (75°C). The IPG strip was pressed carefully with a spatula onto the surface of the SDS gel to achieve complete contact. The agarose was allowed to solidify for at least 5 min. This procedure was repeated for the remaining IPG strips. The gel cassettes were inserted in the electrophoresis apparatus (Protean Xi chamber, Biorad) and the electrophoresis started. The SDS-PAGE gels could be run for 4-5 h or overnight.

Running conditions for one vertical SDS gel (15 °C):

1 h 200 V, 20 mA, 30 W

3-4 h 800 V, 40 mA, 50 W

Overnight running conditions for one vertical SDS gels (15 °C):

18 h 100-150 V, 15 mA, 50 W

The run was terminated when the Bromophenol Blue tracking dye had migrated off the lower end of the gel. The cassettes was opened carefully with a spatula. A spatula was also used to remove the agarose overlay from the polyacrylamide gel. The gel was peeled off the glass plate carefully, lifted by the lower edge and placed in a tray containing fixing solution. Fixing and protein staining were then continued.

3.5 Visualization of biomolecules and imaging tools

3.5.1 Nucleic acids

DNA fragments in agarose gels are visualized by staining with ethidium bromide. Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows a very convenient detection of DNA or RNA fragments in gels. The gel was stained after electrophoresis by soaking 10 min in a dilute solution of ethidium bromide (3 μg/ ml), and then rinsed well with water. To visualize DNA, the gel was placed on a ultraviolet transilluminator. Examination or photography of DNA should be taken shortly after the end of electrophoresis because the DNA bands will diffuse within the gel.

Ethidium bromide can also be incorporated into the gel or added to the samples of DNA before loading, however binding of ethidium bromide to DNA has some influence on the DNA mobility.

3.5.2 Coomassie Blue staining

After electrophoresis the gel was soaked in Coomassie staining solution for 45 min with gentle agitation at RT. The gel was then destained by soaking in the destaining solution with gentle agitation. The destaining solution should be changed several times. The destaining was continued until the protein bands are seen without background staining of the gel. The gel can be stored for some time in deionized water. It might be convenient to carefully transfer the gel to a sealable bag and dried for longer-term storage.

3.5.3 Colloidal Coomassie staining

Colloidal Coomassie staining was used because it is more sensitive than normal Coomassie staining, and the proteins can be analysed by mass spectrometry.

With Colloidal Coomassie Kit

The Colloidal Coomassie staining was done with the Colloidal Coomassie kit from Invitrogen. It is based on the method from Neuhoff [Neuhoff et al., 1988]. After electrophoresis, the gel was soaked in fixing solution for at least 2 h or overnight. After rinsing with distilled water, the gel was further incubated in Colloidal Coomassie staining solution mixed as described in the manual of the manufacterer for 3-12 h with gentle agitation. The gel was destained by rinsing with deionized water until the protein bands were seen without background staining of the gel.

With home-made Colloidal Coomassie stain solution

After electrophoresis, the gel was soaked in fixing solution (40% ethanol, 10% acetic acid) for 1-2 h or overnight. After rinsing with distilled water for 2 × 10 min, the gel was further incubated in Colloidal Coomassie staining solution overnight with gentle agitation. The gel was destained by rinsing with deionized water until the protein bands were seen without background staining of the gel.

3.5.4 Silver staining according to Blum

It is a standard silver staining method modified for 2-D gels [Blum et al., 1987]. It is relatively rapid and sensitive (~1 ng). Since proteins are not crosslinked with glutaraldehyde, it is also compatible with a subsequent mass spectrometric analysis, although it is not as suitable as Colloidal Coomassie.

All steps were done with gentle agitation at RT. All solutions are freshly made with deionized water. For staining one acrylamide gel (20 × 20 × 0.075 cm), about 500 ml of the following solutions were required.

Purpose	Solution (in 500 ml deionized water)	Time
Fixing	40% ethanol, 10% acetic acid	> 2h or overnight
Wash	30% ethanol	3 × 20 min
Sensitizer	0.1 g $Na_2S_2O_3$ (0.02%)	1 min
Wash	deionized water	3 × 20 sec
Silver staining	1 g $AgNO_3$ (0.2%) + 125 µl (0.03%) formaldehyde 37%	20 min
Wash	deionized water	2 × 20 sec
Development	15 g Na_2CO_3 (3%) + 250 µl (0.05%) formaldehyde 37%	2-5 min or until protein spots reach required sensitivity
Wash	deionized water	3 × 20 sec
Stop	5 g (1%) glycine	10 min
Wash	deionized water	3 × 20 sec

- Do not touch the gel with fingers. Always wear gloves or use forceps.
- This method can also be used for staining RNA in PAA gels.
- alternative fixation for small proteins: 50% methanol and 10% acetic acid

3.5.5 Silver staining according to Heukeshoven and Dernick

This staining method is particulary used for visualizing basic proteins [Heukeshoven and Dernick, 1988]. By this method, proteins are crosslinked with glutaraldehyde, so that protein spots can not be used for analysis by mass spectrometry.

All steps were done with gentle agitation at RT. All solutions are freshly made with deionized water. For staining one acrylamide gel (20 × 20 × 0.075 cm), about 500 ml of the following solutions were required.

Purpose	Solution (in 500 ml deionized water)	Time
Fixing	40% ethanol, 10% acetic acid	30 min
Sensitizer	30% ethanol, 64.5 g Na-acetate 5×H_2O (or 39 g Na-acetate), 10 ml glutardialdehyde, 1 g $Na_2S_2O_3$	1 h
Fixing/ Wash	30% ethanol, 5% acetic acid	> 1 h or overnight
Wash	deionized water	6-8 × 15 min
Silver staining	0.5 g $AgNO_3$ + 250 µl formaldehyde 37%	30 min
Wash	deionized water	2 × 20 sec
Development	15 g Na_2CO_3 + 125 µl formaldehyde 37%	1-3 min or until protein spots reach required sensitivity
Wash	deionized water	2 × 20 sec
Stop	5 g (1%) glycine	10 min
Wash	deionized water	3 × 20 sec

- Do not touch the gel with fingers. Always wear gloves or use forceps.

3.5.6 Ponceau staining

This staining is a quick and easy method to visualize proteins after transferring to nitrocellulose membranes. Ponceau is a reversible protein dye allowing one to check the quality of the transfer. The blot was stained in the Ponceau solution for 5 min with gentle agitation. The Ponceau solution is reusable for several times. The blot was destained by rinsing in distilled water, until the protein bands showed up well. If the transfer was efficient, the proteins could be seen as pale pink bands. The molecular weight markers could be permanently marked using a pen even when the membrane was still wet. To get a permanent record of the gel, the ponceau stained blot can be copied.

3.5.7 Phosphor Imager screens

The Phosphor Imager system was developed by Molecular Dynamics for qualitative and quantitative analysis of radioactive probes. The system is approximately ten times more sensitive than x-ray film [Phosphor Imager user manual, 1999, Molecular Dynamics]. The commercially available "Imaging Plates: IPs" are made up of a crystal layer composed of $BaFBr:Eu^{2+}$, contained within an organic binder. Rays, which are emitted from high energy sources, e.g. x-rays, UV-rays, gamma rays, beta particles, excite an electron within the Eu^{2+}-ions, which then shifts from the valence band into the conduction band. The excited electron is trapped in the "F-center" of the $BaFBr^{-}$-complex and the Eu^{3+} remains in its oxidized condition. The excited $BaFBr^{-}$-complex possesses a distinct absorption at 600 nm. By exciting the complex with a Helium-Neon Lasers (630 nm), the electron returns into the valence band. Thus, the Eu^{3+} atoms are again reduced to Eu^{2+}. During this process, a photon (390 nm) is emitted [Johnston et al., 1990].

The intensity of the luminescence is measured in relation to the position of the scanning laser beam and the output is saved as a digital image, yielding a quantitative representation of the initial energy-emitting source.

3.6 Computer based evaluation of data

3.6.1 Image Quant and Image Reader

These are software tools that functions in combination with the phosphor imager scanners; Image Quant in combination with the phosphor imager of Molecular Dynamics, and Image Reader V1.8E in combination with the phosphor imager from FUJIFILM. The tools transcribes the scanned image to digital data, delivering a tif image file in 16 bit format.

3.6.2 VisualGrid

The software program "VisualGrid™, Implemented by Markus Kietzmann with contributions from David Bancroft and Igor Ivanov, Copyrighted and Licensed by GPC AG Genome Pharmaceuticals Corporation 1998 – 1999" was applied for the analysis of the image *.tif files.

Protocol:

Western blots or 2-D gels were scanned and save as *.tif or *.bmp, then inverted by using the software program Adobe Photoshop to a dark background. A virtual grid of 2 × 2 squares was placed over the Western blot signals or protein spots from 2-D gels, so that two signals, which were to be compared, are in each square. For each signal, a mask was set, which defined the spot-area to be measured by the program.

Aside from the average signal, the program calculated background and various quantile values of the signal and the background. Of all the calculated values, only the average value was used for further analyses.

4 Results

4.1 Expression of HMW2

To isolate sufficient material of HMW2 for doing structural and biochemical studies, the HMW2 protein was expressed in *Escherichia coli*.

4.1.1 Expression of the His-tagged HMW2 in *E. coli*

a) Cloning and expression of the C-terminal His-tagged HMW2 in E. coli

The gene *hmw2* fused 6×his tag at the 3' end was constructed and used for overexpression in *E. coli*. It was cloned into the *Nco*I/*Bgl*II site of the vector pQE60 (Qiagen), which added a sequence for a 6×his tag to the 3' end of the cloned gene. *E. coli* strain XL1-blue (Stratagene) was transformed with the gene construct.

The *hmw2* gene contains four UGA triplets, coding for tryptophan in *M. pneumoniae* but for a stop codon in other organisms (Fig. 4.1). Therefore, to express HMW2 in other organisms than *M. pneumoniae*, all UGA codons of the gene *hmw2* were changed to UGG [Boonmee, unpublished].

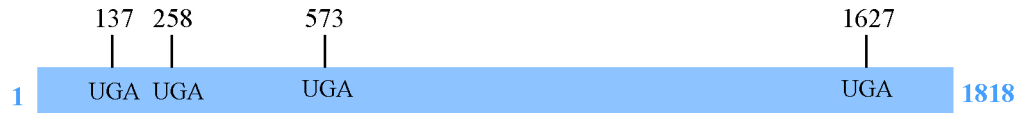

Fig. 4.1: Schematic illustration of the HMW2 protein. The positions of UGAs numbers shown here are amino acid positions.

The success of the mutagenesis could be monitored by analyzing the translation products of the stepwise mutated *hmw2* gene in *E. coli* by Western blot. After conversion of all four UGA codons, the full-length HMW2 protein could be detected in a band indicating a molar mass of 216 kDa (Fig. 4.2).

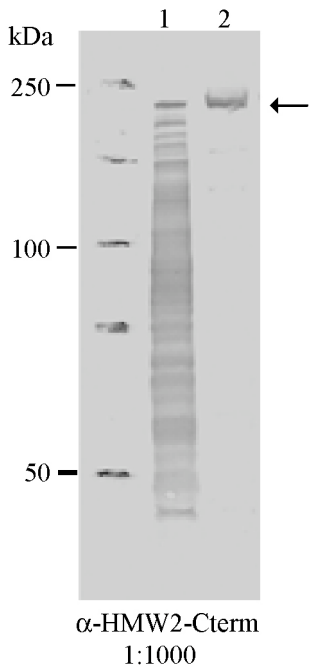

Fig. 4.2: Western blot analysis of expressed full-length HMW2 in *E. coli* and *M. pneumoniae* WT was used as a control (7.5% SDS-PAA gel).
α-HMW2-Cterm, dilution1:1000.
lane 1 = *E. coli*
lane 2 = *M. pneumoniae*

The full-length HMW2 protein was synthesized at very low concentrations and seemed to be degraded very fast in *E. coli*. It was only visible after Western blotting but not by staining with Coomassie Blue. It was not possible to isolate sufficient amount of HMW2 under standart condition and therefor I tried to optimize conditions for the expression.

Optimization of the expression of the C-terminal His-tagged HMW2 in E. coli

Variation of the Inducing time

The expression of the His-tagged HMW2 in *E. coli* was done under standard condition as followed: the diluted overnight culture (≈1:20 ; OD_{600} = 0.2) was grown for two hours, then IPTG was added at a final concentration of 1 mM and incubated for four more hours. Since HMW2 seemed to be degraded during the four hours of induction, the induction time was reduced to two hours. To compensate for the shorter induction period, the noninduced bacteria were now grown for four hours instead of two, to increase the number of cells. Total protein extracts of *E. coli* containg the His-tagged HMW2 expressed within two and four hours were compared (Fig. 4.3). Total protein lysat were seperated on a 7.5% SDS-PAA gel, blotted on nitrocellulose membran and probed with an antiserum which was directed against the HMW2-C-terminal region (α-HMW2-Cterm).

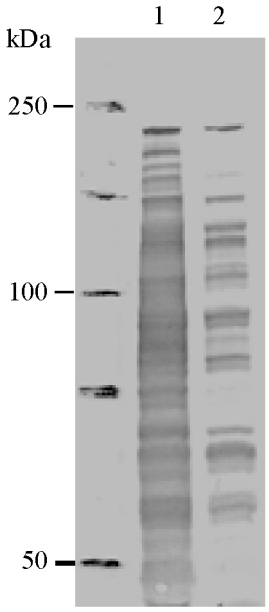

Fig. 4.3: Western blot analysis of HMW2 synthesized in *E. coli* (7.5% SDS-PAA gel). The induction time was compared between four hours and two hours. α-HMW2-Cterm, dilution 1:1000.
1 = four hours
2 = two hours

The Western blot analysis revealed that the amount of fusion protein expressed within two hours was less than that from the four hours induction, but the degradation of the fusion protein was reduced. But even the after two hours induction many degraded HMW2 proteins could be still detected. This indicated that HMW2 was not very stable in *E. coli.* In addition, the amount of HMW2 was very low, since the protein could be only detected by Western blotting but not by staining the gel with Coomassie Blue or silver stain.

Changing the host strain

To improve the yield of HMW2 in *E. coli*, several host strains were tested. The QIA*express* System (Qiagen), to which pQE60 vector belongs, recommens the *E. coli* strain M15[pREP4] which permits high-level expression. In our lab, *E. coli* strain XL1 Blue (Stratagene) is normally used. It harbours a single chromosomal copy of the *lacIq* gene, producing enough *lac* repressor to efficiently block transcription. This *E. coli* strain is ideal for storing and propagating pQE plasmids because it does not contain additional plasmid like the strain M15[pREP4]. Other *E. coli* strains that also have a $lacI^q$ phenotype are JM109 and TG1. These strains (XL1 Blue, JM109 and TG1) can be used as expression hosts for expressing nontoxic proteins, but they may be less efficient than the M15[pREP4] strain and expression is regulated less tightly than in strains harboring the pREP4 plasmid. If the expressed protein is toxic to the cell, 'leaky' expression before induction may result in poor culture growth or in the selection of mutants which grow faster than bacteria containing the correct plasmid. To test differences in expression, the

E. coli strain XL1-blue, M15[pREP4], JM109 and TG1 were transformed with the cloned his-tagged *hmw2* and the expression of the His-tagged HMW2 in these strains was monitored by SDS-PAGE and Western blotting (Fig. 4.4). A 'leaky' expression of the fusion protein was observed in all strain. *E. coli* strain M15[pREP4] showed the most tight control of the expresssion, since one could see that the fusion protein is less expressed in the uninduced sample. Therefore, this strain was chosen for expression of the HMW2 fusion protein in further experiments.

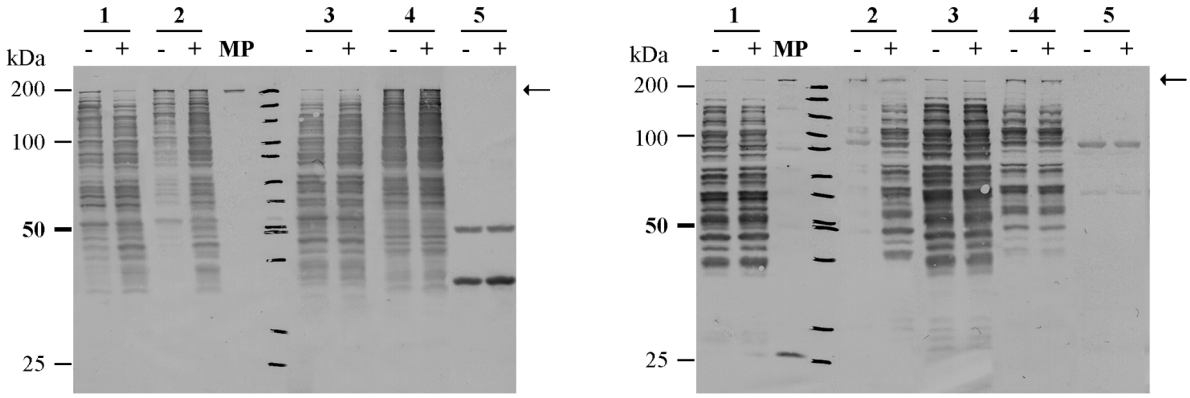

Fig. 4.4: Western blot analysis of HMW2 expressed in various *E. coli* strains (12.5% SDS-PAA gel).
1 = XL1-blue; 2 = M15[pREP4]; 3 = JM109; 4 = TG1; 5 = BL21;
+ = induced; ─ = uninduced; for control with nontransformed *E. coli* see Fig. 4.5.

Reducing temperature for growth and induction

Since the results on expression of HMW2 were still not satisfactory, several growth parameter were changed in particular temperature and time of induction. *E.coli* M15[pREP4] containing the *hmw2* construct was grown overnight at 37°C in YT medium containing 2% glucose. The overnight culture was diluted to OD_{600} = 0.2 and grown at 37 °C in YT medium containing 1% glucose until the culture reached an OD_{600} = 1. YT medium containing 1% glucose was added to dilute the *E. coli* culture to OD_{600} = 0.4. They were grown for two hours at 15 °C, then IPTG was added to final concentration of 0.1mM. The bacteria were incubated overnight (≈ 20 hours) at 15 °C. The expression of the HMW2 fusion protein was monitored by Western blot analysis using α-HMW2-Cterm to detect the fusion protein (Fig. 4.5). No significant change in reduction of protein degradation was observed between cell induced at 37 °C or 15 °C. It seemed that the yield of HMW2 fusion protein was higher in the sample from *E. coli* grown at 15 °C than *E. coli* grown at 37 °C. Therefore, I decided to express this protein at 15 °C.

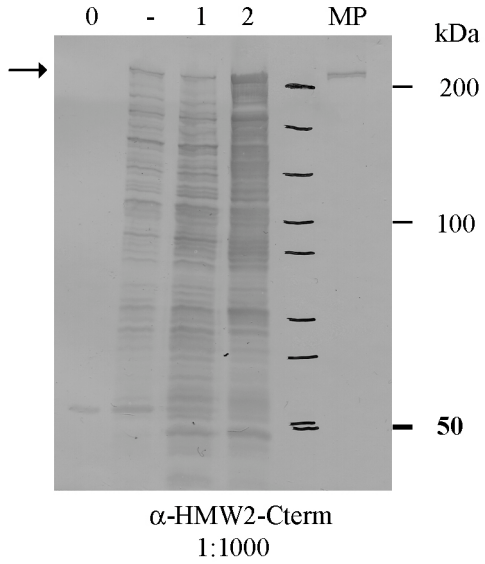

Fig. 4.5: Western blot analysis of HMW2 expressed in *E. coli* strain M15[pREP4] (7.5% SDS-PAA gel). The inducing temperature was compared.
0 = *E. coli* M15[pREP4].
– = *E. coli* containing HWM2 (not induced)
1 = *E. coli* containing HWM2 induced at 37°C
2 = *E. coli* containing HWM2 induced at 15°C
MP = *M. pneumoniae* WT

c) Purification of the C-terminal His-tagged HMW2 fusion protein

Solubility tests of cell pellets containing the HMW2 fusion protein were done with various lysis buffers (native/ denaturing conditions). As seen by SDS-PAGE, the fusion protein was insoluble applying native conditions. To test the various purification procedures, the pellet from 500 ml of *E. coli* culture was used. The purification of the His-tagged HMW2 was successful only by using paramagnetic, precharged nickel particles (MagneHis™ Ni-Particles, Promega) under denaturing condition (6 M Guanidium hydrochloride). The success of the purification was monitored by SDS-PAGE and Western blotting using α-HMW2-Cterm (Fig. 4.6). Although the purification was effective, the amount of isolated expressed protein was so low that renaturation experiments to see polymerization of HMW2 could not be done succesfully.

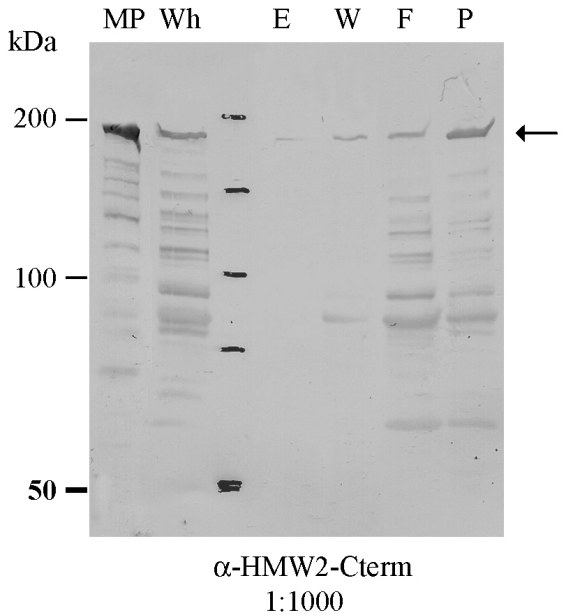

Fig. 4.6: Western blot analyses of various fractions from the purification of His-tagged HMW2 in *E. coli* (12,5% SDS-PAA gel) (α-HMW2-Cterm, dilution1:1000).
MP = *M. pneumoniae* WT (control)
Wh = whole cell extract
E = eluat
W = wash
F = flow through
P = pellet from the cell lysat.

4.1.2 Expression of the His-tagged HMW2 in *M. pneumoniae*

HMW2 was unstable and degraded very fast in *E.coli*, so that it was impossible to isolate enough material for further structural studies. The condition in *E.coli* might be not optimal for the stability of HMW2. Therefore, expression in its original host *M. pneumoniae* was chosen to overcome this problem.

a) C-terminal fusion of a poly His tag

Cloning of the 3' end his-tagged *hmw2* construct

The expression of the His-tagged HMW2 should be regulated by the expression unit of the *clpB* gene from *M. pneumoniae* (this unit will be called throughout this thesis *clpB* promotor, since the only signal conserved in the unit is the -10 region of the promotor).

Since attempts to ligate the small (300 bp) fragment containing the *clpB* promotor and the large (5.8 kbp) fragment containing the whole gene *hmw2* failed, a short *Eco*RI fragment of the *hmw2* gene encoding the first 128 aa (384 bp) was ligated to a PCR product of the *clpB* promotor. The gene *hmw2* (4 UGGs) cloned in the vector pQE60 was used as a source for generating this construct. This construct contained the gene *hmw2* followed by additional six histidine codons at the 3' end. The new construct containing the *clpB* promotor upstream of the ATG start codon of *hmw2* was then amplified by PCR. Codons for additional three histidines were added in a primer used for the PCR, so that the final PCR product contains a gene coding for nine histidines at the 3' end. The obtained PCR product was restricted with *Bam*HI and cloned into the *Bam*HI site of the IS*256*R element of Tn*4001*mod of the vector pKV74. This vector contains a modified transposon Tn*4001*mod [Hedreyda et al., 1993], which can be used as carrier for integrating foreign DNA into the genome of *M. pneumoniae* The cloning strategy is illustrated in Fig. 4.7.

E. coli colonies which were transformed with the ligation product grew very slowly and the size of the colonies was unusual small. No positive clones were found after searching almost 2,000 colonies by colony hybridization. It was previously observed that a gene *hmw2*, of which all four UGA codons were changed to UGG was not very well expressed and seemed to be degraded very fast in *E.coli* (Fig. 4.2). The full-length HMW2 protein is probably toxic for *E.coli* and could therefor increase the generation time and cause the formation of those unusual small colonies which made a finding of positives clones even more difficult. Therefore, a clone habouring the modified gene *hmw2* under the control of the *clpB* promotor might not be easily identified, since the *clpB* promotor can not be regulated, causing a constitutive production of HMW2 in *E.coli*.

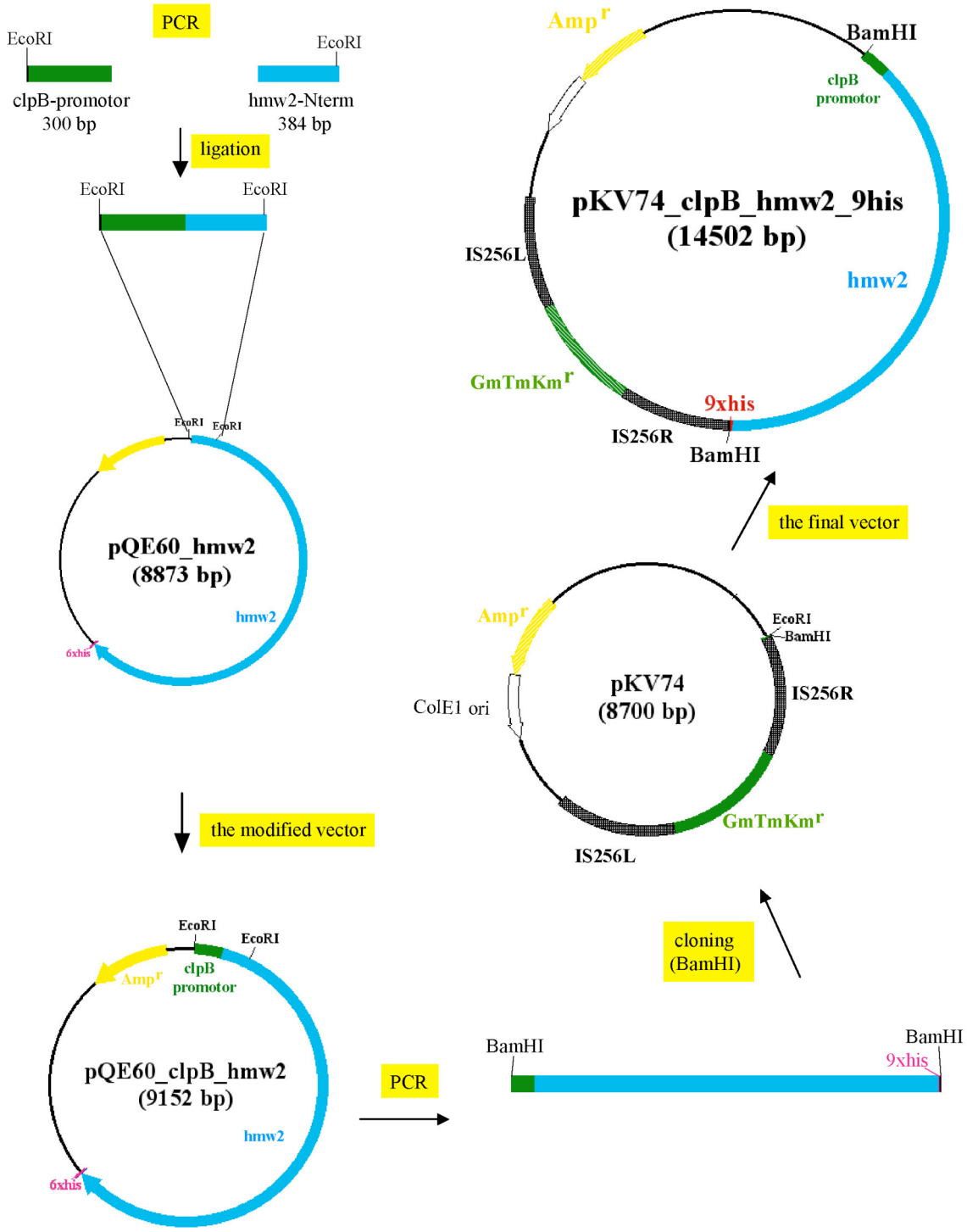

Fig. 4.7: Schematic illustration of the synthesis of the gene *hmw2* fused to DNAfragment encoding nine histidines at the 3' end and of cloning it into the vector pKV74.

Hence, to inhibit synthesis of HMW2 in *E. coli*, a *Bcl*I fragment of the new *hmw2* construct which contained two UGGs was exchanged against a *Bcl*I fragment of the construct of *hmw2* which contains two UGAs. The cloning of the PCR products of the *clpB* promotor and the his-tagged *hmw2* was done as explained above. This time, six positive clones out of 450 clones were found by colony hybridization. Fig. 4.8 shows a scheme of the final *hmw2* construct in pKV74.

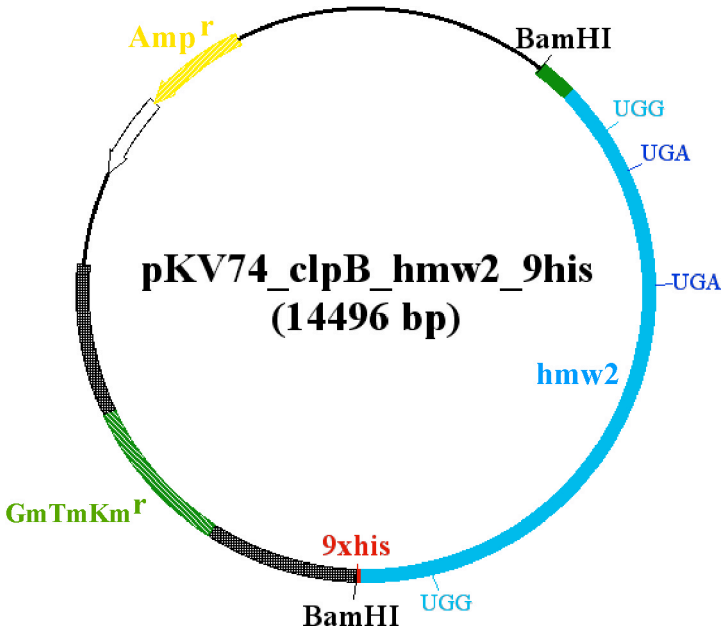

Fig. 4.8: The final construct of gene *hmw2* in pKV74.

Expression of the C-terminal His-tagged HMW2 in *M. pneumoniae*

The modified *hmw2* was integrated into the genome of *M. pneumoniae* WT by transposon insertion with Tn*4001*mod. By monitoring the expression of the C-terminal His-tagged HMW2 in *M. pneumoniae*, a single protein of about 27 kDa (which I will call HMW2-s), instead of the expected 215 kDa, was observed in protein extracts of transformants, which reacted positively with α - poly His antiserum (Fig. 4.9). By Western blotting using α–HMW2-Cterm, two protein bands were observed in the low molecular mass region, a protein of 25 kDa and a 27 kDa. The latter corresponded to the single protein band in Western blot probed with α - poly His antiserum which represented the expressed His-tagged HMW2 in *M. pneumoniae*. The 25-kDa protein band corresponded to the HMW2-s of the wild type *M. pneumoniae.*

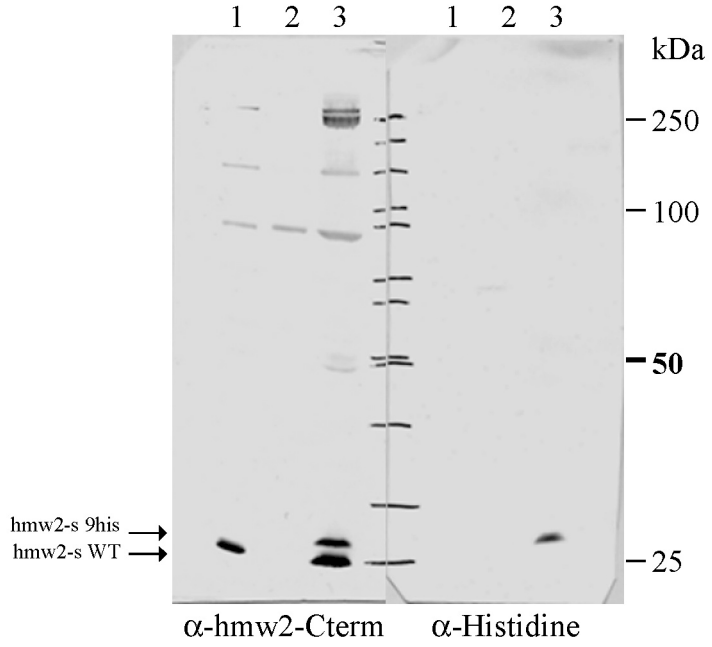

Fig.4.9: Western blot of *M. pneumoniae* WT(1), *M. pneumoniae* mutant A3 (2) and a selected WT transformant (3). Five microgram of each total protein extract was seperated on a 10 % SDS-PAA gel (antisera: α-HMW-Cterm 1:1000; α-Histidine 1:3000).

A detailed analysis of the introduced his-tagged *hmw2* gene showed a deletion of four nucleotides at position 4708-4711 of the gene *hmw2* preventing the synthesis of the mature full-length HMW2 protein (Fig. 4.10) but this frame shift mutant turned out to be very helpful for the isolation of a pure preparation of the the HMW2-s to be used for biochemical characterizations of this protein (see chapter 4.2).

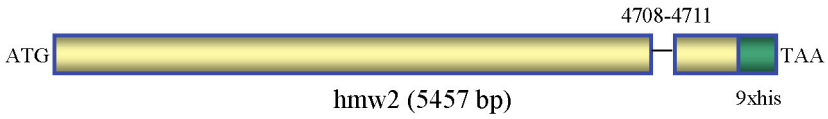

Fig. 4.10: Schematic drawing of the gene *hmw2* fused with a DNA sequence encoding nine histidines. The numbers above the bar give the position of the four bp deletion by the nucleotide numbers.

b) N-terminal fusion of a poly His tag

As an alternative construct, the His tag was fused to the N-terminus of HMW2. The expression of the N-terminal His-tagged HMW2 was regulated by the promotor of the *clpB* gene from *M. pneumoniae*.

For this propose, a sequence coding for methionine followed by 9 histidine was added to the 5' end of the *hmw2 Eco*RI fragment. The *clpB* promotor was amplified by PCR and ligated with 5' end of an *Eco*RI fragment of the *hmw2* gene (384 bp). The ligation product of both gene fragments was amplified by PCR and cloned into the *Eco*RI site of the vector pQE60 resulting in a construct containing the complete gene *hmw2* with two UGA codons.

The final construct contained the *clpB* promotor followed by a sequence coding for one methionine and nine histidines upstream of the *hmw2* gene. It was amplified by PCR using primers adding a *Bam*HI restriction site to both ends of the final PCR product. The PCR product with blunt ends was cloned into the *Sma*I site of the IS*256*R element of Tn*4001*mod of the vector pKV104 [Hahn et al., 1999]. The cloning strategy is illustrated in Fig 4.11.

Recently, new plasmids were developed in our lab (pCT461 and pMT850) and the gene construct of the modified *hmw2* was recloned into these new vectors. The vectors were much smaller than the pKV vector. Their integration in the genome was stable, because the transposase was not part of the transposon, and recloning of the inserted transposon was simple.

The insert was cut out of the construct cloned in pKV104 vector by *Bam*HI and recloned into the *Bam*HI site of both new vectors. *M. pneumoniae* WT was transformed with the gene construct in pMT850. Sine the pCT461 plasmid contains a chloramphenicol-resistence gene, it allowed a convenient selection of the cloned gene in *M. pneumoniae hmw2* mutants which were already gentamicin-resistant.

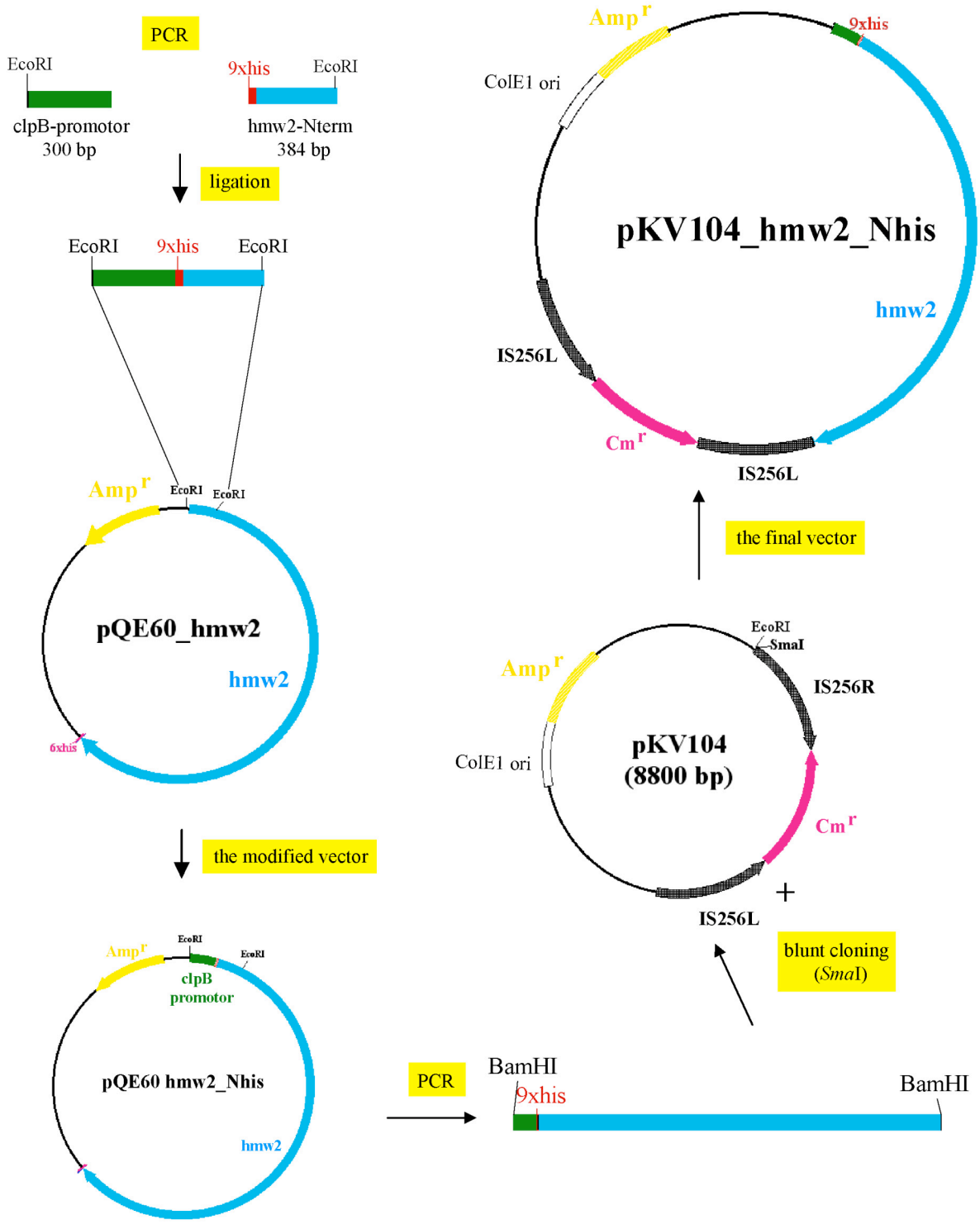

Fig. 4.11: Schematic illustration of the synthesis of the gene *hmw2* fused with a DNA fragment encoding nine histidine at the 5' end and of cloning into the pKV104.

It was shown by Western blotting that the HMW2 fusion protein was produced in the transformant of *M. pneumoniae* mutant A3, but the amount of the protein was too low to be detected with anti-polyHis antiserum (Fig. 4.12). The HMW2 protein was not expressed not very well by the *M. pneumoniae clpB* promotor, although it was shown by transcriptional analysis that this promotor belongs to one the strongest promotors of *M. pneumoniae* [Weiner et al., 2000]. The expression of HMW2 in *M. pneumoniae* might be somehow related with the cell status or growth phase. Since the level of expression was so low, a preparative purification of the HMW2 protein was not possible.

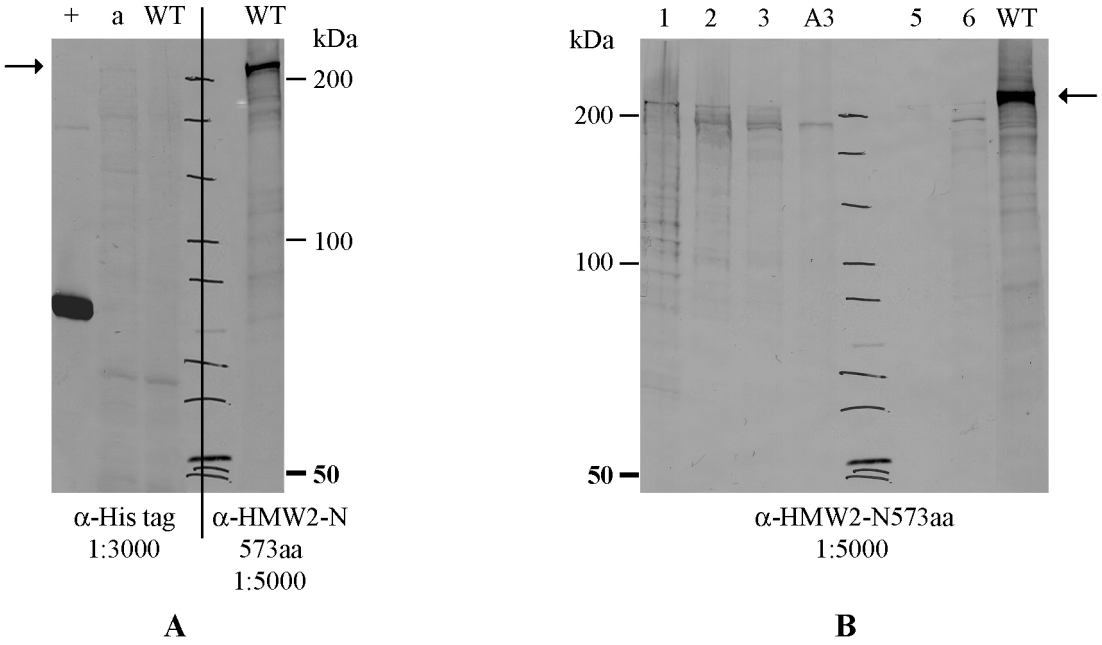

Fig. 4.12: A) Western Blot analysis of total protein extracts of the selected transformant of *M. pneumoniae* WT using an antiserum directed against His tag and the 573aa N-terminal region of HMW2. + = His-tagged fusion protein used as a positive control ; WT = *M. pneumoniae* WT ; a = transformant of *M. pneumoniae* WT

B) Western Blot analysis of total protein extracts of selected *M. pneumoniae* mutant A3 transformants using α-HMW2-N573aa. A3 = *M. pneumoniae* mutant A3 ; WT = *M. pneumoniae* WT ; 1-6 = transformants of *M. pneumoniae* mutant A3

4.2 Determining the N-terminus of HMW2-s

The function of HMW2-s is still unclear, as well as the mode of its synthesis. It could be generated by processing full-length HMW2 or by an internal new start within the *hmw*2 gene. Although the protein analysis of the described deletion mutant (previous chapter, Fig. 4.9 and 4.10) favored an internal new start and made the possibility of HMW2-s being a processing product of HMW2 rather unlikely. As a first step, the N-terminus of HMW2-s was determined by Edman sequencing.

4.2.1 Purification of the C-terminal His-tagged HMW2-s from *M. pneumoniae*

For the N-terminal sequencing approach, the protein had to be purified from *M. pneumoniae*, blotted on PVDF membrane and stained with colloidal Coomassie Blue or amido black. The purification of His-tagged HMW2-s was successful by applying denaturing conditions (6 M guanidium hydrochloride) and using paramagnetic, precharged nickel particles. The success of this purification procedure was monitored by Western blotting using antiserum directed against the C-terminal region of HMW2 (Fig. 4.13). As can be seen on Western blots, the purification was effective, since none of the His-tagged HMW2-s could be detected in the flow-through or in the washing-fraction. The eluatant was precipitated by methanol-chloroform [Wessel and Fluegge, 1984]. After seperation by 12.5% SDS-PAGE, the protein was transferred on a PVDF membrane and stained by colloidal Coomassie Blue. The N-terminal sequencing analyses were done both by Dr. H. Heid (DKFZ, Heidelberg) and by Dr. H. Hippe (Chromatec GmbH, Greifswald).

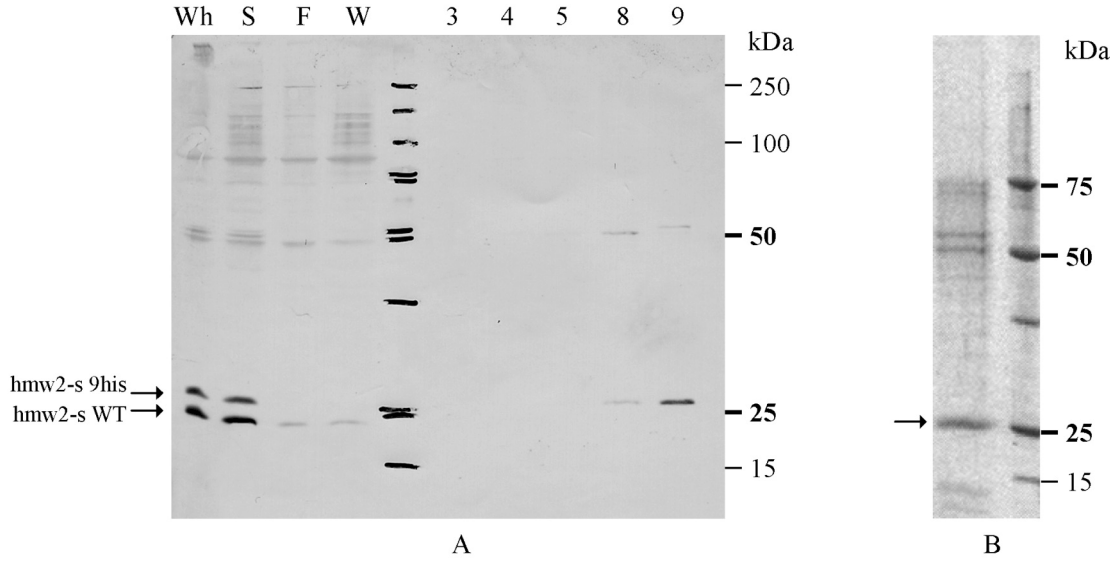

Fig. 4.13: A) Western blotting of different fractions from the purification of HMW2-s (12,5% SDS-PAA gel). Wh = whole cell extract; S = supernatant; F = flow through; W = wash; 3-5; 8-9 = number of collected fraction. (α-HMW2-Cterm, 1:1000)
B) 12.5% SDS-PAA gel of the purified HMW2-s, blotted on PVDF membran and stained with Coomassie Blue. The arrows point to HMW2-s.

However, the Edman sequencing was not successfull, despite optimizing the isolation procedures, repeating the experiments several times, and giving the protein to two different laboratories for an Edman sequencing. We concluded that the N-terminus of this HMW2-s might be chemically blocked.

4.2.2 Construction, expression and purification of three recombinant derivatives of HMW2-s and their application as size markers for HMW2-s

As an alternative method, I tried to determine the N-terminus of HMW2-s by mass spectrometry (MS), but the predicted N-terminal region for HMW2-s contained many arginines (R) and lysines (K), which caused the productions of too many small peptide fragments that could not be analyzed by mass spectrometry (MS). The molecular mass of HMW2-s is about 25,000 Da, as determined by its migration in a SDS-PAA gel. Three different recombinant HMW2-s-like proteins was synthesized. They had a predicted molecular weights of 22,927 (23,750); 24,692 (25,515) and 27,888 (28,711), the number in the bracket shows a predicted molecular weight after adding 6×His tag to their C-termini. I will call them throughout my thesis 24 kDa, 26 kDa and 29 kDa, respectively. Their C-termini were kept constant but the N-termini were different (Fig. 4.14). We expected one of them should migrate very close to the HMW2-s. A comperison of

migration of the recombinant proteins and of HMW2-s should give a rather precise information on the N-terminus of HMW2-s. One difference between these recombinant proteins and the HMW2-s is that the HMW2-s derived from the experiment in chapter 4.1.2 contained nine Histidines at its C-terminus. This caused a difference of about 412 Da because of the additional three Histidines.

MPYPYPWFYPQQKQEDSSNQRHLFEQQLQRMQQRYENELTELR1
MLPPPLFKNGNDMNYPYPYPWFYPQQKQEDSSNQRHLFEQQLQRMQQRYENELTELR2
MAVEQQYQAETFLKTRNADLEKNDNKHLPPPLFKNGNDMNYPYPYPWFYPQQKQEDSSNQRHLFEQQLQRMQQRYENELTELR3

Fig. 4.14: Amino acid sequences of the N-terminal regions of the three recombinant HMW2-s-like proteins. The molecular mass of HMW2-s is about 25,000 Da according to its migration in a SDS-PAA gel. Positions of a possible trypsin cleavage are marked in pink and blue: K = Lysin, R = Arginin., 1 = recombinant protein 24 kDa, 2 = recombinant protein 26 kDa, 3 = recombinant protein 29 kDa.

Genes coding for these three recombinant proteins were amplified by PCR and cloned into *Nco*I/ *Bgl*II site of vector pQE60, which added the sequence for a His-tag (6×histidines) to the C-terminus of a cloned gene. *E. coli* strain XL1-blue (Stratagene) were transformed with the three gene constructs. Their expression was under control of a *lac* operon and therefore inducible with IPTG. After adding IPTG to the *E. coli* culture, the bacteria were further incubated and harvested after 4 hours. The expression of fusion proteins was monitored by SDS-PAGE (Fig. 4.15).

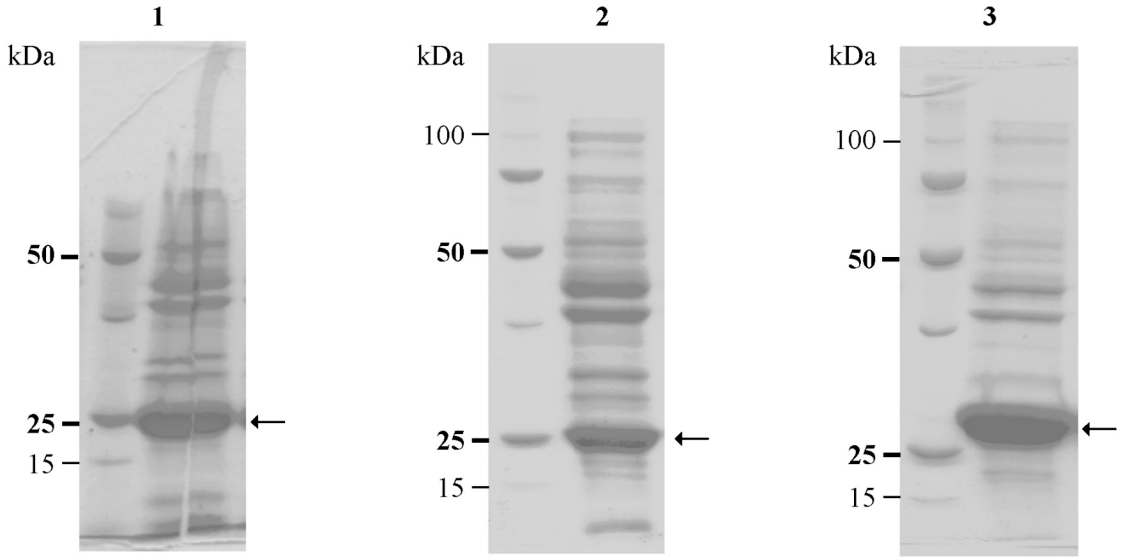

Fig. 4.15: Coomassie Blue stained gels (12.5% SDS-PAA gel) of total protein extract of *E. coli* containing three recombinant proteins. 1 = recombinant 24 kDa, 2 = recombinant 26 kDa, 3 = recombinant 29 kDa.

All three fusion proteins were expressed at a very high level, which facilitated their purification and MS-analysis. They were purified by IMAC using a nickle-charged resin under denaturing conditions (6 M guanidium hydrochloride). The migration of the purified fusion proteins was compared with each other and the natural HMW2-s by SDS-PAGE and Western blotting, respectively (Fig. 4.16). The recombinant 24 kDa protein migrated similarly as HMW2-s, indicating that the N-terminus of both proteins were not far apart.

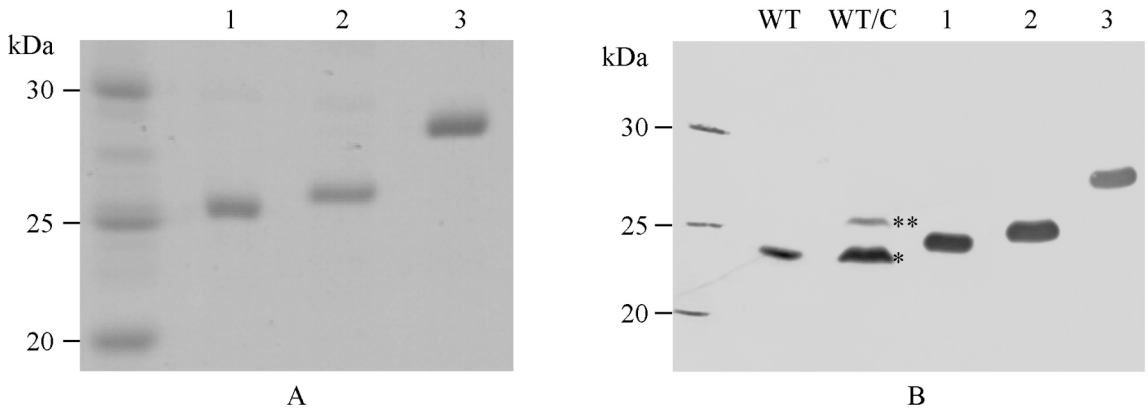

Fig. 4.16: (A) Coomassie Blue stained SDS-PAA gel of the three different recombinant proteins. **(B)** Western blot of three recombinant HMW2-s-like proteins and a new recombinant HMW2-s protein containing the same sequence as the His-taggd HMW2-s from *M. pneumoniae*; 1 = recombinant protein 24 kDa; 2 = recombinant protein 26 kDa; 3 = recombinant protein 29 kDa, WT/C = *M. pneumoniae* WT transformant with his-tagged HMW2-s , WT = *M. pneumoniae* WT, * = wild-type HMW2-s, ** = 9x his-tagged HMW2-s.

4.2.3 Mass spectrometry (MS) analysis of the obtained peptide fragments

The three recombinant HMW2-s proteins and the purified His-tagged HMW2-s from *M. pneumoniae* were analyzed by MS (Dr. T. Ruppert, ZMBH, Heidelberg). The mass spectrometric analyses were done by A. Bosserhof and Dr. T. Ruppert. The signal from the His-tagged HMW2-s isolated from *M. pneumoniae* was relatively weak when compared to the three recombinant HMW2-s-liked proteins. However, one peptide fragment which did not correspond to a predicted fragment derived from tryptic proteolysis was observed (Fig. 4.17). An additional variation of this peptide fragment was also found and had been identified as an oxidized species of this peptide fragment by MALDI-TOF (matrix-assisted laser desorption ionization–time-of-flight) mass spectrometry. The oxidized peptide fragment (* red) appeared as a slightly later peak in the MS compared with the authentic peptide fragment (* black) (Fig. 4.17D).

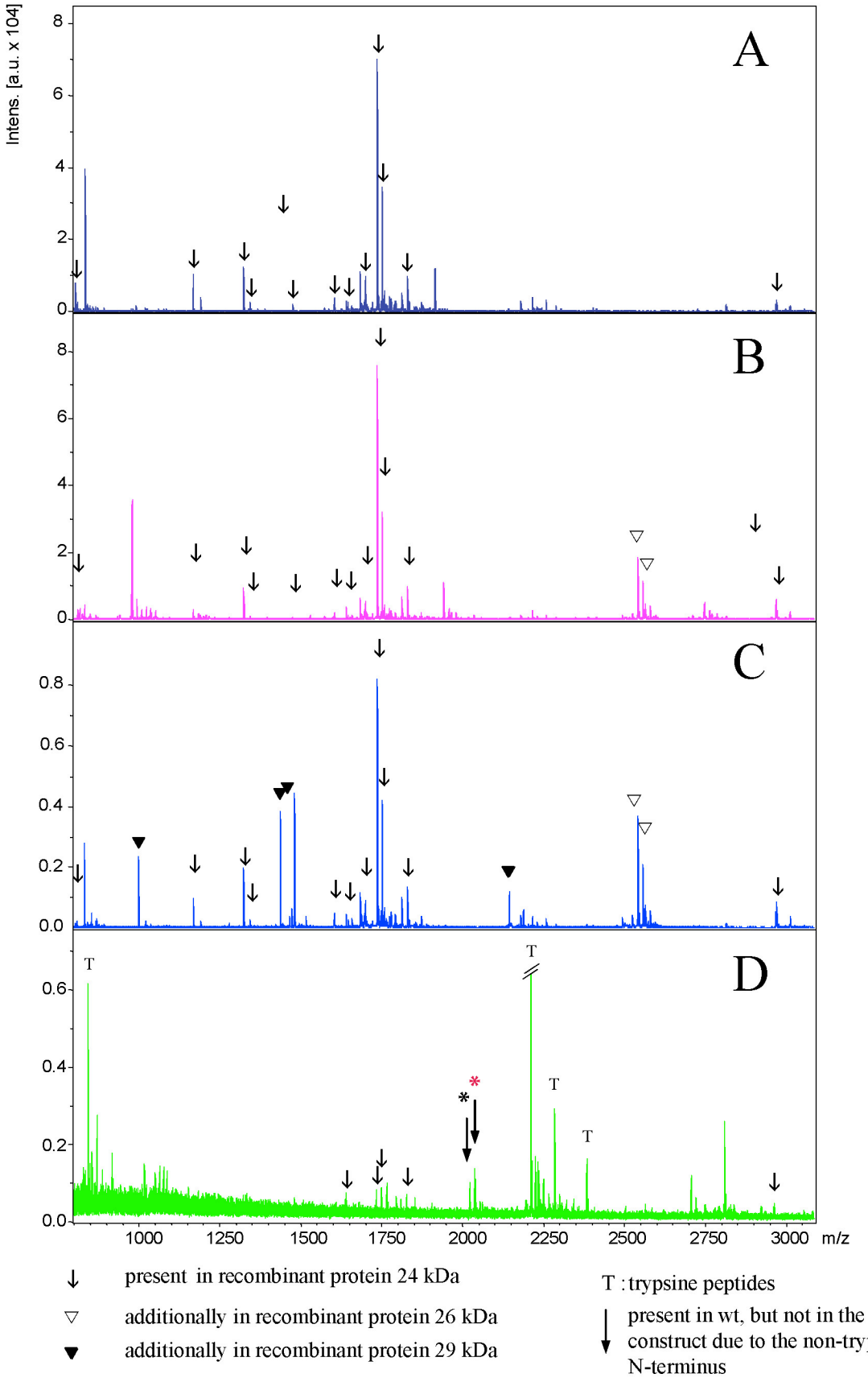

Fig. 4.17: Mass spectrogram of all recombinant proteins.
A = MS of recombinant 24 kDa; B = MS of recombinant 25 kDa;
C = MS of recombinant 29 kDa; D = MS of the His-tagged HMW2-s from *M. pneumoniae* extract.

The oxidation site has been determined to be the N-terminal methionine residue by sequencing of this peptide fragment by MALDI-TOF mass spectrometry, where the methionine was oxidized to methionine sulfoxide. The presence of methionine sulfoxide is commonly observed in gel electrophoresis [Person et. al, 2001]. Signals for peptides containing a single partially oxidized methionine residue will appear in the mass spectrum at mass values corresponding to both the protonated non-oxidized molecular weight (MH^+) and shifted by +16 u mass ($+M_{ox}$) [Person et. al, 2001]. An oxidized methionine in this peptide fragment indicated that the N-terminus of HMW2-s is methionine (4.18).

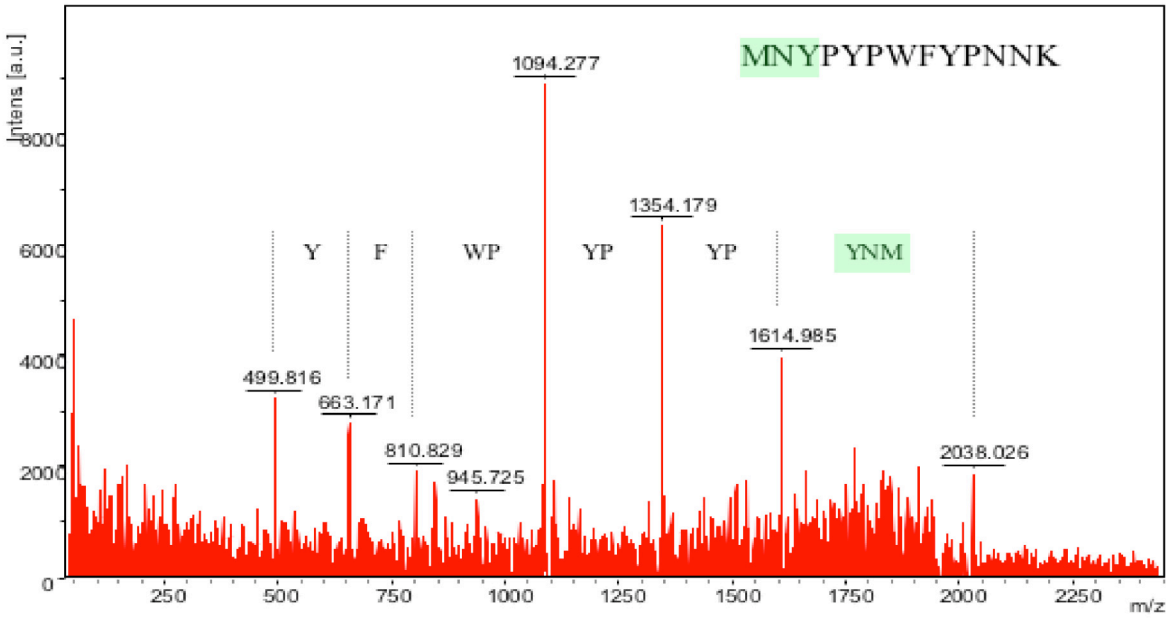

Fig. 4.18: The peptide fragement containing oxidized amino acid from His-tagged HMW2-s from *M. pneumoniae* (see Fig. 4.17 D, * red) was sequenced. Mass spectrogram shows amino acid sequence of this peptide fragment.

Similar pattern of peptide fragments were observed analyzing the three recombinant HMW2-s proteins (see Fig. 4.17). The sequence of the recombinant 24 kDa protein was almost identified with the sequence of HMW2-s from *M. pneumoniae*, except two amino acids were missing (Fig. 4.19).

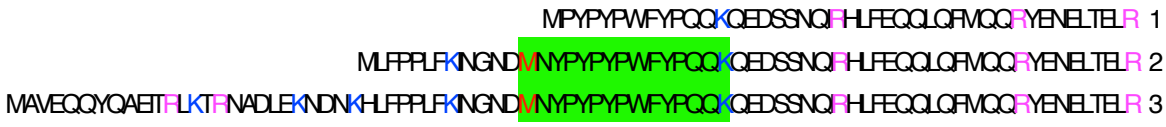

Fig. 4.19: Amino acid sequences of N-terminal regions of the three recombinant HMW2-s-like proteins. The molecular mass of HMW2-s is about 25 kDa according to its migration in a SDS-PAA gel. Positions of a possible trypsin cleavage are marked in pink and blue.The peptide fragment of the N-terminus found in MS-analysis is marked in green. K = Lysin ; R = Arginin. 1 = recombinant protein 24 kDa 2 = recombinant protein 26 kDa 3 = recombinant protein 29 kDa.

4.2.4 Comparison of the peptide fragment patterns between the His-tagged HMW2-s from *M. pneumoniae* and recombinant HMW2-s protein of the same size

To prove the result from the MS analysis and to determine unambigously the N-terminus of HMW2-s, a new recombinant protein was constructed, which should have exactly the same sequence as the His-tagged HMW2-s from *M. pneumoniae*. This new recombinant protein was prepared as described above (see 4.1.2). It was expressed in *E. coli* at a very high level. By MS analysis, it showed exactly the same peptide fragment pattern as the His-tagged HMW2-s from *M. pneumoniae* but with stronger signal (Fig. 4.20). This confirmed that the N-terminus of the His-tagged HMW2-s, which was isolated from *M. pneumoniae*, was correct. Therefore, the molecular weight of the native HMW2-s (without His tag) is 23,204.

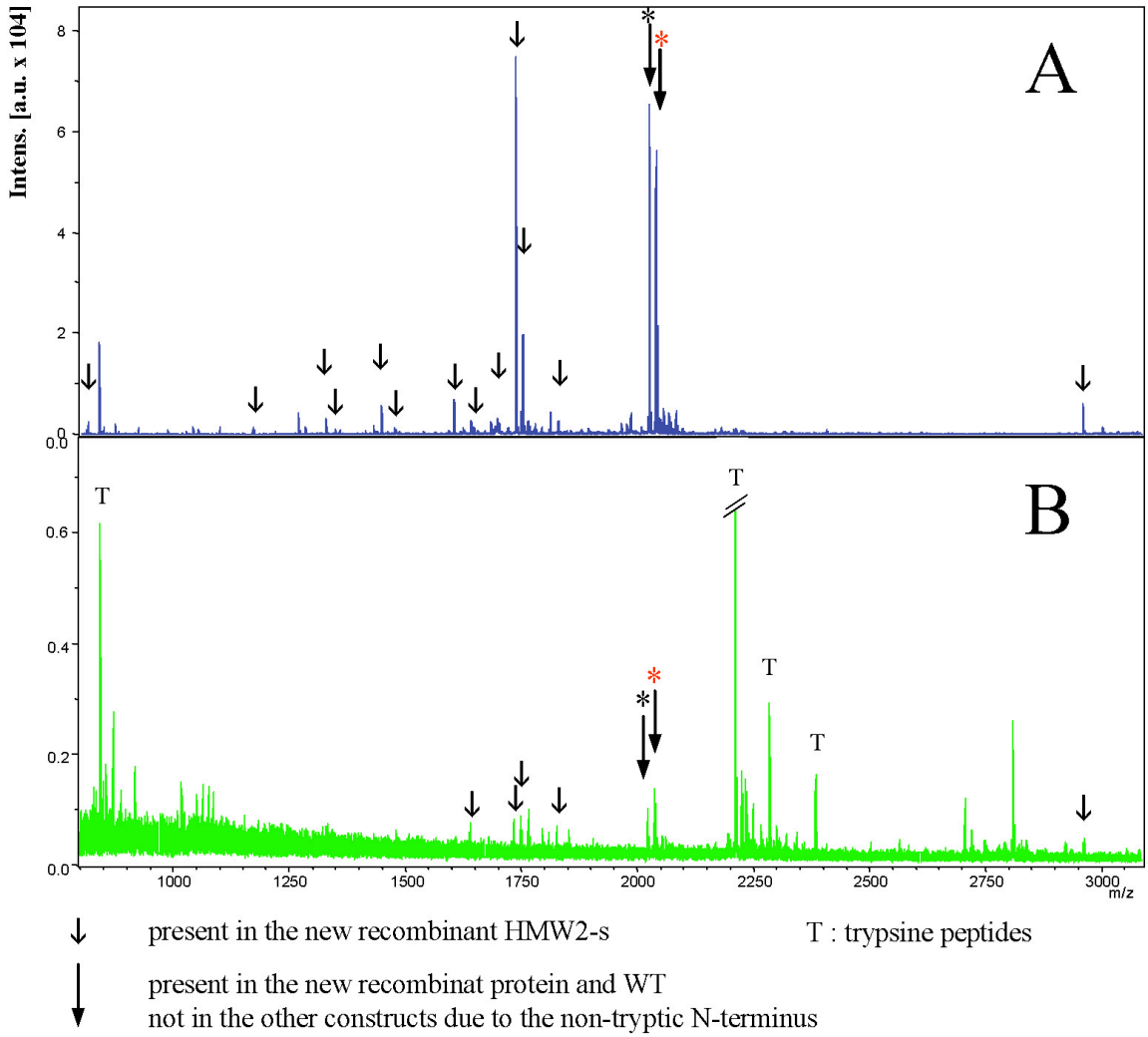

Fig. 4.20: MS analysis of the new recombinant (A), it showed exactly the same peptide fragment patterns as the His-tagged HMW2-s from *M. pneumoniae* (B) but with stronger signal.
* = the oxidized peptide fragment. * = the authentic peptide fragment.

4.3 In vivo synthesis of HMW2 s -

As described in chapter 4.2, the N-terminus of HMW2-s was identified. It starts with a methionine, which suggested that HMW2-s is produced by an internal new start rather than by proteolytic cleavage. To answer this question precisely, the following constructs of the gene *hmw2-s* were made: *hmw2-s* with 300 bp nucleotide upstream of the identified ATG start codon and the same construct with a modified (ATT) internal start codon. Both constructs contained a sequence coding for mRFP and a 7×His tag at their 3' end (Fig. 4.21).

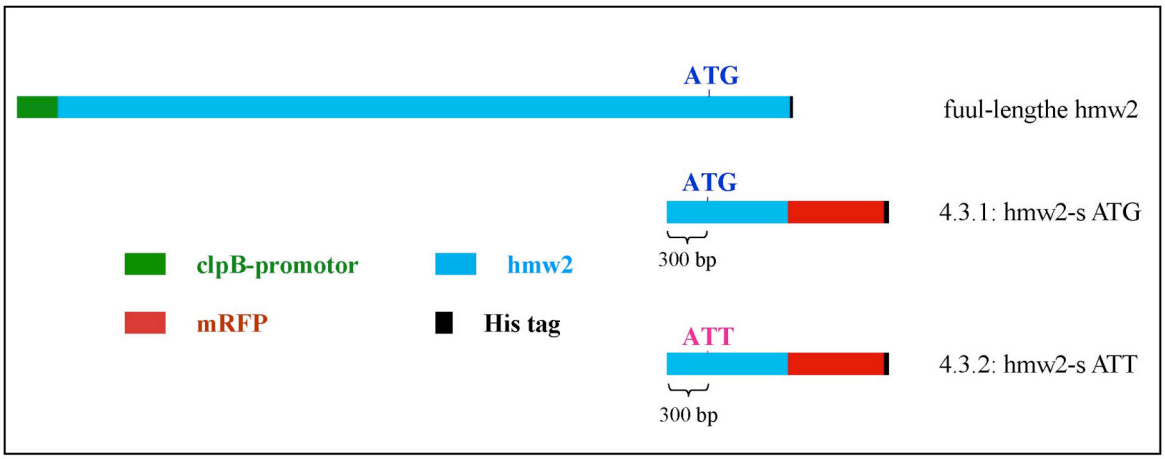

Fig. 4.21: Schematic illustration of the *hmw2-s* gene constructs described in this chapter. For comparison, the complete *hmw2* gene is added.

4.3.1 Construction of a HMW2-s fusion protein

If HMW2-s can be synthesized independently from the complete HMW2, then only the corresponding DNA sequence at the 3' end of the gene *hmw2* should be sufficient to encode HMW2-s. Therefore, a sequence coding for HMW2-s with additional 300 bp upstream from the start codon of HMW2-s was cloned into a *Bam*HI / *Sma*I site of pMT-Red vector (Fig. 4.22). Those 300 nucleotides were chosen as expression unit for the HMW2-s. The pMT-Red vector is a modified mini transposon, which can be used as carrier for integrating foreign DNA into the genome of *M. pneumoniae.* It provided a gene coding for mRFP (a monomer form of red fluorescence protein) followed by a 7×His tag, so that the complete gene construct contained a sequence coding for the *hmw2-s* and an expression unit consisisting of 300 bp upstream of *hmw2-s* and the *mrfp* gene modified with a 7×*his* tag at its 3' end. The modified *hmw2-s* was integrated into the genome of *M. pneumoniae* WT by transposon insertion.

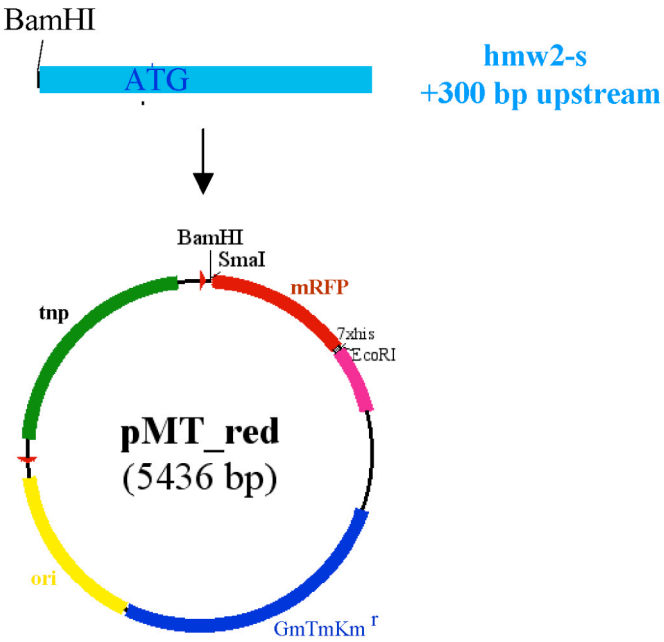

Fig. 4.22: Schematic illustration of cloning the artifical *hmw2-s* gene with additional 300 nucleotides upstream from its start codon. The gene was cloned into the *Bam*HI/ *Sma*I site of pMT-Red vector.

The expression of HMW2-s was monitored by Western blotting. A protein of about 52 kDa was observed in all transformants, which reacted positively with poly-His antiserum (Fig. 4.23). The observed molar mass of 52 kDa agreed very well with the expected molecular weight (49,391) of the fusion protein consisting of HMW2-s, mRFP and a 7×His tag.

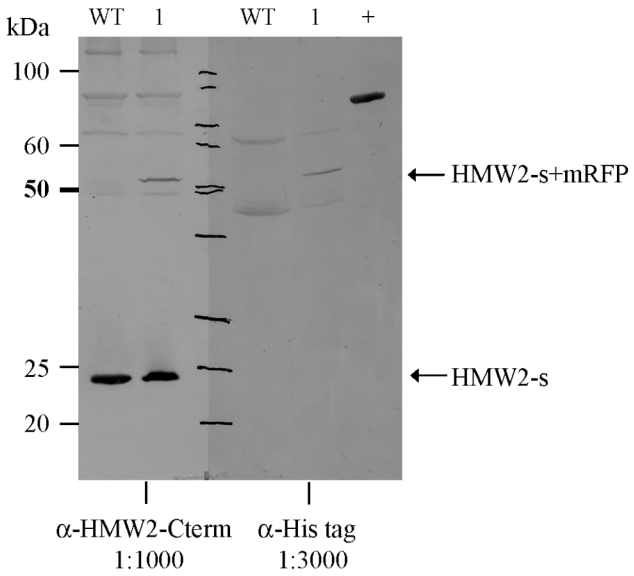

Fig. 4.23: Western blot analysis of total protein extracts of the selected *M. pneumoniae* WT transformant haboring a *hmw2-s* gene construct (12.5 % SDS-PAA gel). Name of antisera and the dilution used are given below the picture.Expected protein bands are indicated by arrows.
WT = *M. pneumoniae* WT, 1 = transformant of *M. pneumoniae* WT haboring *hmw*2-s gene construct; + = positive control (a fusion protein with His tag).

The results of this analyses showed that HMW2-s can be synthesized under *in vivo* condition without the sequence of gene *hmw2* and verified the hypothesis of an internal new start within complete gene *hmw2*.

4.3.2 Construction of a HMW2-s fusion protein with a modified (ATT) internal ATG start codon

As a negative control for the previous experiment (4.3.1), a modified *hmw2-s gene* was synthesized. It contained exactly the same gene fragment as described in Fig. 4.22. The only difference was that the internal ATG start codon was changed into ATT. For this aim, the plasmid pQE60 haboring an insert of the gene *hmw2* was used for further genetic manipulations. The next step was to mutate of the ATG start codon for HMW2-s, which is located at codon position 1620 and change it to ATT. This was done by PCR mediated site-directed mutagenesis, by which the desired mutation is incorporated in the primers used for the PCR amplification. These primers are generally 25-35 bases long and carry the nucleotide which has to be changed in the middle of their sequences (Fig. 4.24). The 'mutation' was finally incorporated into both DNA strands during PCR-cycles.

Fig. 4.24: Schematic illustration of the primers used in mutagenesis-PCR. The primers bind complementary at the gene hmw2 and contain the desired point mutation in the middle of their sequence.

This plasmid containing the modified gene *hmw2* with ATT codon instead of ATG was then used as template for the PCR of *hmw2-s.* The PCR product containing a sequence coding for HMW2-s and additional 300 bp upstream from its modified (ATT) start codon of HMW2-s was cloned into the *Bam*HI/ *Sma*I site of pMT-Red vector. The modified *hmw2-s* was integrated into the genome of *M. pneumoniae* WT by transposon insertion. It was shown by Western blot analysis of the total mixture of all transformants that an HMW2-s fusion protein was not produced (Fig. 4.25A), although the modified *hmw2-s* was integrated in genome of *M. pneumoniae* (Fig. 4.25B), which was confirmed by Southern blot analysis. Genomic DNAs of the transformants were digested with the restriction enzyme *Xmn*I, seperated on 1% agarose gel and transferred overnight to a nylon membrane. The membran was incubated with a Digoxygenin-labeled probe of the *mrfp* gene. The blot showed that the transformants contained the gene construct (Fig. 4.25B).

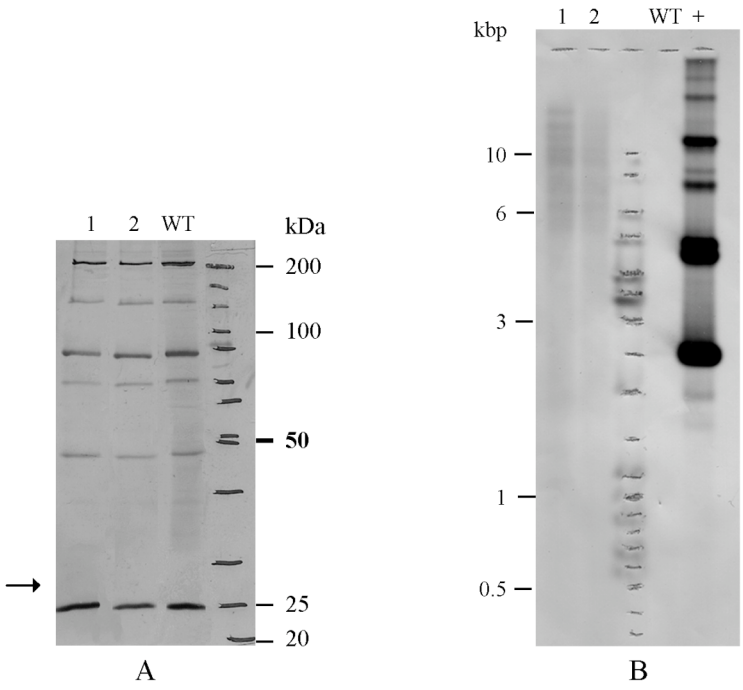

Fig. 4.25: A) Western blot analysis of total protein extracts of the *M. pneumoniae* WT mixture transformants haboring a *hmw*2-s gene construct with a modified (ATT) start codon (1 and 2). Expected protein bands are indicated by arrows.
B) Southern Blot analysis of the mixture transformants of *M. pneumoniae* WT. A DIG-labeled PCR product of *mrfp* was used for detection. WT = *M. pneumoniae* WT;
1-2 = transformants mixture + = positive control (pMT-red)

As expected for the analysis of a mixture of transformants, many different DNA fragments were showed and not a single band. Since the DNA containg the modified *hmw2-s* gene was proven to be correct by sequencing, we concluded from the negative results of the Western blot, that the methionine at position 1620 is indeed crucial for the synthesis of HMW2-s. The experimentally identified translation products of *hmw2* and its truncated derivatives, *hmw2-s* (+ATG) and *hmw2-s* (-ATG) are summarized in Fig. 4.26.

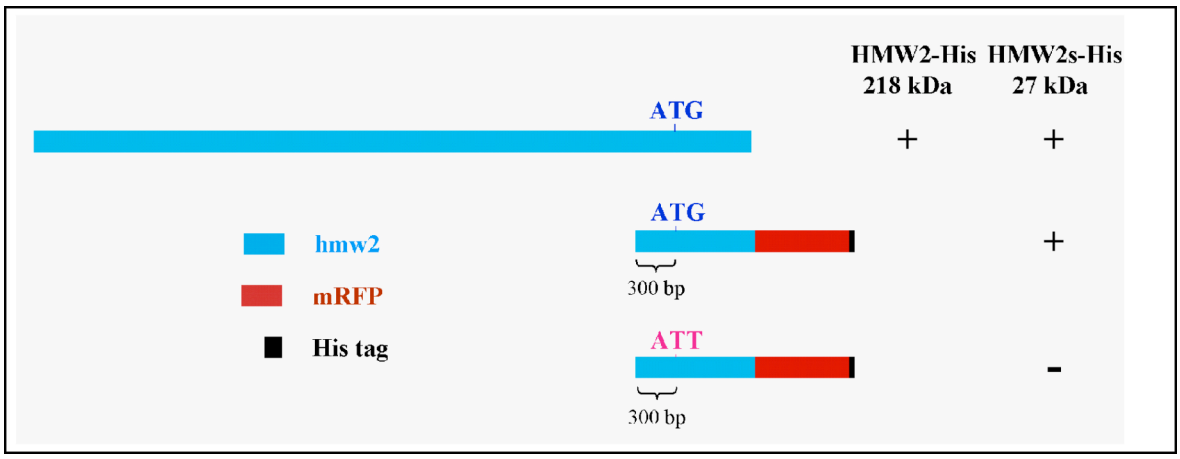

Fig. 4.26: Schematic drawing of the *hmw2* constructs used for the analysis of HMW2-s and the recording of the identified proteins.

4.4 Characterization of the *M. pneumoniae* mutant A3

The hmw2 gene was identified as an important genetic locus, P65 operon, associated with *M. pneumoniae* cytadherence by transposon mutagenesis with Tn*4001* [Krause et.al,1997]. It contains four genes in the following order: MPN309 (*orfp65*), MPN310 (*hmw2, previously orf216*), MPN311 (*orfp41*), and MPN312 (*orfp24*)* flanked by a promoter-like and a terminator-like sequence (Fig. 4.27). MPN310 encodes the protein HMW2. Each transposon insertion mapped to *orfp216 (hmw2)* affected the level of expression of all genes of the P65 operon.

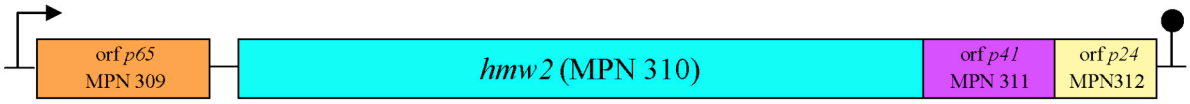

Fig. 4.27: Organization of the *Mycoplasma pneumoniae* P65 operon.

Six mutants with defects in gene *hmw2* were isolated, analyzed and their sites of insertion into the *M. pneumoniae* genome identified [Krause et.al,1997]. The *M. pneumoniae* A3 used (in this work) was kindly provided by Prof. Krause (University of Georgia, USA). To confirm the identity of the mutant and to characterize it in more detail the following experiments have been done: 1) determination of the site of insertion of Tn*4001* within MPN310; 2) analysis of turnover of proteins which might interact with HMW2 by Western blotting and comparative two-dimensional (2-D) gelelectrophoresis.

4.4.1 Determination the site of insertion of Tn*4001* in the genome of the *M. pneumoniae* mutant A3

The site of insertion was analyzed by cloning and sequencing the DNA fragment carrying the transition between either end of Tn*4001* and the genomic DNA. For this purpose, genomic DNA of *M. pneumoniae* A3 was restricted with restriction endonuclease *Hind*III. This enzym cuts the IS-element (IS*256*L) at nucleotide position 1027. The restricted genomic DNA was first ligated into the vector pBC, which was linearized with *Hind*III, and the ligation products used to transform *E. coli* strain TOP10. The obtained colonies were transfered to nitrocellulose membranes and screened for positive clones by hybridizing the

* The names in brackets refer back to the nomenclature [Krause et. al, 1997], while the capital letters MPN indicate the gene numbers according to the re-annotation of the *M. pneumoniae* genome sequence [Dandekar et.al, 2000].

membranes at 60 °C for 16 hours with a DIG-(Digoxygenin) labeled PCR product (1234 bp) of the IS-element (IS*256*L). Plasmids of the clones showing a positive signal were analyzed with *Hind*III. The clones, which contained only one *Hind*III-fragment besides the vector were sequenced with the primers 7221 and 8050. The sequence analysis revealed an integration site at genome position 370306-370307 within the *hmw2* gene (gene position 4839-4840), confirming that we are indeed working with *M. pneumoniae* A3 (Fig. 4.28).

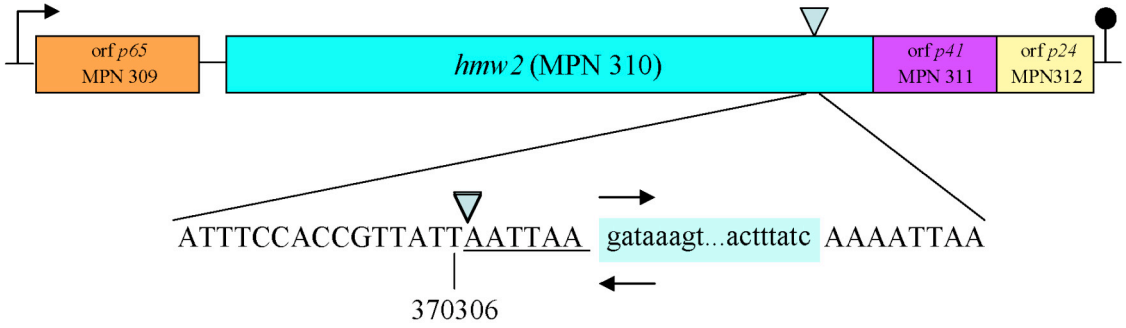

Fig. 4.28: Location of Tn*4001* insertion in *M. pneumoniae* A3. Triangles point to Tn*4001* insertions. The nucleotide sequences at both junctions of mycoplasma and Tn*4001* DNA were examined. Uppercase letters correspond to the sequence of gene *hmw2*, while the nucleotide number corresponding to the junction site in genom of the *hmw2* mutant is indicated (370306). Lowercase letters in the blue box correspond to the nucleotide sequence of IS*256*L of Tn*4001*. The arrow above the box show the direction of the IS*256*L. The 6-bp doublication of genomic DNA, the consequence of the transposon insertion, is underlined.

4.4.2 Comparison of the expression profiles of selected genes in *M. pneumoniae* WT and *M. pneumoniae* mutant A3 by Western blot analysis

It has been shown previously that in *M. pneumoniae* A3, HMW1 and HMW3, when compared with WT, appeared in lower concentrations [Krause et al., 1982]. This observation was explained by a reduced half life of these proteins in the absence of HMW2. In this present work, I extended analyses to such proteins, which, based on the result of the two-hybrid analysis (for detail see next chapter), could be possible candidates for interacting directly with HMW2. The relative difference in concentration of the following proteins (table 4.1) in whole protein extracts of *M. pneumoniae* WT and A3 were determined by evaluating the signal intensities of specific protein bands in Western blots (Fig. 4.29) with the program Visual Grid. It allowed manual adjustment for the grid position overlayed on the images. It gave intensity values of the measured spots, so that I could analyze relative quantitative changes of individual protein spots based on their intensity value (Fig. 4.30).

Table 4.1: List of antisera used in Western blot analysis of *M. pneumoniae* WT and A3

MPN number	gene/protein	length of recognized amino acid (aa)	Position of recognized amino acid (aa)	Antiserum Number	dilution
MPN227	EF-G	82	-	66708	1:3000
MPN310	HMW2-Cterm	320	1459-1778	88256	1:1000
MPN310	HMW2-N573aa	573	1-573	MX-94	1:5000
MPN447	HMW1	122	135-257	84267	1:5000
MPN452	HMW3	118	44-162	65376	1:5000
MPN309	P65	18	167-185	66578	1:3000
MPN426	P115	13	971-982	35	1:1000
MPN297	H10_orf 149	150	1-150	MX-95	1:5000
MPN311	P41	254	49-302	86630	1:500
MPN312	P24	126	74-199	85952	1:250
MPN141	P1	68		45790	1:1000
MPN142	P90	223		43558	1:1000
MPN142	P40	146		42326	1:1000
–	Triton X-100 insoluble Fraction	–	–	60631	1:5000

Elongation factor G was used as a measure for equal protein concentration in both protein extracts. The protein HMW1, HMW3 and the P65 operon-encoded proteins P65, P41, P24 were included in this analysis as controls for proteins which were already known to be differently expressed in *M. pneumoniae* A3 [Krause et al., 1982, 1997].

Total protein extracts of *M. pneumoniae* WT and A3 were separated in 12.5%, 10% or 7.5% SDS-PAA gels, depending on the expected size of the proteins, transferred to nitrocellulose membranes and tested with the corresponding monospecific antisera (Table 4.1). The concentrations of the extracts were adjusted, so that the same amount of protein was always compared. As internal standard, the elongation factor G was included in the analyses. The almost identical signals indicated that the protein concentration in both extracts were very similar (Fig. 4.29).

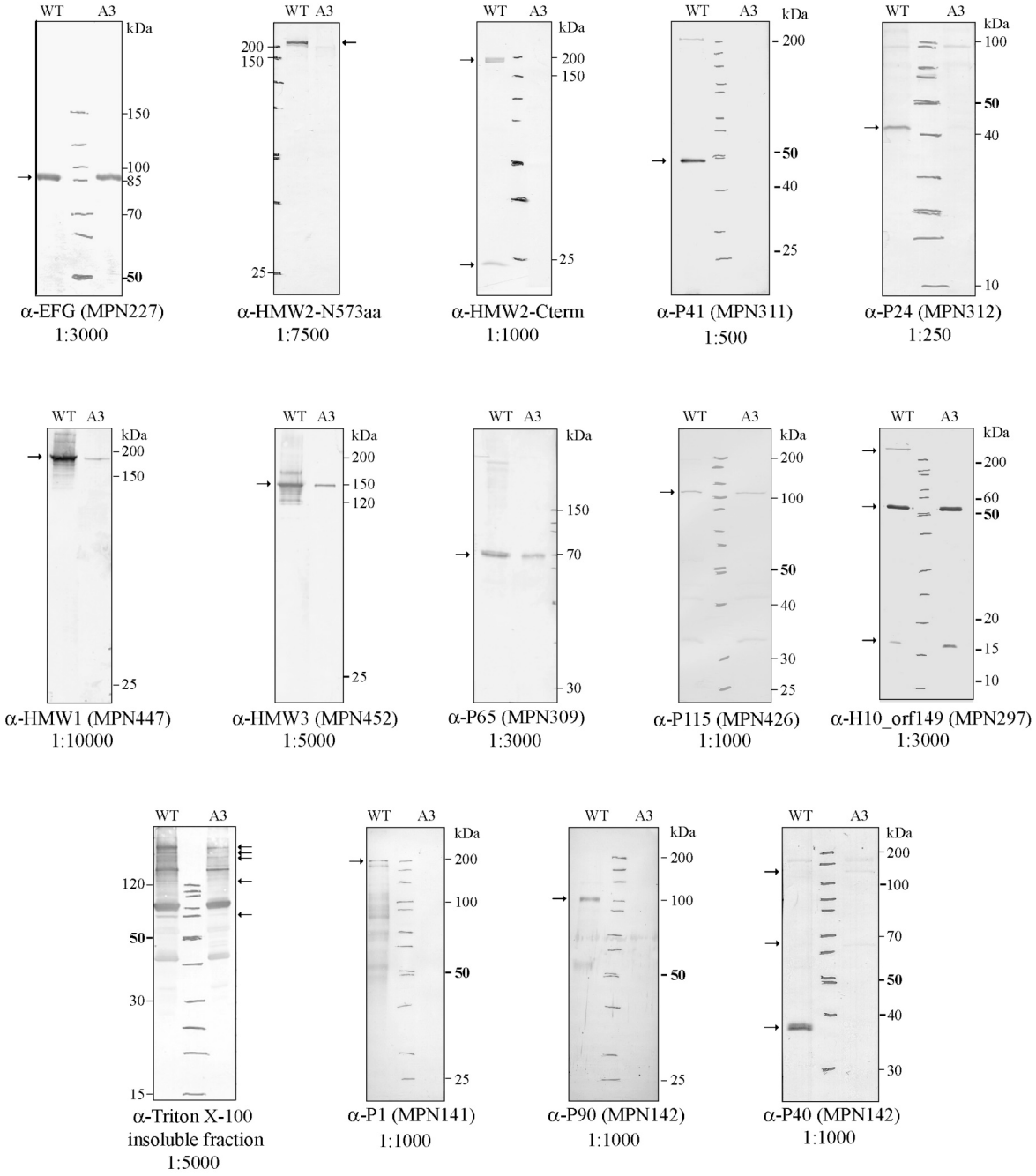

Fig. 4.29: Western blot analyses of expression profiles of selected genes in *M. pneumoniae* WT and *M. pneumoniae* A3. Names of antisera and dilution used are given below each blot. Expected protein bands are indicated by arrows.

The gene *hmw2* encodes two proteins, one with the molecular mass of about 216 kDa and another one of about 25 kDa. The latter one is synthesized from an internal AUG codon in the same reading frame as HMW2 (For details see chapter 4.2). The antiserum which was made against the C-terminal end of HMW2 [Proft, 1994] recognized the complete HMW2

and another protein of about 25 kDa. Both protein bands were not present in extracts of *M. pneumoniae* A3 (Fig. 4.29, upper panel).

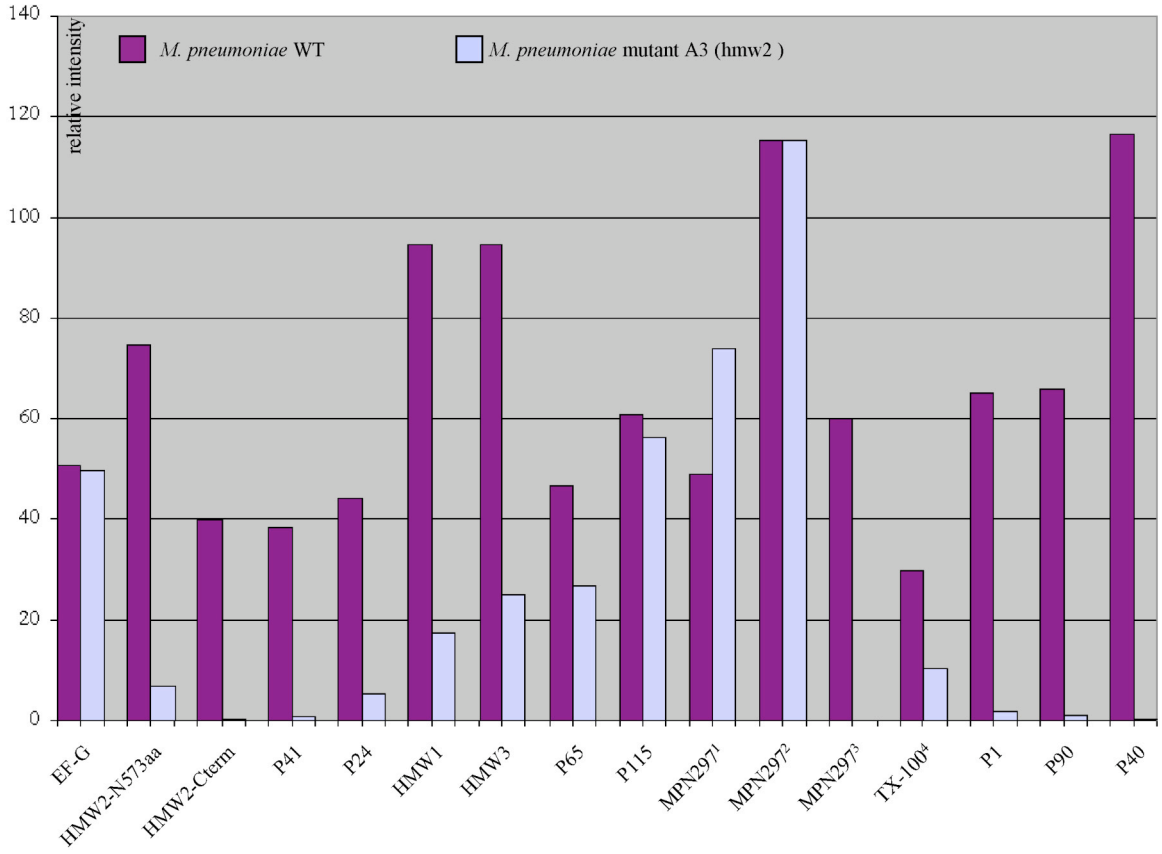

Fig. 4.30: Summary of the intensity values of selected protein from M. pneumoniae WT and A3. Fourteen antisera were used in this experiment (Table 4.1). 1 = monomer, 2 = trimer, 3 = multimer, 4 = average value of signal intensity of five selected protein bands (Fig. 4.29, arrows)

Using antiserum directed against the N-terminal region of HMW2, a very faint protein band with a slightly reduced molecular mass compared to wild type HMW2 was observed (Fig. 4.29, upper panel). This reduction was caused by the insertion of Tn*4001* in the gene *hmw2* at gene position 4839-4840 (Fig. 4.28). This led an early termination of the HMW2 protein due to a switch of the reading frame. Annotation of the DNA sequence predicted that the HMW2 of the mutant A3 should be 206 amino acids shorter causing a reduction of molecular mass of about 20 kDa, as was indeed observed in SDS-PAA gels.

The specific antiserum against P41 reacted strongly with a 47-kDa protein in *M. pneumoniae* WT profile (Fig. 4.29, upper panel). The Western blot analyses confirmed the published results on the reduction in concentration or absence of the proteins HMW1, HMW3, P65, P41 and P24 (Table 4.2).

The most significant new results concerned the proteins P1, P40 and P90. These proteins could not be detected indicating that they were rapidly degraded in the absence of HMW2.

These finding support results from two-hybrid experiments (for detail see chapter 4.5), which suggested that P1 and HMW2 interact indirectly with each other.

The results concerning the proteins from the Triton X-100 insoluble fraction and the gene products of MPN297 were not so clear. In *M. pneumoniae* WT, three different forms of the proposed 17-kDa protein of MPN297 were found with molar masses of 17 kDa, 51 kDa and about 200 kDa. The two latter proteins could be multimeric forms of the original gene product. While the proposed monomeric form was prevalent in *M. pneumoniae* A3, the

51-kDa protein, maybe a trimer, was distributed almost equally in both samples, but the 200-kDa protein was only visible in *M. pneumoniae* WT. The interpretation of these result is difficult, but we have here the first example, where a protein that interacts directly with HMW2 (for details see chapter 4.5) is not fast degraded in the absence of HMW2.

Finally, the general comparison of the Triton X-100 insoluble fractions confirmed that some proteins were underrepresented in *M. pneumoniae* A3. But their identity was not further determined.

Table 4.2: Summary of Western blot analyses of expression of selected proteins in *M. pneumoniae* A3

MPN number	gene/ protein	Signal in Western blot	remarks
MPN309	P65	++	P65 operon, upstream from *hmw2*
MPN311	P24	—	P65 operon, downstream from *hmw2*
MPN312	P41	—	P65 operon, downstream from *hmw2*
MPN452	HMW3	+	
MPN447	HMW1	+	interacting partner from two-hybrid pilot experiment (see chapter 5.5)
MPN426	P115	0	interacting partner from two-hybrid pilot experiment (see chapter 5.5)
MPN297	MPN297	0	interacting partner from two-hybrid pilot experiment (see chapter 5.5)
MPN141	P1	—	localizes at tip structure
MPN142	P90	—	localizes at tip structure
MPN142	P40	—	localizes at tip structure

0 = reduction not significant; ++ = slightly reduced; + = strongly reduced; — = not detectable

4.4.3 Comparison of the expression profiles of selected genes in *M. pneumoniae* WT and *M. pneumoniae* mutant A3 by two-dimensional (2-D) gel electrophoresis

It was shown by Western blot analyses that the expression of many proteins was effected in *M. pneumoniae* cells lacking HMW2. Some of those proteins were known to directly interact with HMW2, but also proteins were identified in this study, like P1, P40 and P90, of which changes in protein concentration depending on the presence of HMW2 have not been observed before. To find other possible interacting proteins, 2-D gel electrophoresis was applied to compare changes of protein concentration between total soluble protein extracts of *M. pneumoniae* WT and *M. pneumoniae* A3. Two-dimensional gel electrophoresis was chosen for several reasons: 1) the protein spots of two gels can be compared by image overlay; 2) it represents a global analysis visualizing about 50% of the expressed protein of *M. pneumoniae*; 3) many of the protein spots have been identified and can be traced to their corresponding gene; 4) the analyses are highly reproducible.

For the comparative 2-D gel anlysis, 80 µg of total protein extracts of *M. pneumoniae* WT and A3 were separated in the first dimension by charge using an immobilized pH gradient from 3-10 and then according to their molecular mass in the second dimension by SDS-PAGE. The gels were silver stained for viewing the protein spots. Image analysis was done using Adobe Photoshop software version 5.0 and signal intensities were measured with the program Visual Grid (GPC Biotech AG). It allows manual adjustment for the grid position overlayed on the images. The useful feature of this software is that it calculates intensity values of the gray-level intensity of the spots, so that one can analyze the change of protein spots quantitatively based on their intensity value.

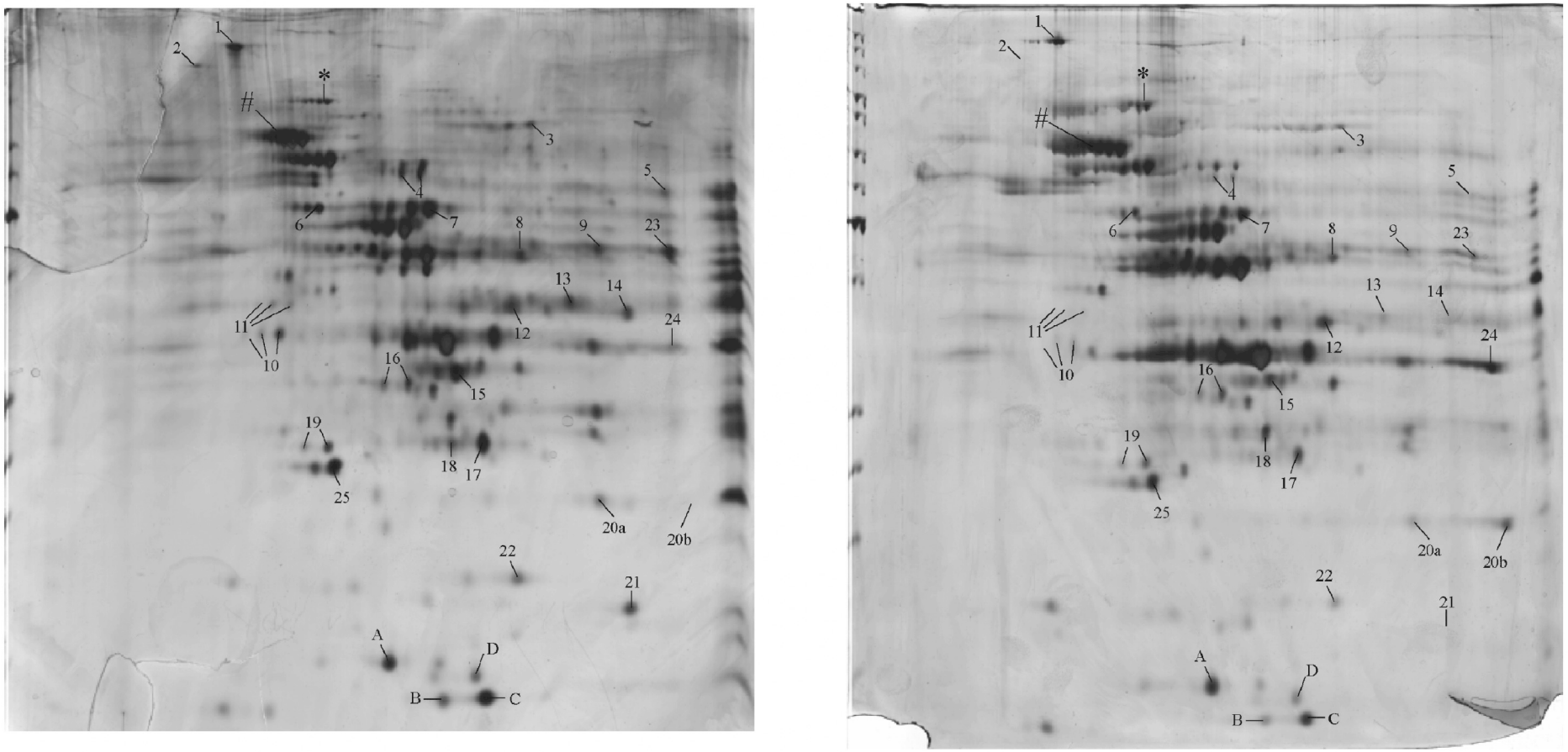

Fig. 4.31: Analytical 2-D gels of *M. pneumoniae* WT and A3. About 80 µg protein extracts were seperated on an immobilized pH gradient from 3 to 10 and a vertical SDS-polyacrylamide gel (12.5%) (20×20 cm). The proteins were silver-stained [Blum, 1987]. Only proteins which were reduced in the mutant A3 are marked and numbered. The assignment of those protein spots to genes were based on data from a previous *M. pneumoniae* proteome analysis [Ueberle et al., 2002,]. The differently expressed proteins are listed in table 4.3.

By overlapping both protein profiles, the difference between both strains could be easily determined. All protein spots with significantly changed signals were marked and numbered (Fig. 4.31). Assignment of those protein spots to gene products were based on data from a previously published *M. pneumoniae* proteome analysis [Ueberle et al., 2002]. All gene products that were reduced in *M. pneumoniae* A3 are listed in table 4.3.

Four protein spots (A, B, C, D) of the 2-D gel of *M. pneumoniae* WT were selected and their intensities compared with same protein spots from the 2-D gel of *M. pneumoniae* A3. These four proteins showed only minor change in their signal intensities and were therefor used as standards to establish the baseline. The factor for the baseline was set at 1.42. If the factor of two spot intensities was above this baseline, the differences in the corresponding protein spots were considered as significant.

From the proteins tested by Western blotting (table 4.2) only the reduction of HMW3 could be confirmed by the 2-D gel approach (Fig. 4.31, spot Nr.2). One could clearly see in the 2-D gels that HMW3 is present in the WT protein extract but absent in the protein extract of *M. pneumoniae* A3. The usefulness of EF-G as internal standard for comparing protein concentrations were also confirmed, since the measured factor was 1.21, which was below the set baseline of 1.42.

From a total of 31 analyzed protein, I identified 12 which had values above the baseline. Most of those were enzymes involved in different metabolic activities (Table 4.3). The possible connection between a decrease in protein concentration and absence of HMW2 will be speculated in the discussion section.

The other proteins from the Western blot analyses (Table 4.2). were not separated well enough or could not be analyzed for technical reasons. One has to consider that proteins with more than three transmembran segments and isoelectric points above 10.5 were not seperated by the conditions applied.

Table 4.3: List of total analyzed proteins by comparative 2-D gel electrophoresis. Proteins with significant difference in concentration in *M. pneumoniae* WT and A3 are shown in Bold. Standard proteins are shown in gray. Factor above 1.42 indicates higher concentration in WT, factor below 0.7 42 indicates higher concentration in A3.

Spot -nr.	Factor[1]	Nr[2]	MPN Nr.	ORF	pI	Mr	Annotation
A	1.02	523	MPN314	F10_orf141b	6.02	16,335	Conserved hypothetic
B	1.42	174	MPN668	K05_orf140	7.12	14,882	Conserved hypothetic
C	1.23	174	MPN668	K05_orf140	7.12	14,882	Conserved hypothetic
D	1.25	217	MPN625	C12_orf141	7.04	15,469	Protein C-like family, inducible by osmotic stress ond organic hydroperoxide
1	1.14	366	MPN474	P01_orf1033	4.79	118,078	Coiled-coil-Protein, probably a structure protein involving in cytoskelet
2	**2.10**	**388**	**MPN452**	**H08_orf672**	**4.51**	**73,721**	**Cytadherence-accessory protein (HMW3)**
3	**1.55**	**73**	**MPN082**	**R02_orf648**	**7.55**	**72,378**	**Transketolase 1 (TklB)**
4	**1.55**	**242**	**MPN600**	**D02_orf518**	**6.54**	**57,374**	**ATP-Synthase**
5	**1.78**	**104**	**MPN050**	**D09_orf508**	**8.82**	**56,591**	**Glycerolkinase (glpK)**
6	1.26	244	MPN598	D02_orf475	5.46	52,237	ATP-Synthase
7	1.25	236	MPN606	C12_orf456	6.53	49,228	Enolase (eno)
8	1.26	411	MPN429	A05_orf409	7.49	44,212	Phosphoglyceratkinase (pgk)
9	**1.56**	**309**	**MPN533**	**G12_orf390**	**8.10**	**43,700**	**Acetatkinase (ackA)**
10	1.39	125	MPN029	B01_orf190	4.95	21,810	Elongationfactor P (efp)-homolog
11	**1.75**	**384**	**MPN456**	**H08_orf1055**	**6.68**	**110,538**	**Conserved hypothetic**
12	1.09	535	MPN302	H10_orf328	7.54	35,989	6-Phosphofructokinase (pfk)

1 = intensity value WT: intensity value A3; 2 = number of the corresponding protein spot on the standard 2-D gel of *M. pneumoniae* WT [Ueberle et al., 2002]

Table 4.3; (continued)

Spot-Nr.	factor[1]	Nr.[2]	MPN Nr.	ORF	pI	Mr	Annotation
13	**1.77**	**211**	**MPN631**	**C12_orf298**	**7.99**	**33,645**	**Elongationfactor Ts (tsf)**
14	**1.89**	**641**	**MPN191**	**GT9_orf327**	**7.43**	**36,661**	**RNA-polymerase alpha-core-subunit (rpoA)**
15	1.34	129	MPN025	B01_orf288	6.89	31,039	Fructosebisphosphat-aldolase (tsr)
16	1.20	455	MPN383	A19_orf282	6.40	31,913	HAD-superfamily hydrolase/phosphatase
17	1.40	647	MPN185	GT9_orf215	7.54	24,255	Adenylatkinase (adk)
18	**1.67**	**92**	**MPN062**	**D09_orf238**	**7.20**	**26,284**	**Purinnucleosid-phospholyrase (deoD)**
19	1.21	314	MPN528	G12_orf184	5.46	21.369	Inorganic pyrophosphatase (ppa)
20a	1.31	361	MPN479	P01_orf197	9.51	21,55	Acylcarrier-proteinphosphoesterase
20b	**0.39**	**361**	**MPN479**	**P01_orf197**	**9.51**	**21,55**	**Acylcarrier-proteinphosphoesterase**
21	**2.50**	**516**	**MPN321**	**F10_orf160**	**8.37**	**18,511**	**Dihydrofolat-reductase**
22	**1.42**	**325**	**MPN517**	**G12_orf166a**	**7.48**	**18,869**	**Probably reductase homolog**
23	**1.67**	**103**	**MPN051**	**D09_orf384**	**9.58**	**42,724**	**Glycerol-3-phosphat-dehydrogenase**
24	**0.62**	**168**	**MPN674**	**K05_orf312**	**8.93**	**33,888**	**L-Lactatdehydrogenase (ldh)**
25	1.12	287	MPN555	H03_orf193o	5.72	22.434	Conserved hypothetic
*	1,21	605	MPN227	G07_orf688	5.52	76,499	Elongation factor G (fus)
#	1.09	406	MPN434	A05_orf595	5.20	65.100	Heat shock protein DnaK

1 = intensity value WT: intensity value A3; 2 = number of the corresponding protein spot on the standard 2-D gel of *M. pneumoniae* WT

4.5 Interaction of HMW2 with other proteins in *M. pneumoniae*

According to our present knowledge, the HMW2 protein plays a central role in the formation of a cytoskeleton-like structure [Krause, 1996]. It seems to be the most important component of the *M. pneumoniae* cytoskeleton [Krause, 1996]. The characterization of non-cytadhering transposon-insertion mutants of *M. pneumoniae* confirmed both the interaction among HMW proteins (HMW1, HMW2, HMW3), especially HMW2, and their involvement in cytadherence [Krause, 1996]. During my diploma thesis, I established the yeast two-hybrid system with the aim to identify interaction partners of HMW2. The gene *hmw2* was divided into four fragments (Fig. 4.32) and individual fragments were used as baits for screening a gene bank of *M. pneumoniae* for binding partners. The *M. pneumoniae* gene bank was cloned in *Saccharomyces cerevisiae* by M. Kögl (RZPD, Heidelberg).

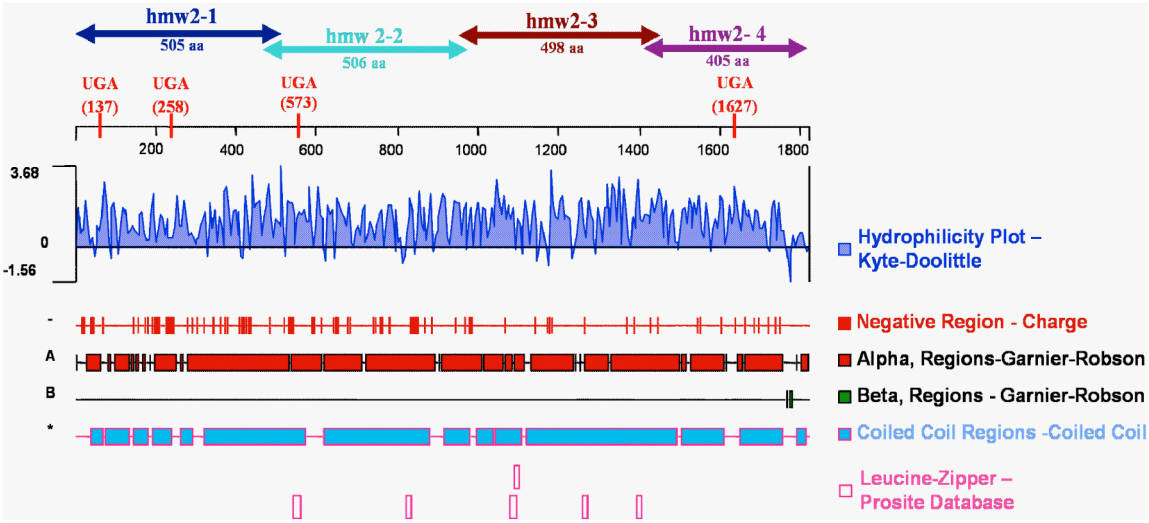

Fig. 4.32: Total profile of the protein HMW2. The positions of the four UGA codons are given in red. For the two-hybrid analysis, the protein was divided into four fragments (named hmw 2-1 to 4). The numbers below the arrows give the size of each fragments in numbers of amino acids.

4.5.1 Results from the two-hybrid screen with HMW2

Two-hybrid screens with HMW2 protein fragments 1 and 2 were not successful because of self-activation of both baits occured (activation of reporter gene without interaction of bait and pray). Positive interactions were found when using HMW2 fragment 3 (HMW2-3) as a bait [Boonmee, 2000]. Possible binding partners found by the two-hybrid screens as binding partners of HMW2-3 are given in Table 4.4.

Table 4.4: The table summarizes the screening results of *hmw2-3* against the *M. pneumoniae* gene bank.

Gene number of interacting protein and ORF name	Length of protein (aa)[a]	Genome position of DNA insert[b]	amino acid position within protein (aa)[c]
MPN 447 HMW1-CTerm.	1018	543786-543369	859-998
MPN 426 P115	982	513876-513361	223-394
MPN 297 H10_orf149	149	351965-352261	11-109
MPN 298 H10_orf119	119	352402-352672	6-96

a) Length of whole protein

b) Fragments of *M. pneumoniae* genomic DNA were cloned randomly into the pray-vector pGAD424. The number shows the corresponding genome positions of DNA fragments found from the screen.

c) Amino acid position of peptide fragments encoded by DNA sequences as shown in column b. They show the interacting region within the whole protein in column a.

4.5.2 Confirmation of two-hybrid screen from the pilot experiment by pairwise tests

To confirm these two-hybrid screen results, selected proteine/ peptide fragments (Table 4.5) were fused to the carboxyl terminus of the Gal4 activation domain (Gal4-AD) and also to the carboxyl terminus of the Gal4 DNA-binding domain (Gal4-BD). The C-terminal region of P1 and HMW2 fragement 4 (HMW2-4) were added in this pairwise test to prove their interaction with other proteins.

Table 4.5: Protein/ peptide fragments used in the pairwise tests.

MPN number	Name of protein/ peptide fragment	Total length of protein (aa)	Length of fragments used in pairwise test(aa)	position of amino acids
MPN 310	HMW2-3	1818	498	944 - 1442
MPN 447	HMW1-Cterm.	1018	399	619 – 1018
MPN 426	P115	983	983	1-983
MPN 297	H10_orf149	149	149	1-149
MPN 298	H10_orf119	119	119	1-199
MPN 141	P1-CTerm.	1606	370	1156-1525
MPN 310	HMW2-4	1818	406	1413-1818

The yeast two-hybrid strain PJ69-4α was co-transformed with plasmids encoding bait and pray fusions of those proteins and the interaction between the fusion proteins was monitored by plating on –LUHA agar (Fig. 4.33). All potential pairwise interactions between those proteins were investigated. They were also tested for non-specific interaction with control plasmids.

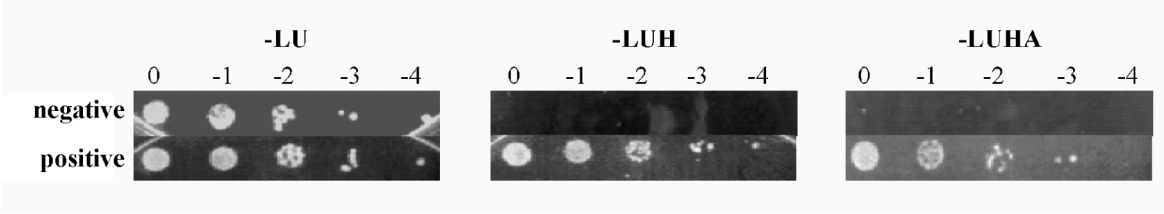

Fig. 4.33: Example of a negative and positive result in a yeast pairwise test. *S. cerevisiae* containing only a bait-(pGBDU) and a pray-(pGAD424) vector was used as a negative control. As positive control, known interacting proteins were used as bait and pray. She2 and She3 proteins were found to interact with each other [Bohl et al., 2000], and therefor were used as positive control in this experiment. Bait vector containing the gene *she2* and a pray vector containing the gene *she3* were co-transformed in *S. cerevisiae* and grown on selective agar medium.

a) MPN310, C-terminal region (HMW2 fragment 3)

Pairwise tests using HMW2-3 as bait and pray are presented in Fig. 4.34. No activation of the reporter genes were observed when HMW2-3 was tested with plasmid vectors containing only GAL4 domains (Fig. 4.34, upper panel). I observed no interaction of HMW2-3 with either P115, the gene product of MPN298, the C-terminal region of P1, HMW2-4 or with itself.

P1 in GAL-AD was auto-active, so that no pairwise test could be done. No yeast clone grew after co-transformation of MPN298 (in GAL4-BD vector) with HMW2-3 (GAL-AD vector), although experiments were repeated and optimized. Therefore, no pairwise test could be done using this gene construct.

However, co-transformation of HMW2-3 with HMW1-C-term and the gene product of MPN297 activated the reporter gene, indicating direct interaction of HMW2-3 with both proteins. The interaction between HMW2-3 and HMW1 was found with HMW2-3 in the GAL-BD vector and HMW1 in GAL-AD vector, which agreed with the results from the previous two-hybrid screen. HMW1 in GAL-BD activated the reporter gene by itself so that it could not be used in a GAL4-based two-hybrid system. Therefore, interaction between HMW2-3 and HMW1 could not be confirmed when proteins were tested with exchanged GAL4 domains.

Co-transformation of HMW2-3 with MPN297 also resulted in an activation of reporter genes. This interaction was observed with HMW2-3 in the GAL4-BD vector and MPN297 in the GAL4-AD vector. By exchanging plasmid, interaction between HMW2-3 and MPN297 could not be confirmed because of a weak auto-activation of MPN297 in the GAL-BD vector (Fig. 4.37, upper panel).

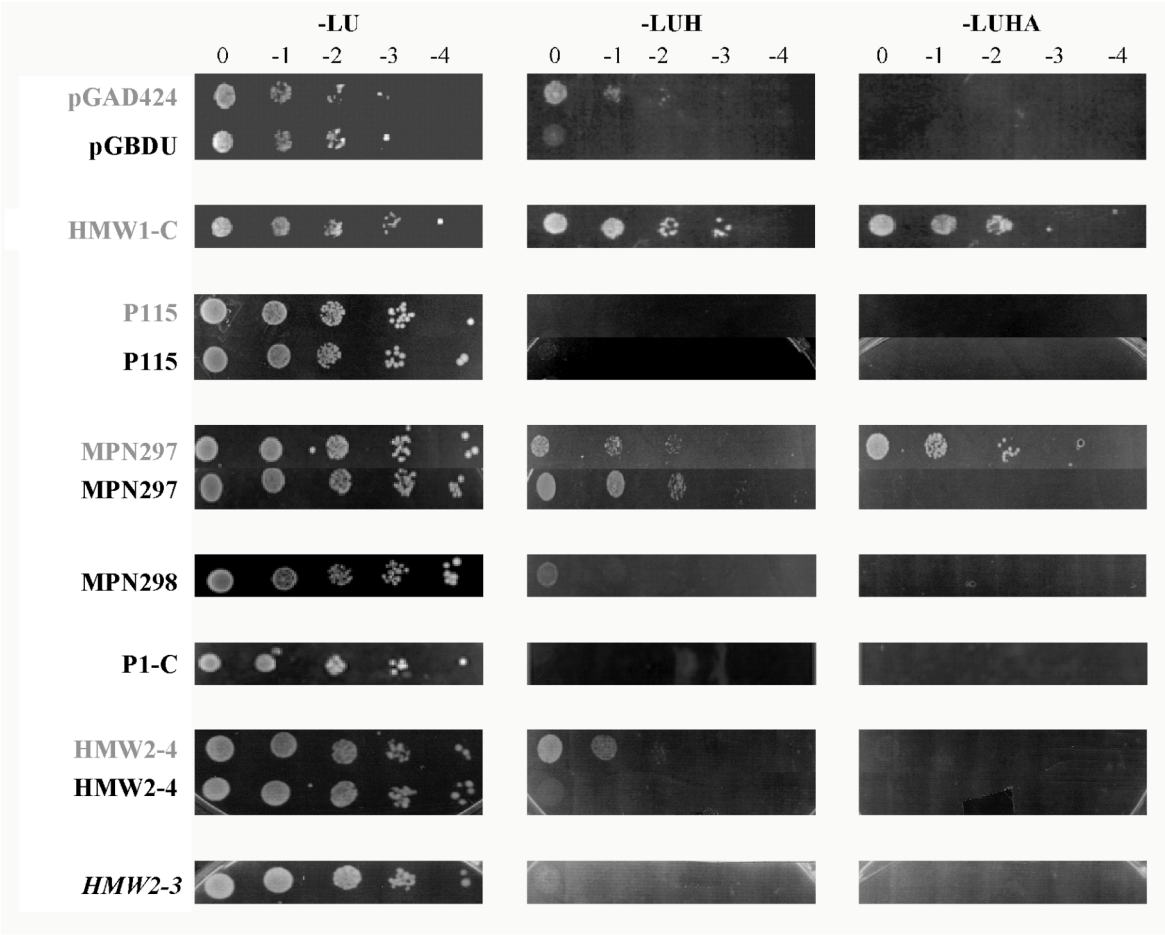

Fig. 4.34: Yeast two-hybrid reporter gene activation in a pairwise test using HMW2-3 as bait and prey. All positive cultures are based on the activation of two independent reporters: His3 and Ade2. Presence of colonies indicates activation of the His3 and Ade2 reporters. Proteins which were tested against HMW2-3 are listed on the left. Constructs in pray vectors are indicated in gray and those in bait vectors are in black. The interaction with itself is shown in *italic*.

b) MPN447 (HMW1-C-terminal region)

Pairwise tests using HMW1 as bait and pray are presented in Fig. 4.35. HMW1-C-terminal region cloned in the GAL-BD vector was autoactivated (Fig. 4.35, upper panel) and therefore could not be used for this purpose. However, two pairwise combinations were observed in the direct mating test with HMW1-C-terminus in GAL-AD vector. It was shown that the HMW1-C-terminal region interacted *in vivo* with HMW2-3 and the C-terminal region of P1. Data from two-hybrid experiments indicated also that interaction between both proteins might take place at their C-termini.

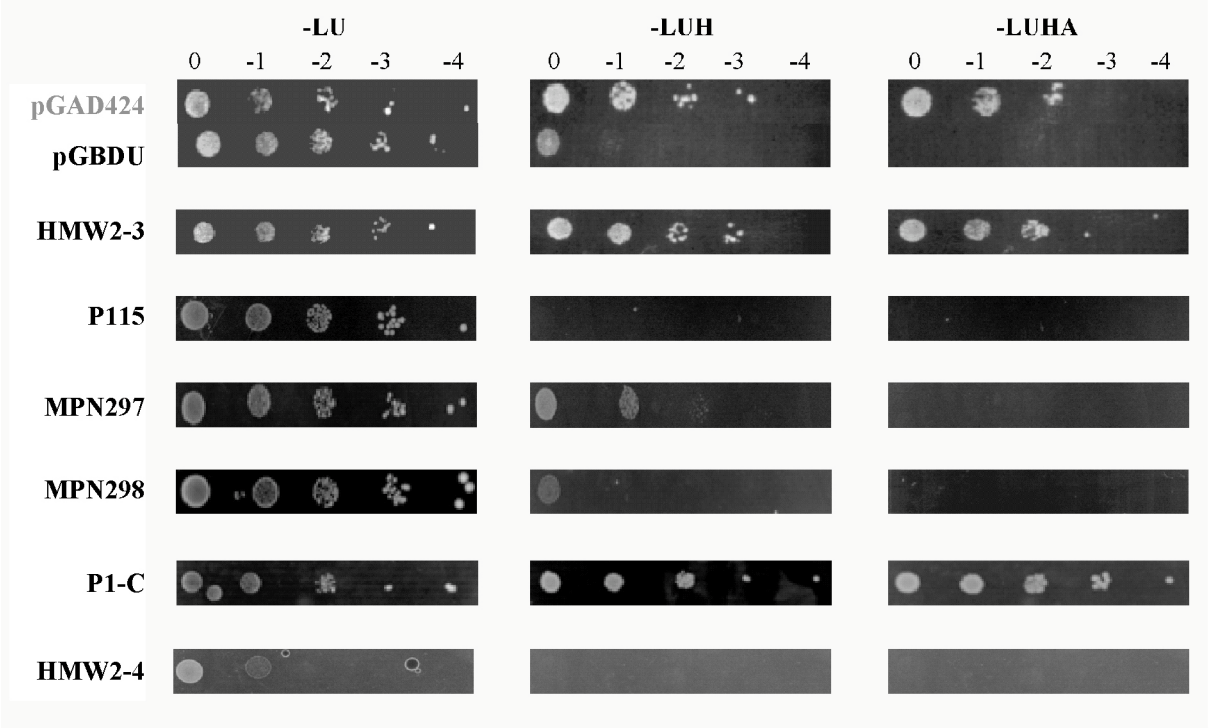

Fig. 4.35: Yeast two-hybrid reporter gene activation in a pairwise test using HMW1-C-terminal region as prey. The construct of hmw1-C-terminal region in a bait vector was found to show auto-activation (ability to cause nonspecific activation of reporters). All positive cultures are based on the activation of two independent reporters: His3 and Ade2. Presence of colonies indicates activation of His3 and Ade2 reporter. Constructs in pray vectors are indicated in gray and those in bait vectors are in black.

c) MPN426 (P115)

Pairwise tests using P115 as bait and pray are presented in Fig. 4.36. No interactions with any proteins were detected when full-length P115 was part of the GAL-BD vector. HMW2-3 did not interact with P115, which disagreed with the results from the initial two-hybrid screen. The difference between the previous two-hybrid screen and this experiment was that in this experiment full-length P115 was used. In the two-hybrid screen pilot experiment, the initial interaction was found with a peptide fragment which contained only the coil-coil domains of P115.

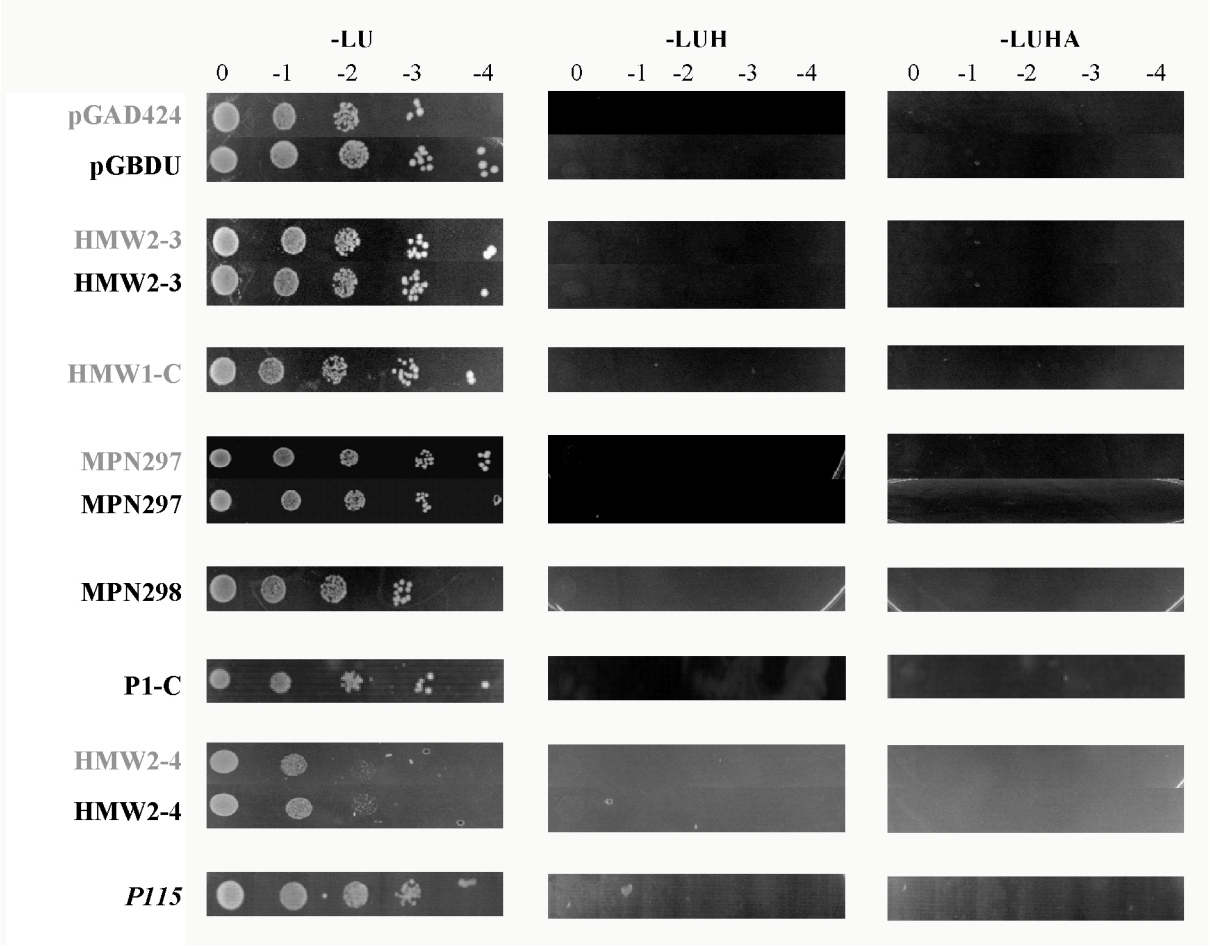

Fig. 4.36: Yeast two-hybrid reporter gene activation in a pairwise test using P115 as bait and prey. All positive cultures are based on the activation of two independent reporters: His3 and Ade2. Presence of colonies indicates activation of His3 and Ade2 reporter. All constructs used were tested for their ability to cause nonspecific activation of reporters and were found not to do so. None of interactions with P115 were found in this test. Constructs in pray vectors are indicated in gray and those in bait vectors are in black. The interaction with itself is shown in *italic.*

d) MPN297

Pairwise tests using gene product of MPN297 as bait and pray are presented in Fig. 4.37. MPN297 is weakly auto-activated in GAL-BD vector (Fig. 4.37, upper panel), so the interaction between MPN297 and HMW2-3 could not be confirmed in the manner of exchanging plasmid. However, a pairwise test in which full-length MPN297 was used as pray, showed that MPN297 binds to the HMW2-3. This result corresponded to the screening result from the previous two-hybrid screen with HMW2-3. Furthermore, it was shown in the pairwise test that MPN297 was able to interact with itself. So this result indicated that they could form a dimer or polymer *in vivo*.

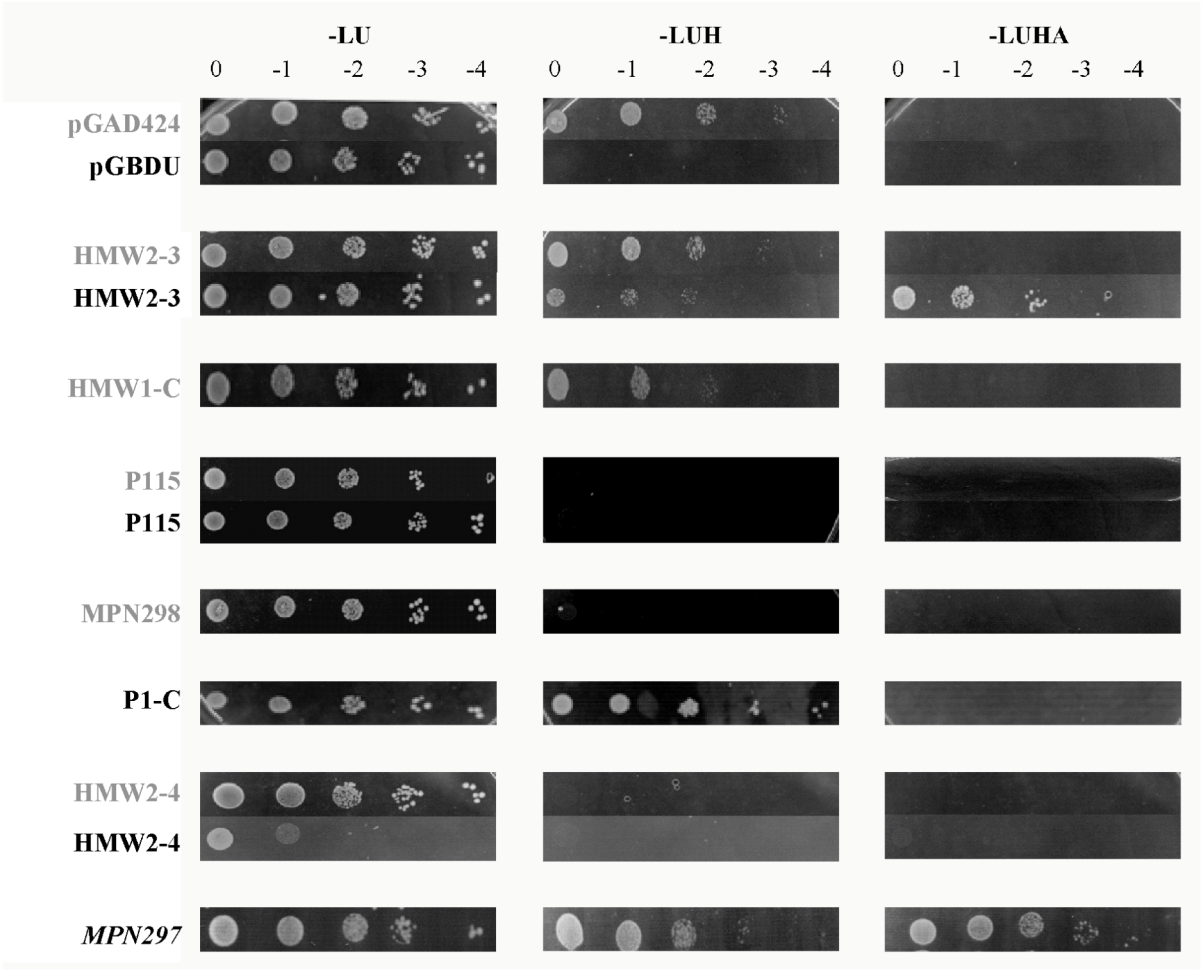

Fig. 4.37: Yeast two-hybrid reporter gene activation by MPN297 protein pairs. Typical results observed on media lacking adenine. Constructs in pray vectors are indicated in gray and those in bait vectors are in black. The interaction with itself is shown in *italic*.

e) MPN298

Pairwise tests using gene product of MPN298 as bait and pray are presented in Fig. 4.38. No interaction with any other proteins, even with HMW2-3, was detected when MPN298 was used in the pairwise test. It did not confirm the data from two-hybrid screen with HMW2-3. The gene MPN298, therefore, seems rather to be a false-positive result from the two-hybrid screen. Transformations of yeast cells with MPN298 in the GAL4-AD vector was not succesful although many parameters were optimized. Therefore no experiment could be done with this gene construct.

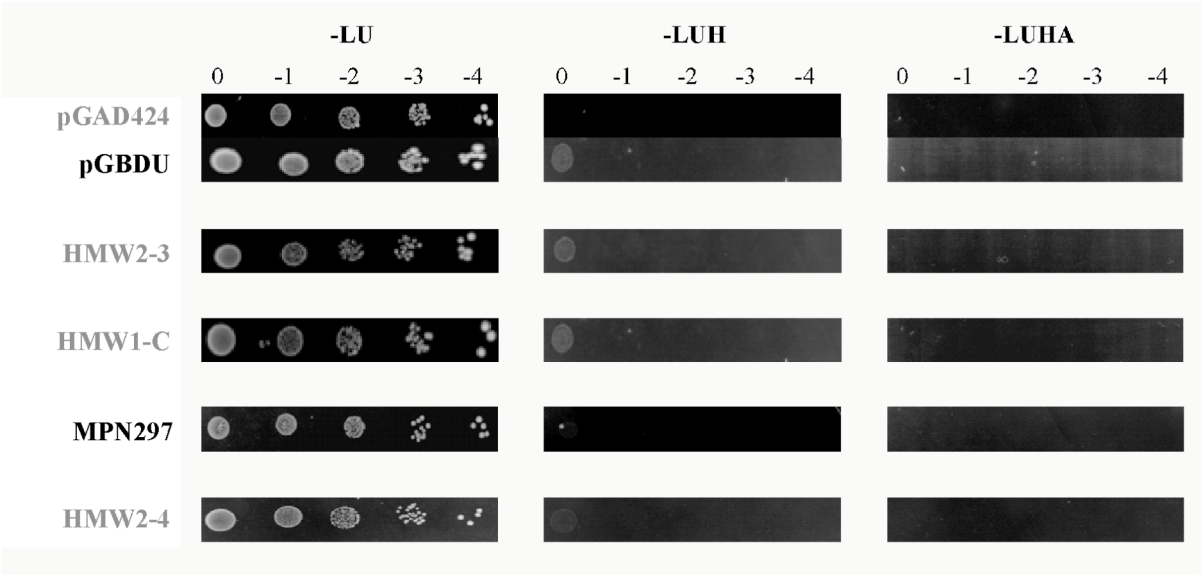

Fig. 4.38: Yeast two-hybrid reporter gene activation in a pairwise test using MPN298 as bait and prey. No positive interaction was detected. Constructs in pray vectors are indicated in gray and those in bait vectors are in black.

f) MPN141 (P1-C-terminal region)

Pairwise tests using P115 as bait and pray are presented in Fig. 4.39. It was shown previously that there are interactions between P1 and HMW1 of *M. pneumoniae* [Layh-Schmitt et al., 2000]. However, it was not possible to determine, which part of the P1 protein interacts with which part of the HMW1 protein.

Therefore, P1, the main adhesin of *M. pneumoniae*, was tested for its interaction with proteins from the list (table 4.4) in the yeast two-hybrid system. The C-terminal region of P1 (amino acid residues 1156-1525) was fused to Gal4-AD and Gal4-BD. The P1 in GAL4-AD vector was strongly auto-activated and could not be used in the pairwise test. However, P1 in GAL-BD interacted clearly with the HMW1- C-terminal region and also interact weakly with MPN297.

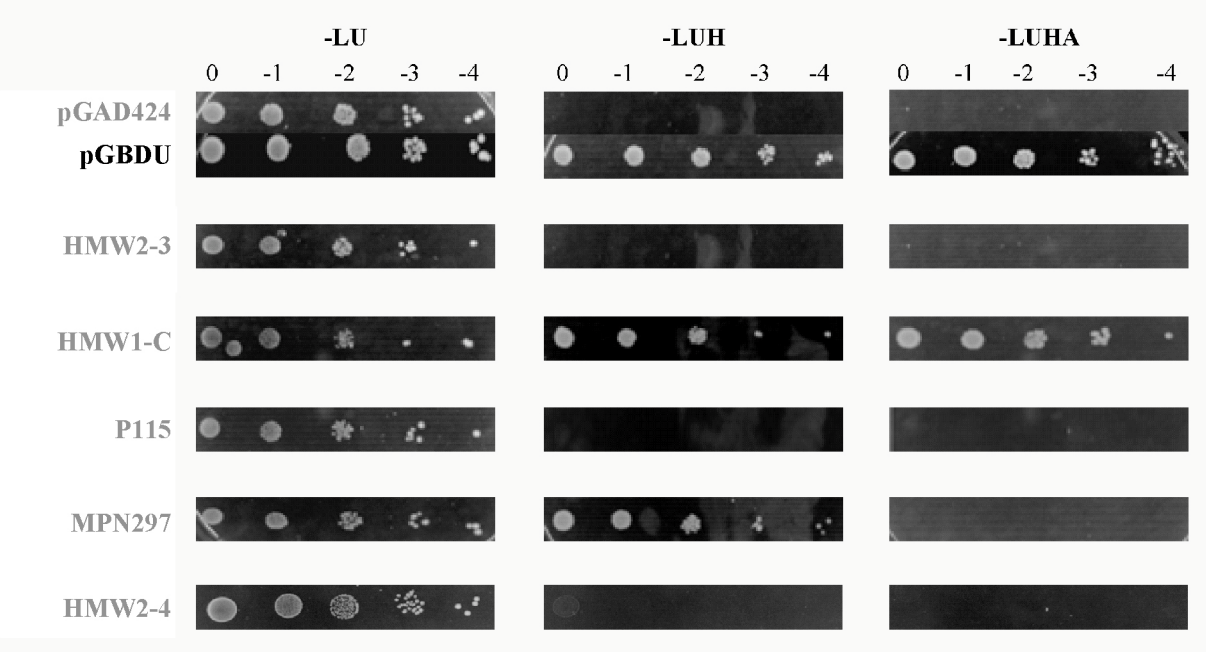

Fig. 4.39: Yeast two-hybrid reporter gene activation in a pairwise test using P1-C-terminal region as bait. The construct of P1-C-terminus in a pray vector was found to show auto-activation (ability to cause nonspecific activation of reporters). All positive cultures are based on the activation of two independent reporters: His3 and Ade2. Presence of colonies indicates activation of His3 and Ade2 reporter. Constructs in pray vectors are indicated in gray and those in bait vectors are in black.

g) MPN310 (HMW2 fragment 4)

Pairwise tests using HMW2-4 as bait and pray are presented in Fig. 4.40. No interaction with any other proteins was detected when HMW2-4 was used in the pairwise test. However, it was shown from the pairwise test that HMW2-4 could bind to itself. So one could assume that HMW2-4 is responsible for dimer- or polymer formation of the complete HMW2 or HMW2-s *in vivo*, since HMW2-s is the subfragment of the HMW2-4 (HMW2-s +208 aa upstream of the N-terminus).

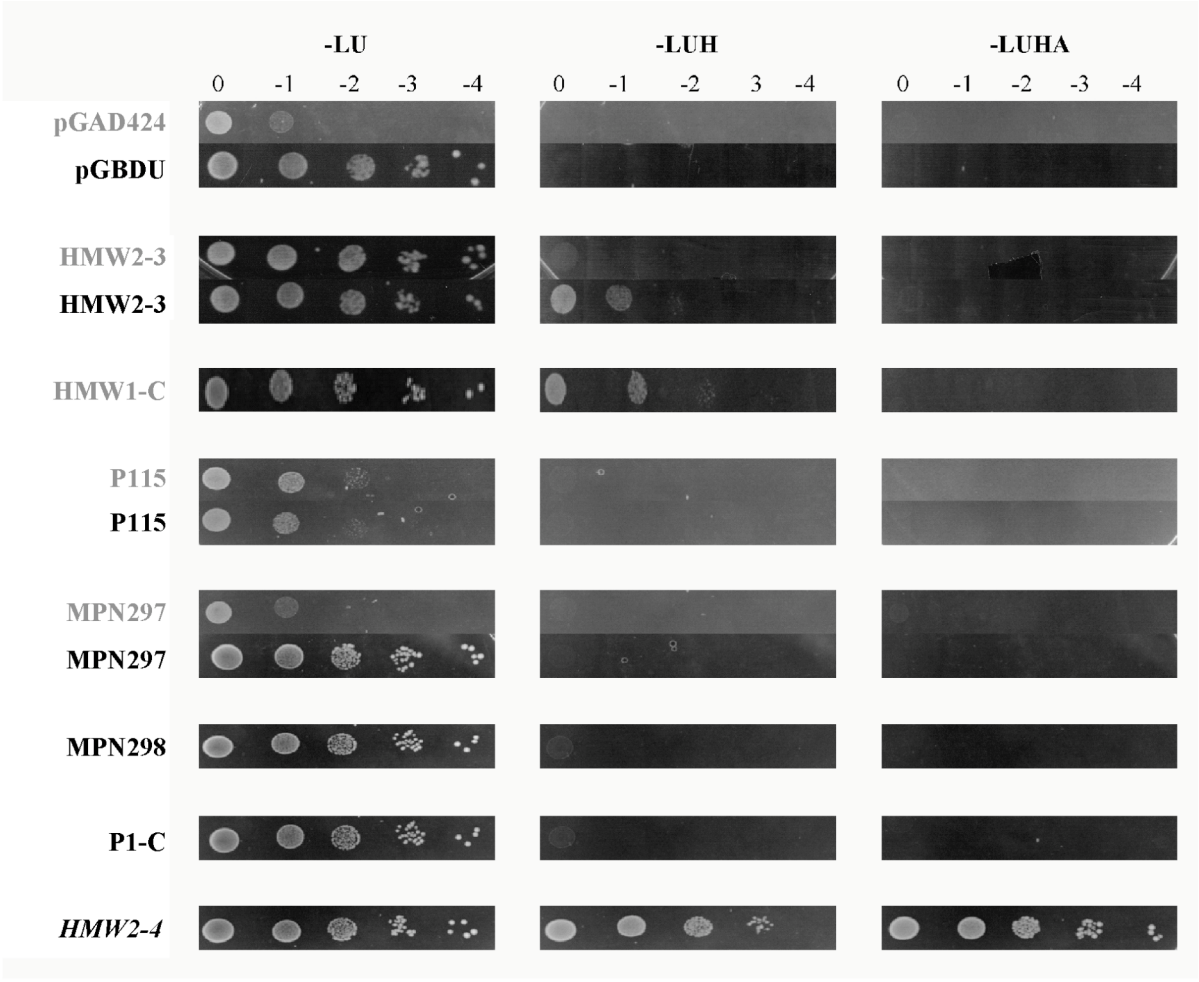

Fig. 4.40: Yeast two-hybrid reporter gene activation in a pairwise test using HMW2-4 as bait and prey. All positive cultures are based on the activation of two independent reporters: His3 and Ade2. Presence of colonies indicates activation of His3 and Ade2 reporter. Proteins which were tested against HMW2-4 are listed on the left. Constructs in pray vectors are indicated in gray and those in bait vectors are in black. The interaction with itself is shown in *italic*

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4.5.3 Summary of the results of the pairwise tests

Results from two-hybrid screening using HMW2-3 as bait should be confirmed in these pairwise experiments. Two interactions were confirmed: interaction of HMW2-3 with the HMW1-C-terminal region and with MPN297. Two other interactions proposed on basis of the previous screen were shown to be rather false-positive results: interaction of HMW2-3 with P115 and MPN298. Nevertheless, some potential interaction pairs were newly identified from pairwise tests, namely the interaction of P1 with the HMW1-C- terminal region and with MPN297. Two proteins were found to have ability to form homopolymers, MPN297 and HMW2-4. The data from yeast two-hybrid pairwise tests are summarized in Table 4.6.

Table 4.6: Summary of the results of pairwise approaches

bait \ pray	pGAD424	HMW2-3	HMW1-C	P115	MPN297	MPN298	P1-C	HMW 2-4
pGBDU	neg	neg	autoactive	neg	neg	neg	autoactive	neg
HMW 2-3	autoactive	autoactive	POS++	autoactive	POS++	0	autoactive	autoactive
HMW 1-C	autoactive	autoactive	autoactive	autoactive	autoactive	autoactive	autoactive	autoactive
P115	neg	neg	autoactive	neg	neg	0	autoactive	neg
MPN297	autoactive	autoactive	autoactive	autoactive	POS++	autoactive	autoactive	autoactive
MPN298	neg	neg	autoactive	neg	0	0	autoactive	neg
P1-C	neg	neg	POS++	neg	POS+	0	autoactive	neg
HMW 2-4	neg	neg	autoactive	neg	neg	0	autoactive	POS++

POS++= positive, strong interaction (growth on –LUHA),
POS+ = positive, weak interaction (growth on –LUH), autoactive = growth on –LUHA without interaction,
autoactive = growth on –LUH without interaction, 0 = transformation not successful.

4.6 Isolation of protein complexes containing the HMW2 fusion protein

It was shown (see chapter 4.5) that HMW2 interacts with several other proteins. To confirm these results by another independent method, I isolated *in vivo* protein complexes containing HMW2 from *M. pneumoniae* by applying the TAP-tag purification method [Puig et al., 2001].

4.6.1 Cloning and expression of the N-terminal TAP-tagged HMW2

The *hmw2* gene was modified with the tap tag to facilate the isolation of HMW2 containing protein complexes. A DNA fragment encoding the TAP tag was fused to the 5' end of the gene *hmw2* (Fig. 4.41).

Fig. 4.41: Schematic illustration of the construction of hmw2 gene fused with N-terminal TAP tag.

The expression of the tap-tagged *hmw2* was regulated by the *clpB* promotor from *M. pneumoniae*. The plasmid pBS1761 carrying the DNA sequence encoding the N-terminal TAP (N-TAP) tag (kindly provided from Dr. Jansen, ZMBH Heidelberg) was used as template for amplifying the N-terminal TAP tag by PCR. The PCR product was then ligated to the 3' blunt end of the PCR product of the *clp*B promotor. The DNA fragment containing both sequences was then amplified again by PCR using the ligation product as template. The PCR product containing the *clp*B promotor followed by the N-terminal TAP tag was then ligated with an *Eco*RI fragment containing the 5' end of the *hmw2* gene (384 bp). The PCR product derived from this ligation reaction containing the *clp*B promotor, followed by the TAP tag and N-terminal *hmw2* (*Eco*RI fragment) was then ligated into the *Eco*RI sites of pQE60 containing gene *hmw2* (original sequence with 4 UGA codons. The entire modified *hmw2*gene was amplified by PCR and the PCR product with the expected size was cloned into the *Sma*I site of the transposon Tn*4001*, which is part of the plasmid pKV104. The cloning strategy is illustrated in Fig. 4.42.

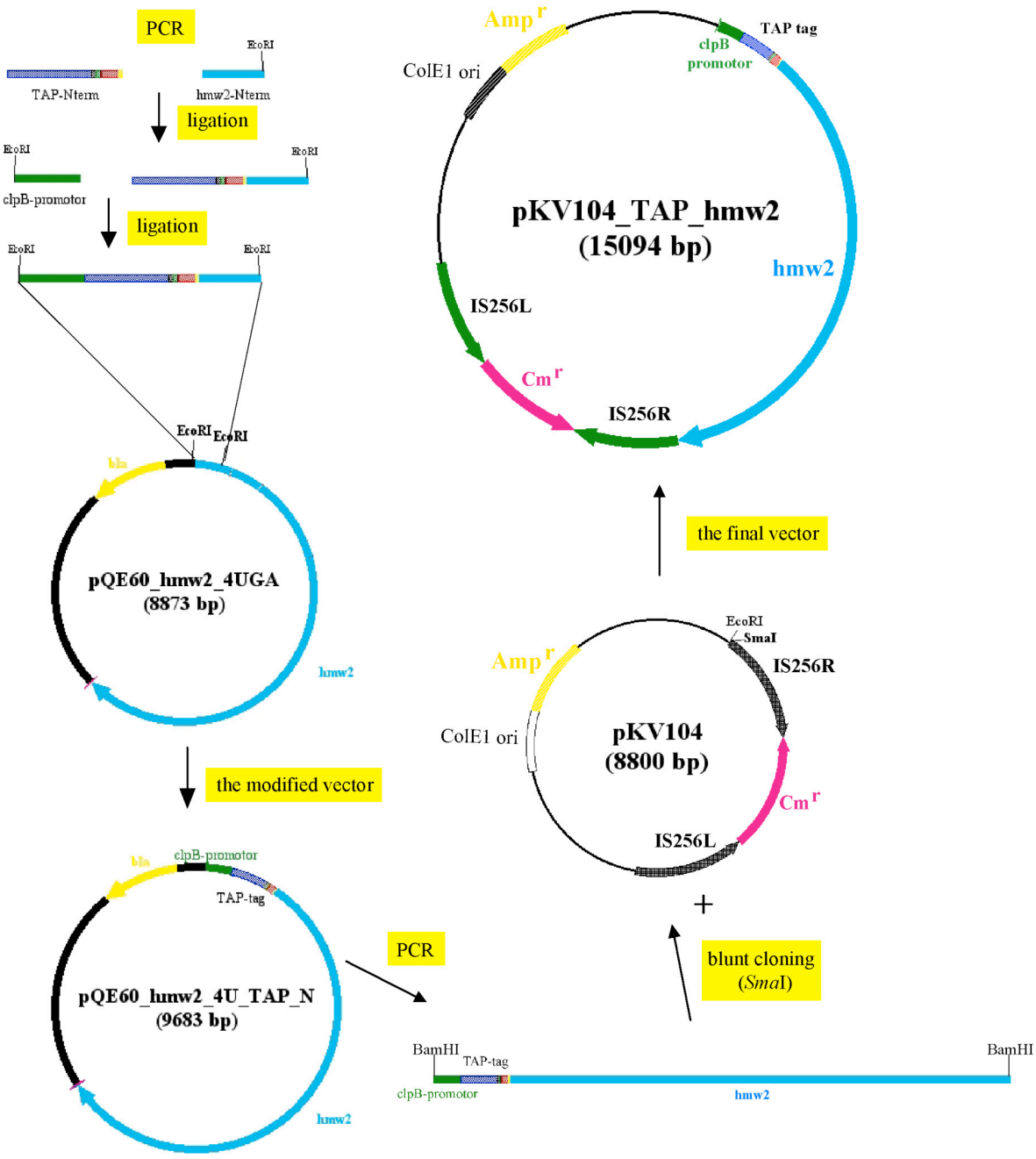


Fig. 4.42: Strategy for cloning the gene fusion of *hmw2*-N-tap-tag into the plasmid pKV104.

After transforming *M. pneumoniae* WT and the *M. pneumoniae* A3, the expression of the TAP-tagged HMW2 could be shown. By Western blotting using antiserum against the N-terminal HMW2, a 235-kDa protein and a 215-kDa protein were observed in protein extracts of *M. pneumoniae* WT transformants and only a 235 kDa protein in *M. pneumoniae* A3 transformants (Fig. 4.43). The 235 kDa protein corresponded to the expected size of the TAP-tagged HMW2. In addition, the IgG-binding domain (in TAP tag) was detected by Western blotting using serum from a mycoplasma-free rabbit as a source of IgG. It reacted positively with the 235 kDa protein in both transformants, confirming the presence of the TAP-tagged HMW2 in these cells.

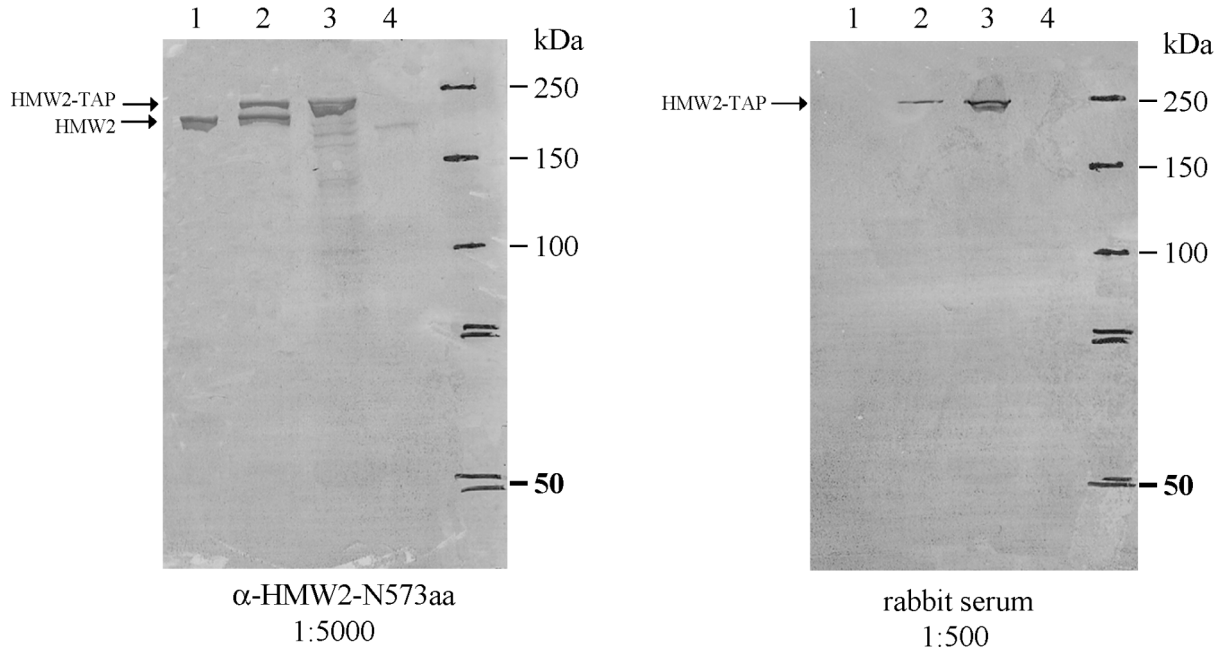


Fig. 4.43: Western blot analysis of expressed TAP - tagged HMW2 in *M. pneumoniae* cells. (7,5 % SDS - PAA gel). 1 = *M. pneumoniae* WT ; 2 = *M. pneumoniae* WT transformant ; 3 = *M. pneumoniae* mutant A3 transformant; 4 = *M. pneumoniae* mutant A3. Anti-HMW2-N573aa antiserum and serum from a mycoplasma-free rabbit were used to detect the protein.

4.6.2 Pilot experiments concerning cell breakage and protein solubility

Various tests were done to fine appropiate conditions for the purification of the TAP-tagged HMW2 and possible protein complexes containing the TAP-tagged HMW2 protein.

Cell breakage

The *M. pneumoniae* WT transformant Nr.1 was resuspended with the buffer recommended for general purification of TAP-tagged protein (IPP150: 10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% Nonodet (NP-40)) [Puig et al., 2001] and then the cells were broken by sonification, french press or shaking with glass beads (1 mm , or 0.1 mm diameter). After centrifugation, the distribution of the TAP-tagged HMW2 in supernatant and pellet was monitored by SDS-PAGE and Western blotting (Fig. 4.44). The TAP-tagged HMW2 was partly found in the supernatant of cell extracts prepared by sonification, but only traces of the fusion protein were detected in the supernatant after preparing with both size of glass beads. The TAP-tagged HMW2 could also not be detected in the supernatant from cell extracts treated with french press. Therefore, based on the results from these experiments, sonification was chosen as method for preparing cell extracts for the purification of protein complexes.

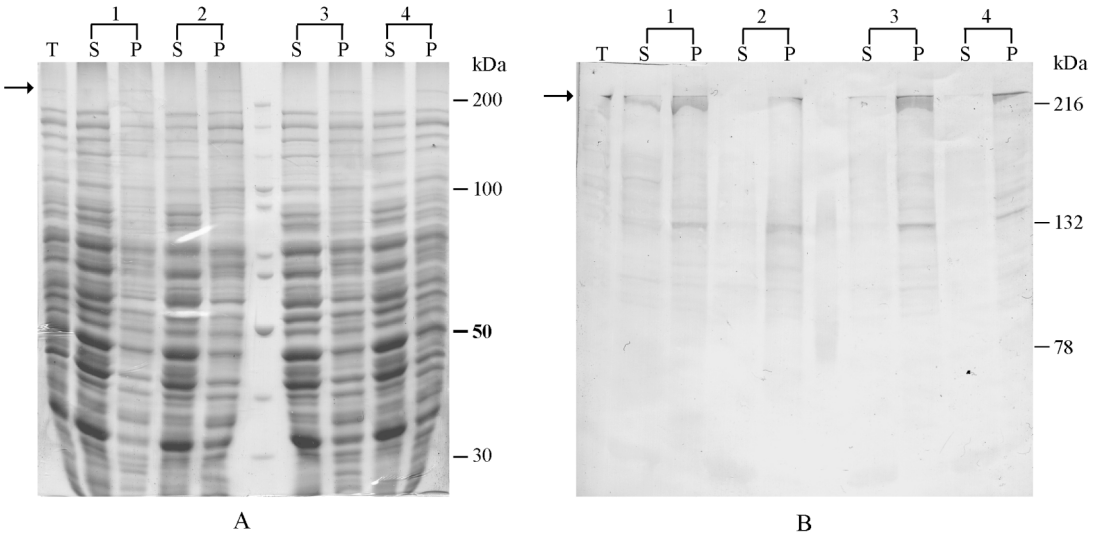


Fig. 4.44: Coomassie Blue stained gel (A) and Western Blot (B) of total protein extract of *M. pneumoniae* mutant A3 transformant containing TAP-tagged HMW2. Various methods for cell breakage were tested. (10 % SDS-PAA gel).

1 = sonification
2 = french press
3 = glass beads (1 mm diameter)
4 = glass beads (0.1 mm diameter)

T = untreated total protein extract
S = supernatant
P = pellet

Protein solubility

The HMW2 protein is a relative large protein and not soluble in buffer without detergents or chaotropic substances. Therefore, IPP150 buffer was modified by adding different components in final concentrations as indicated: 5 M potassium chloride, 1% digitonin, 0.5% NP-40, 0.5% Tween-20, 0.5% SDS, 2% DMSO. The *M. pneumoniae* WT transformant (TAP-tag$^+$)was resuspended with those buffers and the individual cell suspensions were sonicated and separated in pellet and supernatant by ultracentrifugation. Supernatant and pellet of each cell suspension were loaded on a 7.5 % SDS-PAA gel. Distribution of the TAP-tagged HMW2 was monitored by Western blotting (Fig. 4.45). The fusion protein was only soluble in buffers containing 5 M potassium chloride or 0.5% SDS.

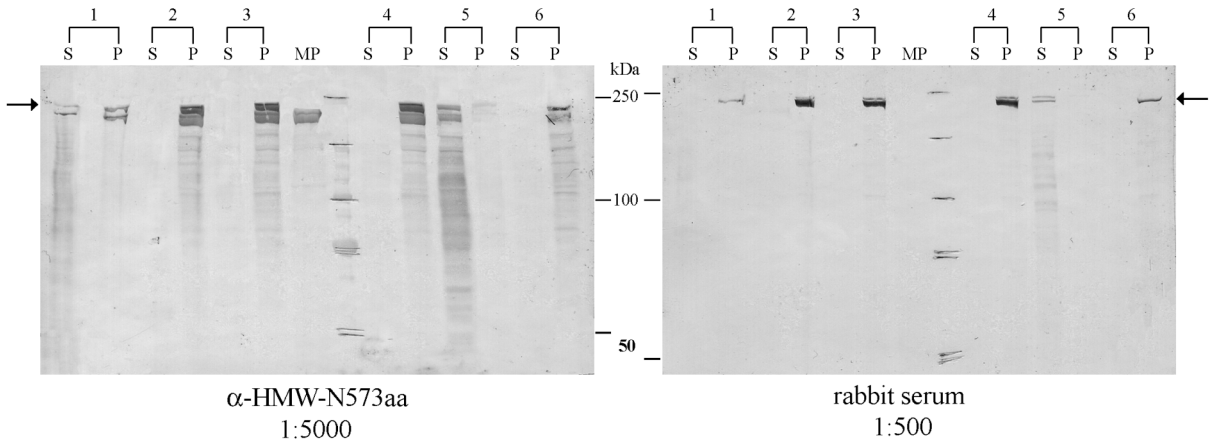


Fig. 4.45: Western Blot of a total protein extract of *M. pneumoniae* mutant A3 transformant containing TAP-tagged HMW2 (7.5% SDS-PAA gel). Various modifications of the IPP150 buffer were compared. 1 = 5M potassium chloride; 2 = 1% digitonin; 3 = 0.5% NP-40; 4 = 0.5% Tween-20; 5 = 0.5% SDS; 6 = 2% DMSO; S = supernatant; P = pellet

Binding affinity of the TAP-tagged HMW2 to IgG beads under selected buffer conditions

IPP150 buffers containing 5M potassium or 0.5% SDS were used to treat the cell extract of *M. pneumoniae* A3 transformant as described. Cell extract treated with an unmodified IPP150 buffer was used as a control. The supernatant was incubated with Dynabeads MyOne® Carboxylic Acid (Dynatech) coupled with rabbit-IgG (Sigma-Aldrid). All fractions of each step were monitored by Western blotting (Fig. 4.46) using antiserum against the N-terminal region HMW2.

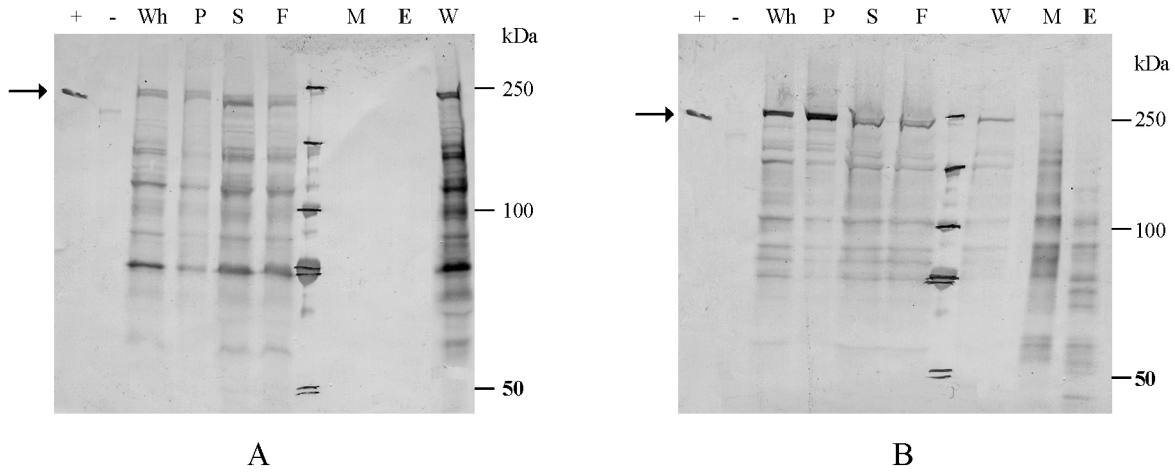


Fig. 4.46: Western blotting of fractions from the first-step-purification test of TAP-tagged HMW2 using buffer containing 5M potassium chloride (A) or 0.5% SDS (B) (7.5% SDS-PAA gel).
+ = *M. pneumoniae* WT; – = *M. pneumoniae* mutant A3; Wh = whole cell extract; P = pellet; S = supernatant; F = flow through; W = wash; E = eluat; M = magnet beads after elution.

The TAP-tagged HMW2, dissolved in either one of the modified IPP150 buffers, was detected in the flow-through and in the washing buffers but not in eluat fraction. This indicated that the IgG-Protein A interaction did not tolerate these buffer conditions. Similar experiments with IPP150 buffer without addition of detergents or high salt were also not successful.

Test of the TAP-tagged HMW2 solubility in buffer with different concentrations of SDS

Previous tests showed that TAP-tagged HMW2 was soluble in 0.5% SDS. However, this concentration was too high and inhibited binding of the fusion protein to IgG-beads. To find the lowest concentration of SDS, by which TAP-tagged HMW2 is still soluble, the following three concentrations of SDS were tested: 0.05%, 0.15%, 0.45%. After treating cell extracts with those buffers, the distribution of the TAP-tagged HMW2 in supernatant and pellet was monitored by SDS-PAGE and Western blotting (Fig. 4.47).

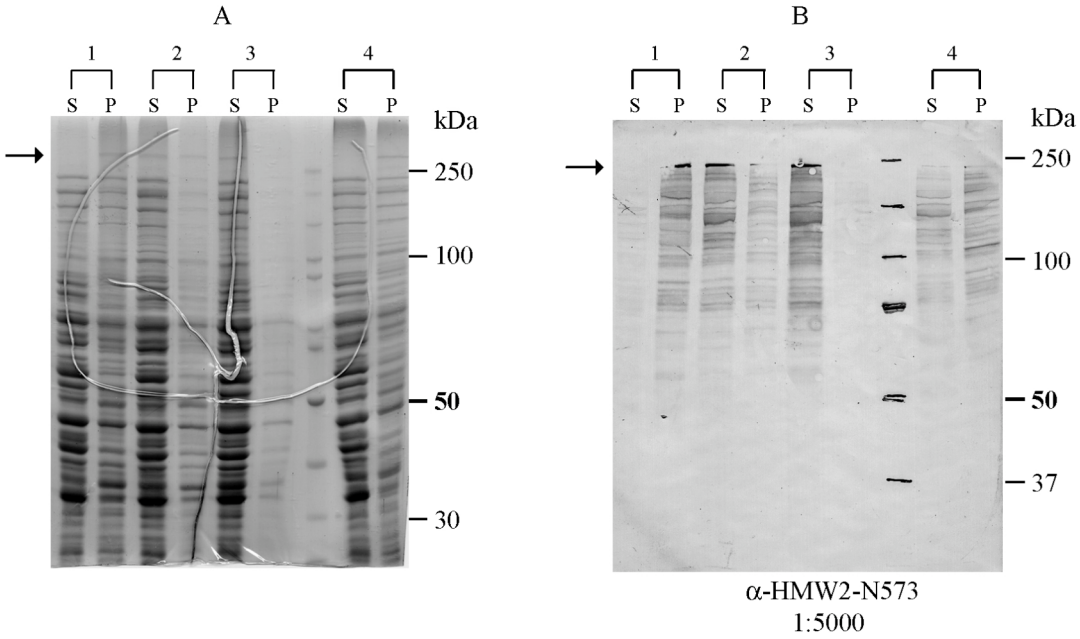


Fig. 4.47: Western Blot of a total protein extract of *M. pneumoniae* mutant A3 transformant containing TAP-tagged HMW2 (10% SDS-PAA gel). The following concentrations of SDS were tested to solubilize the TAP-tagged HMW2. S = supernatant ; P = pellet ; 1 = 0.05 % SDS ; 2 = 0.15 % SDS; 3 = 0.45 % SDS; 4 = 0.5 % NP-40.

Most of the TAP-tagged HMW2 was soluble in buffer contating 0.15% SDS. It has been reported that the IgG-Protein A interaction is taking place in the presence of up to 500 mM NaCl, 1% NP-40 and low concentrations of SDS (0.1%) [Puig et.al, 2001]. Therefore, I applied similar conditions for the purification of the TAP-tagged HMW2, since this protein is soluble at SDS-concentration of about 0.15%.

4.6.3 Purification of the TAP-tagged HMW2 under optimized conditions

Although it was reported that IgG-Protein A interaction is taking place in the presence of up to 500 mM NaCl, 1% NP-40 and 0.1% SDS [Puig et.al, 2001], buffer conditions for each purification step using TAP-tag strategy had to be carefully tested. Detergents (e.g. SDS and NP-40) and high salt concentrations (above 300 mM NaCl) inhibit TEV-protease activity (Invitrogene, technical service). Binding affinity of calmodulin beads is disturbed in the presence of SDS, however, buffers containing up to 1 M NaCl and 0.1% NP-40 can still be used (Stratagene, technical service). Therefore, buffer components for each purification step were individually optimized and adapted to keep the protein complex soluble without destroying it. Considering these aspects, a buffer containg 10 mM Tris-HCl (pH 8) and 250 mM NaCl was used as basic buffer for further purification steps. By adding the chemicals (see "Materials and Methods") and detergents, individual buffers for each step were prepared. The following concentrations of detergents were used:

Cell lysis	0.1% SDS	1% NP-40
First step binding: IgG beads	0.1% SDS	1% NP-40
Washing IgG beads	–	–
Elution with TEV protease	–	–
Second step binding: calmodulin beads	–	0.1% NP40
Elution with EGTA	–	0.1% NP40

As starting material for the purification of the HMW2 containing protein complex(es), cells from a 4500 ml culture of *M.pneumoniae* A3 transformant with *tap*-tagged *hmw2* were used, which yieled about 350 mg of total protein (about 25 mg total protein). As negative control, the mutant A3 containing no TAP-tagged HMW2 was also purified under same conditions. After applying IgG Sepharose (Amersham Bioscience) in the first purification step and washing the calmodulin beads (Stratagene) in the second step, one milliliter of calmodulin elution buffer was added to the calmodulin beads. Five elution fractions of 200 µl each are collected, 100 µl of each fractions were precipitate (methanol/chloroform), separated on SDS-PAA gels and visualized by silver staining (Fig. 4.48).

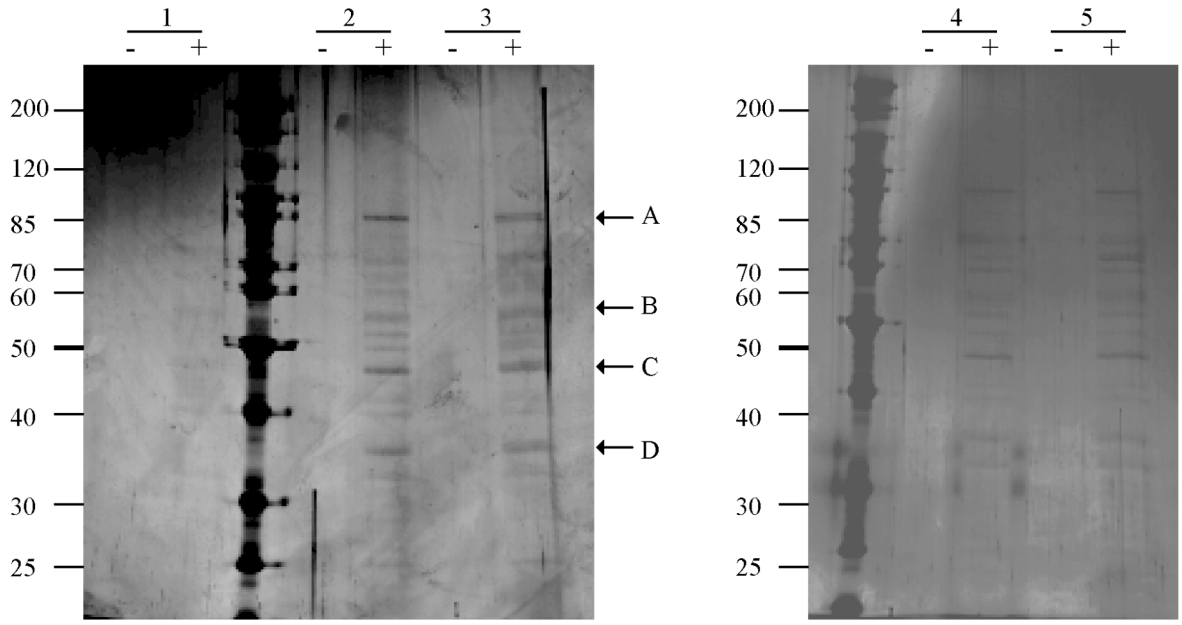


Fig. 4.48: Silver stained gel (10% SDS-PAA gel) of fractions (1-5) from the purification of TAP-tagged HMW2 containing protein complexes. Protein bands (indicated with arrows) were cut and identified by mass spectrometry.
– = fraction from the purification of *M. pneumoniae* mutant A3
+ = fraction from the purification of *M. pneumoniae* mutant A3 transformant containing TAP-tagged HMW2.

After the purification with calmodulin beads, one could see that the purification was effective. No background from any protein band was seen in the negative control, but several proteins remained in the sample containing TAP-tagged HMW2. These proteins could potentially interact with the HMW2 protein. Four protein bands were cut out from fraction Nr.2 and identified by mass spectrometry (MS). The identified proteins are summerized in table 4.7.

Table 4.7: Proteins which were co-purified with TAP-tagged HMW2.

Sample	MPN number	ORF	calculated MW	Molar mass on SDS-PAA gel	Annotation
band A	MPN160	VXpSPT7_ orf377	42,162	85,000	MG147 homolog; conserved hypothetical
	MPN573	D02_orf543	57,589	85,000	heat shock protein GroEL
band B	no result	–	–	–	–
band C	MPN665	K05_orf394	42,590	47,000	elongation factor TU (tuf)
	MPN015	D12_orf285	32,762	47,000	MG011 homolog; conserved hypothetical
	MPN140	E07_orf324	36,461	47,000	ORF4, P1 operon; DHH family phosphoesterases
band D	MPN430	A05_orf337	36,246	36,000	Glycerladehyde-3-phosphate dehydrogenase (Gap)
	MPN015	D12_orf285	32,762	36,000	conserved hypothetical, MG011 homolog
	MPN392	F11_orf327	35,169	36,000	Pyruvate dehydrogenase E_1-beta subunit (PdhB)
	MPN140	E07_orf324	36,461	36,000	orf4, P1 operon; DHH family phosphoesterases

The molar masses of some proteins identified on the bands A-D did not always corresponded to the predicted molecular weight based on the DNA sequence of the gene. Possible reasons could be post-translational modifications of these proteins or a dimerization (e.g. by MPN160).

About 100 µl of the fraction Nr.2 were divided into four aliquots and were separated on a 10% SDS-PAA gel, the proteins were transferred to nitrocellulose membrane and probed with four selected antisera directed against: EF-Tu (as a positive control), HMW2, HMW1 and the gene product of MPN297. Since there were only small amounts of protein in the samples, the Western blot had to be developed with the more sensitive "Extracellular fluid" (ECF) reagent which is based on chemofluorescence. The signal from the blot probed with α-EF-Tu was easily observed, however only traces of TAP-tagged HMW2, HMW1 and the gene product of MPN297 could be detected (Fig. 4.49).

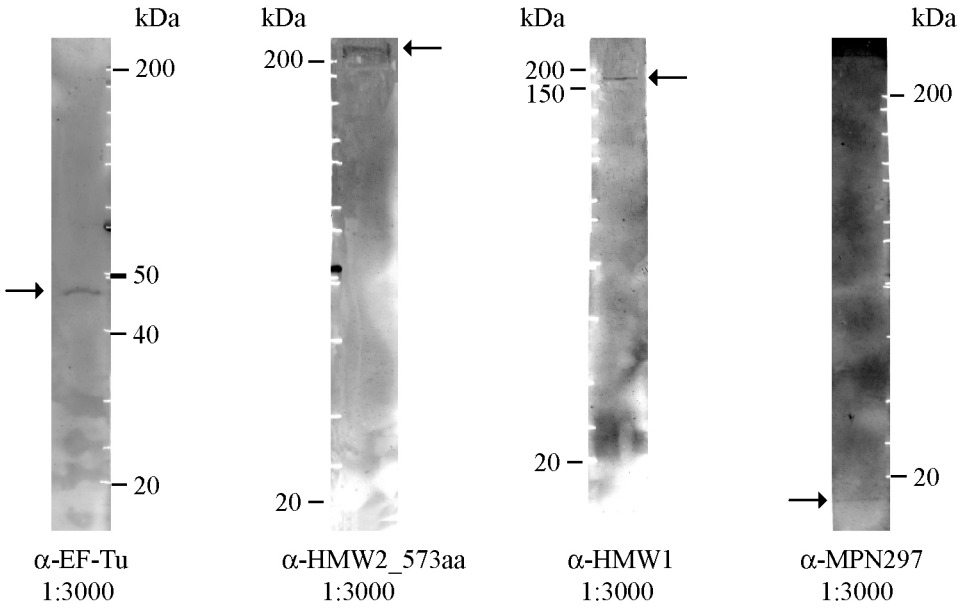


Fig. 4.49: ECF Western blots of fraction Nr.2 (10% SDS-PAA gel) from the purification of TAP-tagged HMW2 containing protein complexes. Names of antisera and dilution used are given below each blot. Identified protein bands are indicated by arrows.

Fraction Nr.3, which showed a similar protein pattern like fraction Nr.2 in silver stained gels was also analyzed by ECF Western blotting. Again, the fraction Nr.3 were divided into four aliquots and separated on 10% SDS-PAA gel, proteins were transferred to nitrocellulose membrane and probed with four selected antisera directed against: HMW2, P1, P90 and P115. In these analyses, P115 showed the strongest signal, and again, HMW2 was seen only as a faint band. In addition, traces of P1 and P90 could be observed in this fraction (Fig. 4.50).

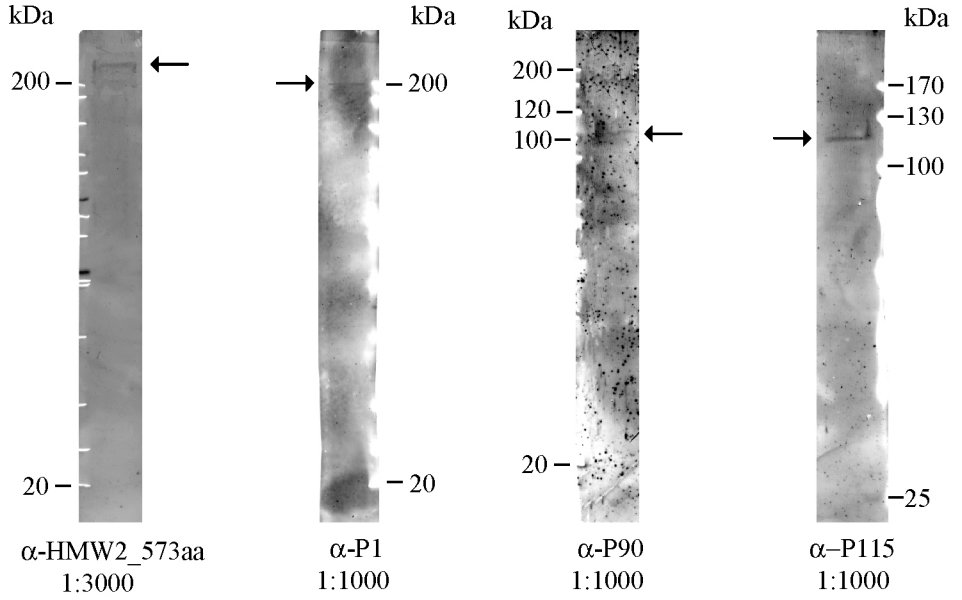


Fig. 4.50: ECF Western blots of fraction Nr.2 (10% SDS-PAA gel) from the purification of TAP-tagged HMW2 containing protein complexes. Names of antisera and dilution used are given below each blot. Identified protein bands are indicated by arrows.

4.7 Localization of HMW2 in *M. pneumoniae* using a new anti-HMW2 antiserum

Several cytadherence-associated proteins HMW1, HMW2, HMW3, P1, P90, P40, P30, and P65 have been reported to be localized at the attachment organelle [Balish et al., 2003; Jordan et al., 2001; Krause, 1996; Krause and Balish 2001; Layh-Schmitt and Herrmann,1994; Stevens and Krause 1991, 1992]. Subcellular location of most of them have been also determined by immunocytochemistry, including P1, P30, P40, P90 [Layh-Schmitt and Herrmann,1994], P65, HMW1 [Stevens and Krause,1991, 1992]. However, the cellular sublocalization of the important protein HMW2 has still not been convincingly shown.

So far, the localization of HMW2 at the tip was indicated by fluorescence microscopy with *M. pneumoniae,* which has been transfomed with a gene fusion expressed a green fluorescence HMW2 (HMW2-GFP) [Balish et al., 2003]. But this did not provide the conclusive evidence for co-localization of HMW2 with the rod, because this method is not sensitive enough for a precise localization. The correlation between the rod structure and HMW2 can only be done precisely by immunoelectron microscopy. The positive fluorescence signal at the tip did only indicate that HMW2 might be somehow connected with the tip structure. There are two HMW2 specific antisera available (Fig. 4.51), one of them recognizes the first 29 amino acids of the N-terminal end [Regula, 1999] and another one directed against 319 amino acids of the C-terminal region of HMW2 [Proft, 1994]. Attempts to define the subcellular location of HMW2 in *M. pneumoniae* cells using those antisera in immunoelectron microscopy failed, since none of these antisera gave satisfying results.

Fig. 4.51: Schematic drawing of HMW2. Antibody against the C-terminal region of HMW2 is shown here in pink and the antibody against the N-terminal region is shown in red bar with a porpotional scale and position. The numbers above the pink and red bars are positions of amino acid of HMW2 recognized by the antibody.

The possible explanation was the well-known low specificity of antisera against coiled-coil structure forming proteins. Even if these antisera react very strongly in Western blotting analyses. In addition, the antiserum against the C-terminal region recognized two proteins, HMW2 and HMW2-s. Therefore, in case of positive results, it would not be clear, whether HMW2 or HMW2-s was colocalized. By using the program coils of percoils

(www.expasy.org) to determine score value of coiled-coild domains within HMW2 protein sequence, it was shown that non coiled-coil regions are found in the N-terminal part of HMW2. Therefore, a reactive new antigen was produced which contained 573 amino acids of the N-terminal region of HMW2. This antiserum should recognize larger regions of HMW2 and be more specific.

4.7.1 Construction of a HMW2 fusion protein for generating a new antiserum

For antiserum production, the antigen had to be as pure as possible and available in sufficient amounts. The *hmw2* gene contains 4 UGA triplets, coding for tryptophan in *M. pneumoniae* (Fig. 4.1) but for a stop codon in other organisms. Therefore, for expression of HMW2 in other organisms, all UGA codons of gene *hmw2* were changed to UGG [Boonmee, unpublished].

It was observed that after converting the first two UGA codons, the resulting HMW2 fragment showed the highest expression (Fig. 4.52). The corresponding protein, 573 amino acids, was considerably larger than the previous antigen used for producing anti-HMW2 antiserum (29 amino acids). Due to its size and its high expression level, this truncated HMW2 was chosen as antigen for the generation of a new antiserum.

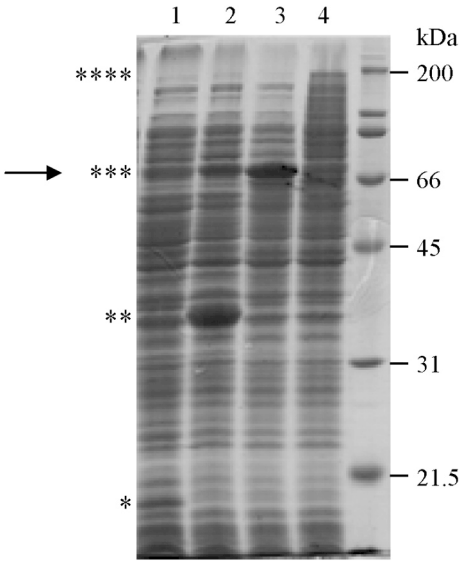


Fig. 4.52: Coomassie Blue stained gel of total protein extracts of *E. coli* tranformed with *hmw2* genes which contained 1, 2, 3 and 4 UGA codons. (10% SDS-PAA gel). Number of * indicates position of fusion protein on each lane. The arrow points to the protein used as antigen.
1 = HMW2 4UGA (20 kDa)
2 = HMW2 3 UGA (32 kDa)
3 = HMW2 2 UGA (68 kDa)
4 = HMW2 1 UGA (185 kDa)

A vector construct of pQE60 with gene *hmw2* (2 UGGs) was used as a template for amplifying the 5' end of the 1722 bp DNA fragment of the gene *hmw2* (which I will call *hmw2*-AG) which terminated at the third UGA codon of the gene. The construction was first cloned into the pQE9 vector which provided a *his* tag to the 5' end of the fused gene (Fig. 4.53).

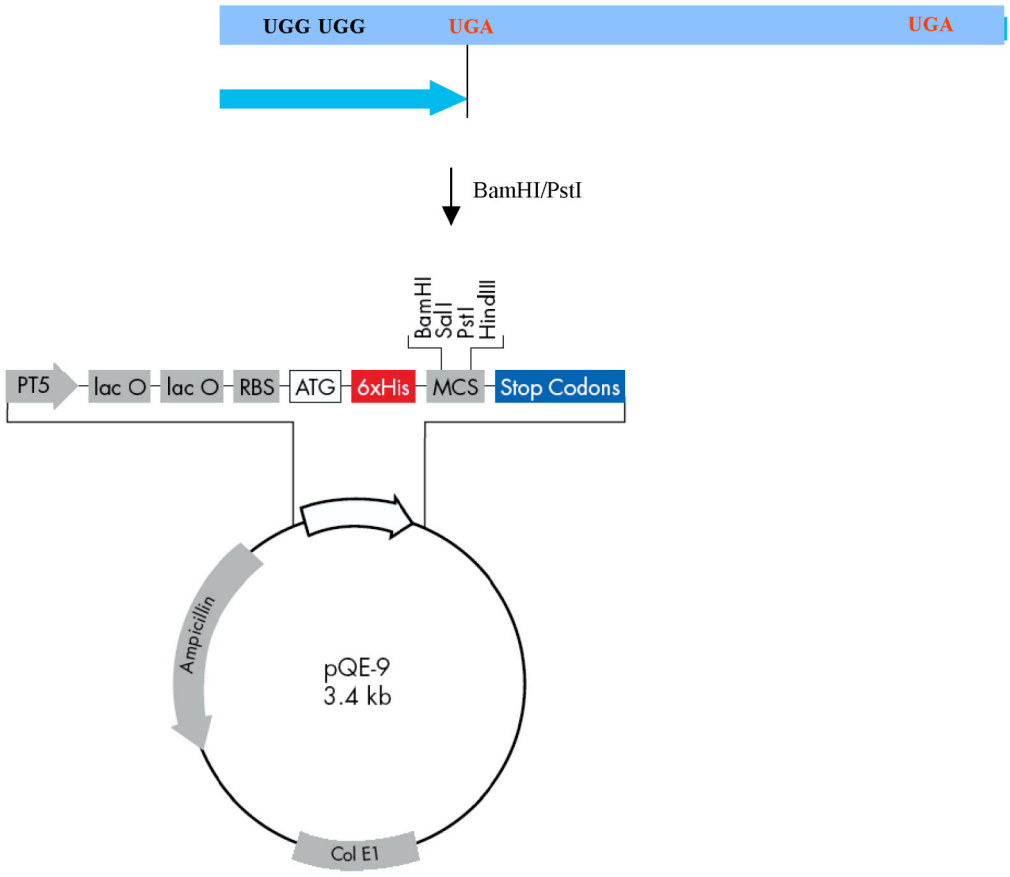


Fig. 4.53: Schematic illustration of the cloning of the *hmw2*-AG into the pQE9 vector.

However, the fusion protein was expressed at a very low level (Fig. 4.54), compared to the fusion protein from the mutagenesis experiment which had no N-terminal His tag. We concluded that,in this case, expression of this protein might be negatively influenced by the N-terminal His tag.

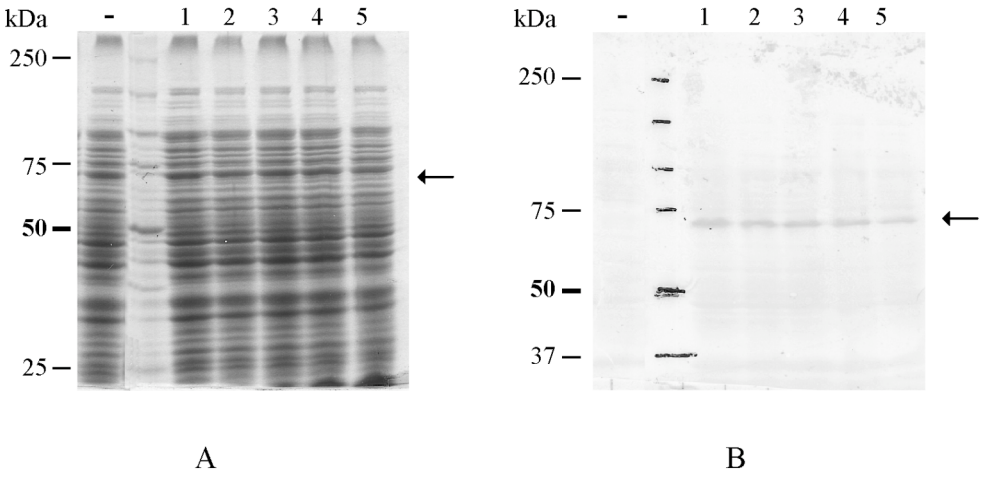


Fig. 4.54: Coomassie Blue stained gel (A) and Western blot (B) of the N-terminal HMW2 (573 aa) expressed in pQE9 vector in *E. coli*. (10 % SDS-PAA gel) (α-HMW2_573aa, dilution 1:3000). Arrows indicate expected fusion protein . Five different clones were tested (1 – 5) , – = transformed *E. coli* , uninduced.

Therefore, I decided to fuse the His tag to the C-terminus of HMW2. To improve the specific binding of our His-tagged protein, we added three more histidines to the C-terminal end of HMW2-AG. An existing vector pQE60 with gene *hmw2* (2 UGGs) was used again as a template for this purpose. *E. coli* XL1-blue strain was transformed with the ligation product. A search for the right clone was done by monitoring protein expressions of each clone. This time, the fusion protein was expressed at a high level and therefor could be produced in large quantities for purification and immunization (Fig. 4.56).

4.7.2 Purification of the HMW2-AG fusion protein by IMAC

Some proteins overexpressed intracellularly in *E. coli* are frequently sequestered into insoluble inclusion bodies. Therefore, to find proper conditions for an efficient purification of HMW2-AG fusion protein, the solubility of the fusion protein was determined. An advantage of His-tagged proteins is that, if they are found in insoluble inclusion bodies, they can be easily solubilized with chemicals such as 6 M guanidium hydrochloride or 8 M urea or with a variety of detergents and be purified on Ni-NTA matrices.Samples of cell pellets containing the HMW2-AG fusion protein were resuspended with various lysis buffers (native/ denaturing conditions). Distributions of the fusion protein were monitored in SDS-PAGE. Under native conditions, most of the fusion protein still remained in the insoluble fraction, therefor a denaturing buffer containing 4M urea was chosen for all processes. Purification of His-tagged HMW2-AG was done by with ÄKTAexplorer™ 10 systems (Amersham Biosciences), using a 5-ml HiTrap Chelating column (Amersham Biosciences) loaded with nickel ions. The ÄKTA explorer™ was connected to a computer installed with the control program UNICORN™, by which all parameters for each purification steps could be modified. The program displayed actual run status and current flow path, so one will have a real-time overview of the system and separation (Fig. 4.55), followed by automatic generation of the purification report with extensive data evaluation wizard and reporting options.

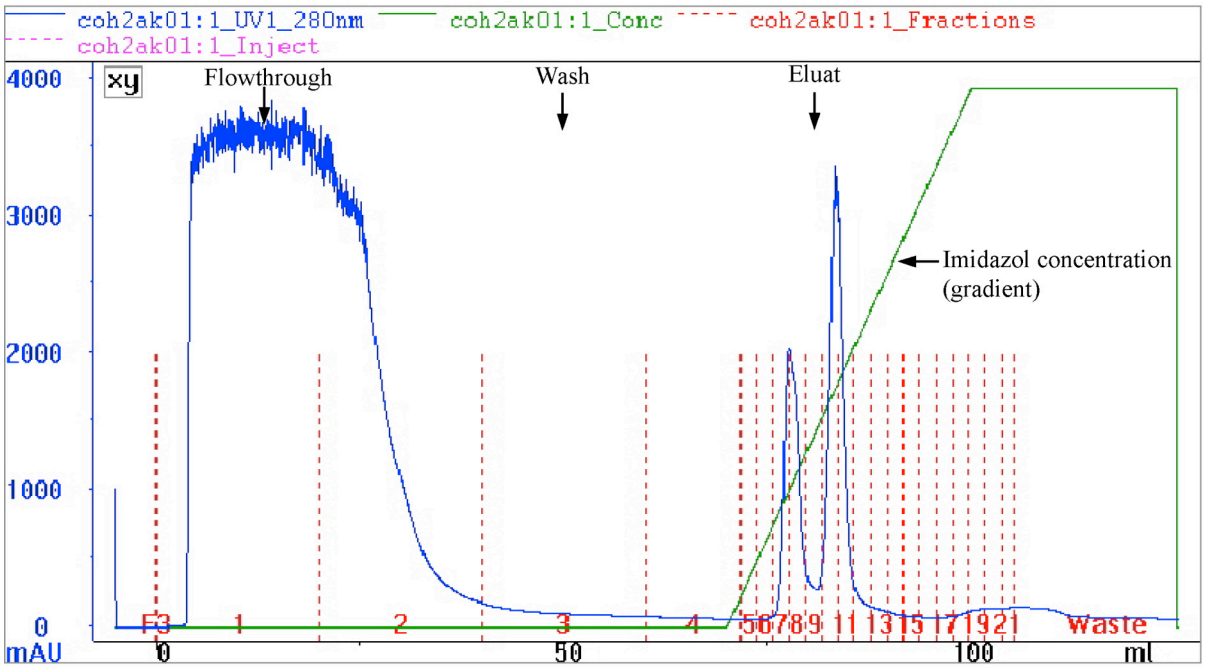


Fig. 4.55: Flow-chart of purification of N-terminal HMW2 by ÄKTAexplorer™.

Samples of eluated fractions which were shown in the program to contain the fusion protein were each loaded on a SDS-PAA gel and visualized by staining with Coomassie Blue (Fig. 4.56). Purifaction was very effective, so that no further purification steps were necessary. Six milligrams of the His-tagged HMW2-AG had been isolated from 500 ml of *E. coli* liquid-culture. The purified protein was precipitated with aceton to get rid of urea, which would interfere with the immunization.

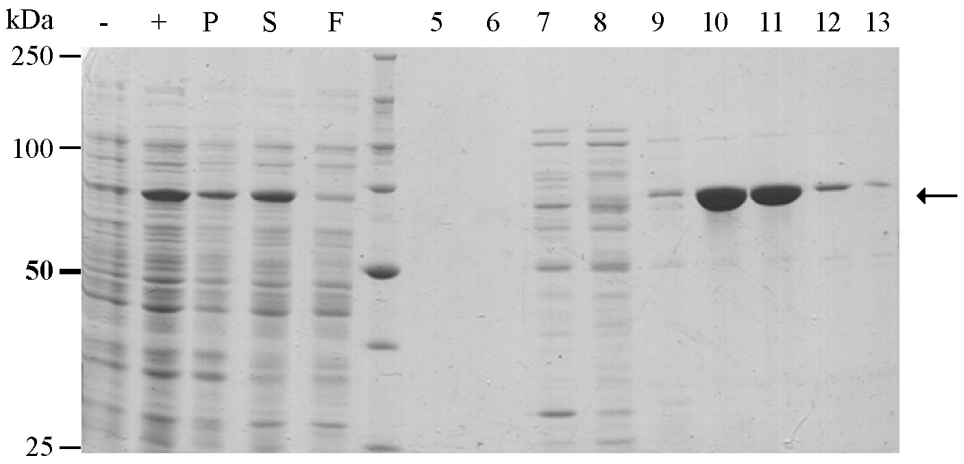


Fig. 4.56: Coomassie Blue stained gel of fractions from each purification step of HMW2-AG (10% SDS-PAA gel). – = whole cell extract of *E. coli* without the fusion protein; + = whole cell extract of *E. coli* containing HMW2-AG; P = pellet; S = supernatant; F = flow through; 5-13 = number of collected fraction.

4.7.3 Tests of the new antiserum

For each immunization 0.6 milligram of His-tagged HMW2-AG was used (for details see "Material and Methods"). After each boost, specificity of the antiserum was tested by Western blotting. Antisera of all boosts were compared with each other and also with the previous N-terminal antiserum (Fig. 4.57).

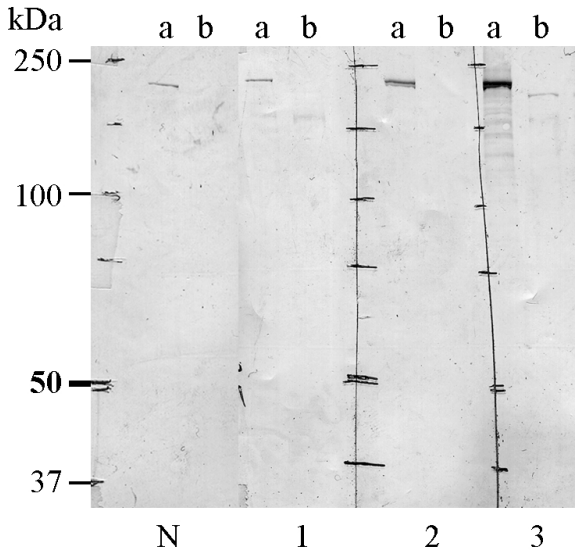


Fig. 4.57: Western blot analysis of a whole cell extract of *M. pneumoniae* WT (a) and of the *M pneumoniae* A3 (b) using various antisera directed against N-terminus of HMW2 protein.
N = antiserum directed against 29 aa [Regula, 1999]
1 = antiserum directed against 573 aa, first boost
2 = antiserum directed against 573 aa, second boost
3 = antiserum directed against 573 aa, third boost

The highest intensity of a reaction with HMW2 was monitored when probed with an antiserum derived from the third boost. This serum can be diluted at least 1: 10,000 and still gave a strong signal (Fig. 4.58).

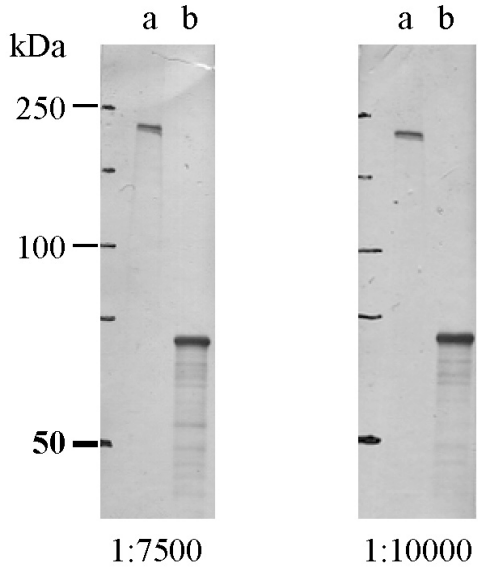


Fig. 4.58: Western blot analysis of a whole cell extract of *M. pneumoniae* WT (a) and the purified N-terminal HMW2 fusion protein (b) using the new antiserum taken after the third boost.

Furthermore, this antiserum showed no cross-reaction with any other proteins of *M. pneumoniae* whole protein extracts, indicating that this newly produced antiserum was very specific.

4.7.4 Localizing HMW2 in *M. pneumoniae* with a new anti-HMW2 antiserum

The new anti-HMW2 antiserum was then used for localization of HMW2 in *M. pneumoniae* cells by immunoelectron microscopy. This was done by Dr. Hegermann (AG Prof. Meyer, Goettingen). A reaction at the proximal region of the rod was observed in labelling experiments of ultrathin cryosections with this new antiserum (Fig. 4.59).

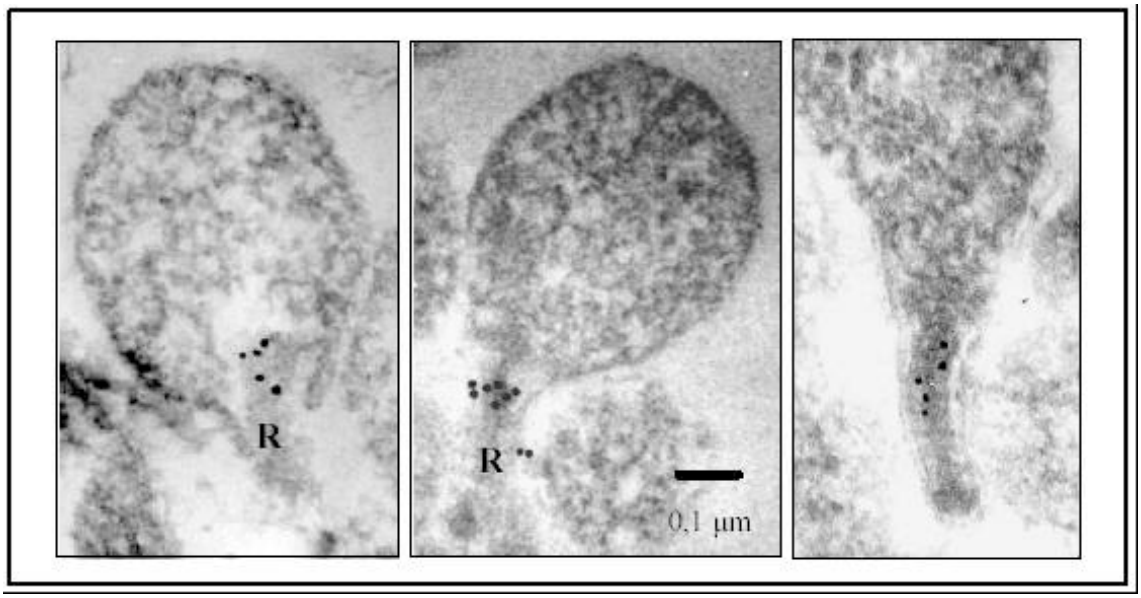


Fig. 4.59: Immuno-gold labelling of ultrathin cryosections of *M. pneumoniae* with a new anti-HMW2 antiserum. Signals in proximal region of rod was detected. R: rod (taken from Hegermann, 2003).

No signal in so called whole-mount-preparations was observed, neither by cells with intact cytoplasma membrane nor by cells after removing their cytoplasma membrane. This leads one to assume that HMW2 is not exposed on the surface and is also not a part of the peripheral cytoskeleton [Hegermann, 2003].

The positive reaction near the proximal region of the rod suggested a participation of HMW2 in the structure of the rod or a participation in the formation of the circular structure at the proximal end of the rod (Fig. 1.3).

5 Discussion

5.1 Expression of HMW2

Expression in *E. coli*

Protein expression in *E. coli* provides the possibility to obtain large quantities of a desired protein which can be used for a wide range of studies including vaccine production, structural, biochemical and cell biological studies. *E. coli* is most commonly used as a host for the expression of recombinant proteins, although a foreign protein, when expressed, often forms an insoluble product, termed an inclusion body or undergoes degradation by proteases. Most of those problems arise from misfolding of the expressed protein, being a main obstacle for expressing protein in large amount in *E. coli*. This might be caused by some noxious properties of the gene product (e.g. instability, misfolding, toxicity to the host, degradation of the 'foreign' mRNA etc.) [Ausubel et al., 2003].

The full-length HMW2 protein was expressed in *E. coli* in so small quantities so that it was only visible by Western blotting. Since a single subfragment of 573 amino acids covering the complete N-terminal region of HMW2 could be expressed very well, it must be either the size of the protein and the extended coiled-coil structure which prevents effective translation or stability in *E. coli* or some sequences in the C-terminal half of HMW2. An indication of instability is the high background of protein bands with lower molar masses observed in SDS-PAA gels combinded with Western blot analyses. This background indicates that HMW2 was degraded very fast in *E.coli*, although a variety of growth parameters was tested, which may provide a greater yield of non-degraded fusion protein in the soluble fraction. Several theories have been advanced to explain the susceptibility of certain proteins to degradation (e.g. the N-end rule [Varshavsky, 1997]), the function of the majority of proteases, and regulation of proteolysis within cells.

Difficulties in expressing large proteins with extended coiled-coil domains have also been observed. For example, attempts to purify full-length Tpr* from various mammalian tissues, or to isolate the full-length N- and C-terminal domains from bacterial or insect expression systems, did not yield sufficiently high amounts of undegraded protein [Brettel, Hase, Herrmann, Krohne, and Cordes, unpublished data; Hase et.al, 2001].

The expression of HMW2 in *E. coli* was regulated by the lac control region. It was observed in Western blot analyses of the expressed HMW2 that this system was not

* protein containing predicted coiled-coil domains. It is a component of nuclear pore complex (NPC)-attached intranuclear filaments [Hase et.al, 2001].

controlled tightly enough. Expressing the HMW2 protein occurred without induction by adding IPTG. In addition to reducing the bacterial growth temperature and induction time, a much tighter controlled expression unit e.g. the "tet system" [Lutz and Bujard, 1997]) could help to reduce degradation of foreign protein expressed in *E. coli* keeping expression turned off until the clone has been grown up to a relative high titer and allowing them only one or two hours of protein synthesis.

Expression in other organisms

Like mitochondria, *Mycoplasma* species utilize the UGA codon to encode tryptophan [Inamine et al., 1990] rather than to serve as a stop codon. In general, *Mycoplasma* genes containing one or more UGA codons will not be expressed due to premature termination in commonly used expression systems that strictly adhere to the universal genetic code. An exception is the very limited readthrough of the UGA stop codon in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium [Parker and Wiley, 1989; Roth, 1970]. Interestingly, although UGA also functions as a termination codon in *Bacillus subtilis*, the efficiency of UGA readthrough in this Gram-positive bacterium is relatively high due to the presence of a tRNA that reads the UGA termination codon as tryptophan [Lovett et al., 1991]. Therefore, UGA readthrough in *B. subtilis* happens much more often than that in other members of the family *Enterobacteriaceae* [Karow et al., 1998].

Furthermore, *B. subtilis* strains with mutations in the structural gene (*prfB*) for release factor 2 (RF2) showed increased readthrough levels by overcoming UGA-mediated termination [Karow et al., 1998]. Based on those observations, Kannan and Baseman expressed in *B. subtilis* the specific UGA-containing genes encoding the P30 adhesin (one UGA) of *Mycoplasma pneumoniae* and methionine sulfoxide reductase (two UGAs) of *Mycoplasma genitalium* [Kannan and Baseman, 2000]. Due to natural UGA suppression, these *Mycoplasma* genes were expressed as full-length proteins, but with a relatively low efficiency.Expression of the P30 adhesin in *E. coli* resulted in two bands, one migrating to the expected position and another one indicating a higher molar mass [Kannan and Baseman, 2000].

Similar experiences have been made with other genes of *Mollicutes*. Multiple protein bands were observed expressing *Mycoplasma hyorhinis* genomic fragments in *E. coli* [Notarnicola et al., 1990]. The *Spiroplasma* gene encoding spiralin contains no UGA codons but also generated multiple recombinant proteins in *E. coli* [Mouches et al, 1985]. Expression of a set of multiple proteins exceeding the coding capacity of a cloned genomic fragment from *Mycoplasma capricolum* has also been described for *E. coli* [Andersen et

al., 1984]. Differences in UGA codon usage and initiation sites for translation in *E. coli* may account for these variations in protein size [Notarnicola et al., 1990].

Besides expression in baterial hosts, expression of HMW2 in *Dictyostelium discoideum* might also be another alternative choice for expression of coiled-coil-rich proteins [Schwarz et.al., 1999]. *Dictyostelium discoideum* is being used as a model organism to investigate the involvement of the actomyosin* cytoskeleton in a variety of cellular tasks. Two novel, unconventional myosins were identified in this organism, MyoK and MyoM [Schwarz et.al., 1999]. This organism was used to express myosins succesfully [Schwarz et.al., 1999]. Since HMW2 has some sequence similarity to the tail region of myosin II heavy chain [Krause et al., 1997], HMW2 might be more stable when expressed in *Dictyostelium discoideum* than in *E. coli*.

Codon usage

As mentioned above, differences in codon usage among organisms cause to a variety of problems concerning heterologous gene expression, since not all 61 codons are used equally. The so-called major codons are those that are used in highly expressed proteins, whereas the minor or rare codons tend to be in genes expressed at a low level. Which of the codons are the rare ones depends strongly on the organism. The codon usage of *E. coli* and *M. pneumoniae* is presented in Table 7.1 (Appendices). For more information on the low usage codons of both organisms see Table 7.2.

Usually, the frequency of the codon usage reflects the abundance of their cognate tRNAs. Therefore, when the codon usage of the target protein differs significantly from the average codon usage of the expression host, this could cause problems during expression such as: decreased mRNA stability (by slowing down translation); premature termination of transcription and/or translation (which leads to a variety of truncated protein products); frameshifts, deletions and misincorporations (e.g. lysine for arginine) and inhibition of protein synthesis and cell growth [Kane, 1995].

As a consequence, the observed levels of expression are often low or there will be no expression at all. Especially in cases were rare codons are present at the 5'-end of the mRNA or in clusters expression levels are low and truncated protein products are found.

Several codons that have been associated with translation problems in *E. coli* are: arginine (AGG, AGA, CGA, CGG), leucine (CUA), isoleucine (AUA), proline (CCC) and glycin (GGA). For instance, the presence of more than 8-9 of the two rarest Arg codons, AGG

* a protein complex composed of actin and myosin. It is a constituent of muscle fibre and is responsible for muscular contractions.

and AGA, particularly back-to-back as a tandem repeat, has prevented over-expression of the SurEα proteins of *Pyrobaculum aerophilum* [Mura et al., 2003].

Interestingly, there are significant differences in the codon usage of the *hmw2* gene (table 7.1, Appendices) when compared to the codon usage of *E. coli*. The most significant difference concerns the CGG codon (coding for arginine). The frequency of this codon in the *hmw2* gene was 10.4 fold higher than in *E. coli.* In addition, the following codons from the *hmw2* gene also differ significantly from the average codon usage of *E.coli*: AAG (lysine, 6.1 fold), CAA (glutamine, 6.1 fold), UUA (leucine, 3.8 fold), CUG (leucine, 2.6 fold), GAA (glutamine, 2.1 fold) and UUG (leucine, 2 fold). These seven codons represent 60.7 % of the complete set of codons from the *hmw2* gene.

Moreover, the codon which is most predominantly presented in the gene *hmw2* (245 out of 1818 codons) is the CUA codon (coding for leucine). It is distributed over the entired sequence of *hmw2*, particularly in the middle region of the gene (Fig. 7.1, Appendix). They are frequently found to be located near each other (e.g. at gene position 2059 and 2074; 2854 and 2860; 3097 and 3118; 3280 and 3298). It was shown that the truncated parts of *hmw2* gene could be expressed very well in *E.coli* (chapter 4.2 and 4.7). Those regions of *hmw2* contain different rare codons, where the CUA codon is dominating in the middle region of the sequence. Based on this observation, both the predominance of the leucine codon CUA in *hmw2* sequence and the significant differences of codon present in *hmw2* gene and the average codon usage in *E. coli* might be the reasons for poor expression of the HMW2 full-length protein in *E. coli*.

To improve the expression levels of HMW2 in *E. coli*, several approaches could be taken:

• Replacing codons that are rarely found in highly expressed *E. coli* genes with more favourable codons throughout the whole gene.

• Co-expressing the genes encoding for a number of the rare codon tRNAs. There are several commercial *E. coli* strains available that encode a number of tRNAs with rare codons gene [Carstens and Waesche, 1999; Schenk et al., 1995; Wakagi et al., 1998].

• Examining the second amino acid after the start methionine. In endogenous *E. coli* proteins not all codons are used to the same extend as the second triplet (following the N-terminal methionine). The most used one is AAA lysine (13.9%) while other codons are not used at all. Looman and colleagues showed that the expression efficiency of a modified *lacZ* gene varies at least 15 fold, depending on the codon at the second position. Thus, choosing the right codon in this position, e.g. changing it into one that is moreoften used in *E.coli*, could improve expression levels [Looman et al., 1987]. Later on, by establishing the

N-end rule, Varshavsky showed convincingly that the half-life of a protein is strongly induced by the amino acid at the second position [Varshavsky, 1997], which is in agreement with the observation of Looman.

• Minimizing the GC content at the 5'-end. A high GC content at the 5'-end of the gene of interest usually leads to the formation of secondary structure in the mRNA. This could result in interrupted translation and cause lower levels of expression. Thus, higher expression levels could be obtained by changing G and C residues at the 5'-end of the coding sequence to A and T residues without changing the amino acids [Voges et al., 2004].

• Addition of a transcription terminator (or an additional one if one is already present).

• Addition of a fusion partner. Fusion of the N-terminus of a heterologous protein to the C-terminus of a highly-expressed fusion partner often results in high level expression of the fusion protein, e.g. a His-tagged derivative of bacteriophage Lambda head protein D (GpHD) [Forrer and Jaussi, 1998] or an N-terminus sequence of human interleukin 1beta (HIL-1beta) [Lee et al., 1999].

• Using protease-deficient host strains. The use of host strains carrying mutations which eliminate the production of proteases can sometimes enhance accumulation of the desired protein by reducing proteolytic degradation. *E. coli* strain BL21, the work horse of *E. coli* expression, is deficient in two proteases encoded by the *lon* (cytoplasmic) and *omp*T (periplasmic) genes.

5.2 Internal start in *hmw2* gene

The existance of HMW2-s was first observed in Western blots of total protein extracts from *M. pneumoniae* when probed with an antiserum which was directed against the C-terminal region of HMW2 [Proft, 1994]. This monospecific antiserum was made by immunization of rabbits with a 320 amino acids long peptide from the C-terminal region of HMW2 (position 1459-1778). It reacted with a 216-kDa protein and a 25-kDa protein (HMW2-s) in Western blot experiments of total protein extracts from *M. pneumoniae* WT but monospecific antibodies directed against a peptide from the N-terminal region of HMW2 reacted only with a 216-kDa protein and not with HMW2-s.

The complete loss of HMW2-s in *M. pneumoniae* A3 (*hmw2*$^-$), of which the *hmw2* gene was inactivated by a transposon insertion close to the 3' end of the gene *hmw2*, raised the possibility that HMW2-s might be a product of an internal translation initiation [Krause et al., 1997]. It was argued that two different ATG codons near the 3' end of *hmw2* at nucleotide positions 4735 and 4858 could initiate start of translation to generate, in frame

with HMW2, a polypeptide of 25 kDa. Exhaustive attempts to determine the N-terminal end or to obtain amino acid sequences from internal regions were unsuccessful [Krause et al., 1997]. In this work, the N-terminus of HMW2-s was sucessfully identified. The result of mass spectrometric analyses revealed that HMW2-s starts with methionine, which is encoded by the ATG codon at position 4858. Results from chapter 4.3, together with observations about HMW2-s in different *hmw2* mutants are summarized in Fig.5.1.

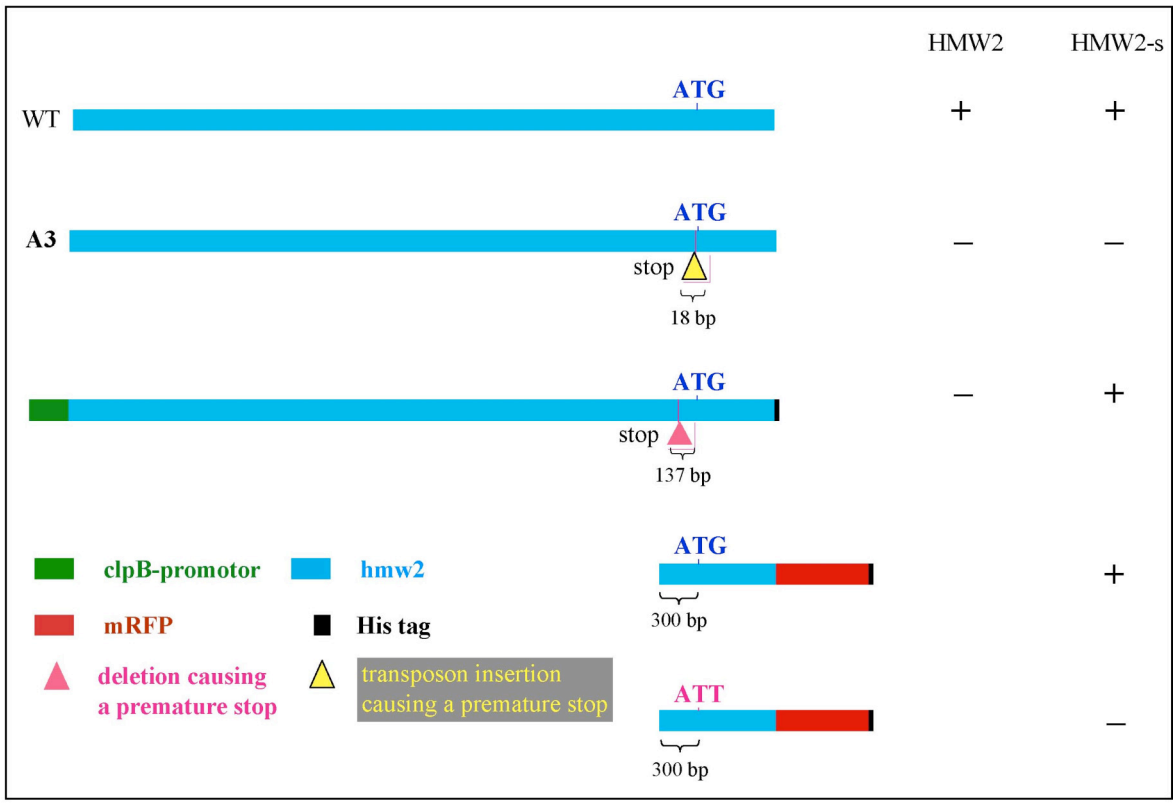


Fig. 5.1: Summary of HMW2 and HMW2-s proteins synthesized in *M. pneumoniae* from natural and artifical *hmw2* genes (see chapter 4.3). WT = *M. pneumoniae* WT; A3 = *M. pneumoniae* mutant A3.

It was shown in chapter 4.3 that HMW2-s can be produced despite deleting 2/3 from the *hmw2* gene. This supports strongly the hypothesis of an internal new start within the *hmw2* gene. The DNA sequence of the 18 nucleotides upstream from ATG start codon of HMW2-s seemed to play critical role for the expression of HMW2-s (Fig. 5.1, A3). A detailed analysis of the sequences in this DNA region showed a potential promotor region (-10) of a *M. pneumoniae* gene (Fig. 5.2). The characteristic features are the conserved -10 region, absence of a conserved -35 region, and a short leader mRNA without a pronounced ribosomal binding site.

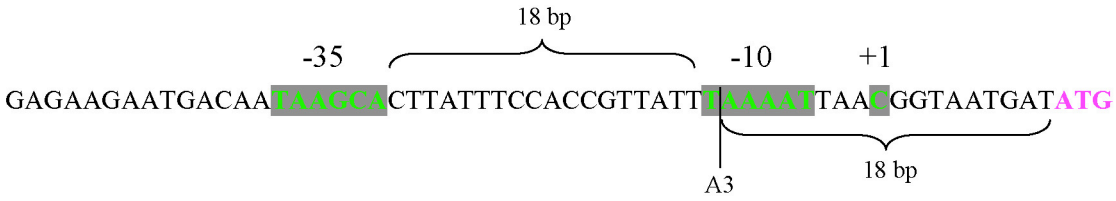


Fig. 5.2: The proposed promotor region for *hmw2-s*.

The sequence TAAAAT of the -10 region is also found in the promotors of the genes MPN083 (lipoprotein), MPN446 (*rps*4), MPN449 (unknown), MPN454 (unknown), MPN 455 (*cta*D), MPN 560 (*arc*A), MPN592 (lipoprotein) and MPN309 (*p65*). The sequence TAAGCA of the -35 region is identical to those of MPN455 (TTAGCA), MPN401 (TTGGCA) and again, the MPN309 (TTTGCA). These data provide additional support that HMW2-s is indeed synthesized by an internal start codon. Moreover, the proposed promotor region shows similarity to the promotor of the P65 operon, of which the gene *hmw2* is part of. Interestingly, this internal start codon seemed to be specifically used by *M. pneumoniae* only, since HMW2-s has never been observed in protein extracts from *E.coli* containing the full-length, UGA free *hmw2* gene (see chapter 4.1).

Internal translational start site also occurs in other organisms, for instance in the *clpA* gene of *E. coli* [Seol et al., 1994, 1995]. The *clpA* gene has dual translational start sites encoding two polypeptides with different sizes, 84 kDa and 65 kDa; referred to as ClpA84 and ClpA65, respectively. The smaller polypeptide is derived from the internal start site [Seol et al., 1994]. It has been suggested that ClpA65 may play an important role in the regulation of the ClpA84 level and hence in the control of ATP-dependent protein breakdown in *E. coli* [Seol et al., 1995].

Although the HMW2 homologue (MG218) is expressed in *Mycoplasma genitalium*, a protein corresponding to HMW2-s has not been identified in that organism [Dhandayuthapani et al., 1999]. Interestingly, the C-terminal region of the *M. genitalium* homologue of HMW2 lacks a predicted trivalent coiled coil region (Fig. 5.3) possibly obviating a HMW2-s-like molecule in this organism. However, the roles of HMW2 and HMW2-s in the biogenesis of the attachment organelle remain poorly defined. To understand the function of HMW2-s, it would be desirable to have a mutant with a complete functional HMW2 but incapable to synthesize HMW2-s.

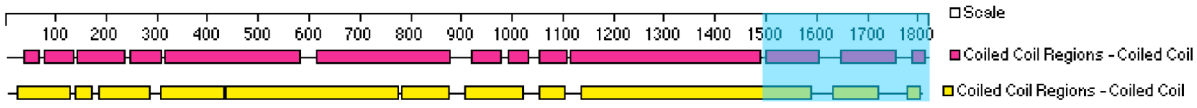


Fig. 5.3: Schematic illustration of the HMW2 homologous protein of *M. genitalium* (yellow) and comparison with the HMW2 of *M. pneumoniae* (pink). The C-terminal region (marked with blue) of the *M. genitalium* homologue of HMW2 lacks a predicted trivalent coiled-coil region.

5.3 Effects of HMW2 on the stability of other proteins from *M. pneumoniae*

The rationale behind the comparative analyses of protein profiles from M. pneumoniae WT and *hmw2*¯ mutants was that proteins which normally interact directly with HMW2 are rapidly degraded if the interacting partner protein HMW2 is absent.

Comparative Western blot analyses:

By comparative Western blot analyses, changes in the concentration of 12 selected proteins, candidates for the interaction with HMW2 (Table 4.1) in *M. pneumoniae* mutant A3 were compared with those from WT. The concentration of five from these proteins (HMW1, HMW3, P65, P41 and P24) have been previously reported to be reduced in the mutant A3 [Krause et al., 1982, 1997]. My data support these earlier notions. The most significant new results concerned three proteins of the P1 operon (P1, P40 and P90). They could not be detected in the protein extracts of the mutant A3. This indicated that they might undergo a rapid degradation in the absence of HMW2. These results supported data from two-hybrid pairwise tests of HMW2 with P1, suggesting that P1 and HMW2 interact at least indirectly with each other by HMW1, which was able to bind to both P1 and HMW2. The loss of all proteins of the P1 complex in *hmw2*- mutants might be a consequence of the missing binding partner HMW1 which has a high turnover rate in the absence of HMW2. Without HMW2, the P1 complex probably can not be formed and inserted properly in the membrane, making those proteins useless for the bacterium and are therefore degraded.

The concentrations of two other proteins, which reacted positively in the two-hybrid screen pilot experiment, P115 and the gene product of MPN297, was also determinded in the mutant A3. No significant changes of their concentrations was observed. P115 was part of an enriched protein complex containing HMW2 indicating that P115 might interact directly or indirectly with HMW2. The protein encoded by MPN297 was shown by two-hybrid experiments to directly interact with HMW2. Although its concentration was not reduced, higher multimeric forms observed in the WT profile could not be observed in the mutant A3. Since this protein was found to interact also with itself, it could be speculated that HMW2 might affect the polymerization of this protein, and therefor monomeric and trimeric forms of this protein accumulated in the mutant A3 but higher multimers were not formed. These results suggest that P115 and the gene product of MPN297 might have at least two functions, of which one is independent of HMW2 and therefor their concentrations are not significantly affected by the absence of HMW2.

Comparative 2-D gel electrophoresis

For a more comprehensive analysis of the effect of HMW2 on other proteins of *M. pneumoniae*, 2-D gel electrophoresis was selected as a method. Changes of protein concentration of individual proteins from *M. pneumoniae* WT and *M. pneumoniae* A3 in total soluble protein extracts were monitored.

Twelve proteins from a total of 41 analyzed proteins were found to have a significant decrease in their concentrations in the mutant A3 (Table 5.1). Most of those were enzymes involved in different metabolic activities. Five of the identified proteins with significant reduction were also found in the Triton X-100 insoluble fraction. The connection of the different enzymes to HMW2 is not clear at all. It could be argued that these enzymes are organized in the bacterium forming the so called enzoskeleton* [Norris et al., 1996] and that HMW2 does participate in this organization.

Table 5.1: Proteins with significant differences in concentration in *M. pneumoniae* WT and A3 detected by comparative 2-D gel electrophoresis

Presence in the Trition X-100 insoluble fragment		**Absence in the Triton X-100 insoluble fragment**	
MPN Number	Annotation	MPN Number	Annotation
MPN082	Transketolase 1 (TklB),	MPN050	Glycerolkinase (glpK)
MPN321	Dihydrofolate reductase (DhfR),	MPN051	Glycerol-3-phosphat-dehydrogenase
MPN452	HMW3	MPN062	Purinnucleosid-phospholyrase (deoD)
MPN456	Conserved hypothetic putative lipoprotein	MPN191	RNA-polymerase alpha-core-subunit (rpoA)
MPN674	1-Lactate dehydrogenase (Ldh)	MPN533	Acetatkinase (ackA)
		MPN479	Acylcarrier-proteinphosphoesterase
		MPN600	ATP-Synthase alpha chain (atpA)
		MPN631	Elongationfactor Ts (tsf)

HMW3 was the only protein of those compared by Western blotting (see also 4.4.1), the reduction of which could be also confirmed by the 2-D gel approach. The other proteins from the Western blot analyses were probably not separated well enough or could not be analyzed for technical reasons. The following limitting factors of the 2-D gel approach have to be considered:

* Metabolic pathways form multi-enzyme complexes which channel substrates and which connect membranes and nucleic acids to create the extensive, cross-linked, intracellular structure termed 'Enzoskeleton'. This enzoskeleton includes eukaryotic-like cytoskeletal structures and elements such as the MukB and FtsZ proteins. The enzoskeleton was proposed to be regulated by calcium and by protein phosphorylation during adaptation to different environments and during the cell cycle [Norris et al., 1996].

About 50 % of *M. pneumoniae* proteins have a basic isoelectric point. With the standard 2-D gel electrophoresis, as described by Görg and coworker, a pH range from 3 to 10, under special conditions to pH 12, can be covered [Görg et al., 1995]. But the basic section is not focussed as well as the acidic section. So, in practical terms, identifications of strongly basic proteins with a pI above 10, are still a problem applying standard 2-D gel electrophoresis. Furthermore, the silver staining according to Blum does not reliably work with basic proteins, i.e. these will be stained only after a long exposure or the staining will be incomplete and does not show a correct comparison.

The separation of particular small proteins (< 6 kDa) und large proteins (> 100 kDa) is also difficult by standard 2-D gel electrophoresis. Small proteins move with the protein front and will not be separated into single spots. They are normally incompletely fixed in the gel and will be washed out [Ueberle, 2001]. Moreover, the accumulation of Sodium dodecylsulfat ions in the gel can cause poor resolution [Harry et al., 2000]. Therefore, for the separation of small proteins special gel systems are required, which separated only proteins with molar masses below 25 kDa.

High molecular proteins are also usually underrepresented in 2-D gels. They are lost in 2-D gels for several reasons: they are badly solubilized under the conditions of separation according to charge in the first dimension. Based on their electrophoretic mobility, they focus very slowly and appear only as a strip, or due to dilution effects, are not visible even with the sensitive silver staining method [Ueberle et al., 2002]. For instance, HMW1, pI 3.79 and a molar mass of 112,200 Da, and HMW2, pI 9.78 and a molar mass of 215,600 Da, are barely detectable, although, based on pI and molecular mass, they should be very well separated.

Another group of proteins, which are also well-known to be underrepresented in 2-D gels, are membrane proteins. The separation of membrane proteins is most critical in 2-D gel electrophoresis. Besides their usually basic pI-values, they are poorly solubilized in the used aqueous buffers due to their hydrophobicity. Membrane proteins with more than three predicted transmembran domains are normally not separated in 2-D gels [Ueberle et al., 2002].

Despite these limiting factors, it was shown here that the 2-D gel electrophoresis is a suitable method gaining in one experiment a fast overview on the protein compositions of most of the soluble proteins of a cell. Although 2-D gel electrophoresis provides very reproducible protein patterns, it gives however small variations which arise from gel to gel. Two-dimensional gel electrophoresis is time consuming, labor intensive and gel-to-gel variations is a major source of error. This makes it sometimes difficult to distinguish real biological differences from variations caused by technical problems. These disadvantages

were solved to some extend by the ETTAN- DIGE system (Amersham Biosciences) recently introduced as an improved method for doing comparative 2-D gel electrophoresis. This system uses size and charge-matched, spectrally resolvable fluorophore to pre-label protein samples to be compared individually, but separating them on a single 2-D gel. This allows comparing proteins from different sources e.g. from a wild type and a mutant of a given species with a much better accuracy and sensibility [Alban, 2003].

5.4 Proteins interacting with HMW2 in the two-hybrid pairwise tests

The yeast two-hybrid system detects direct interactions between pairs of proteins expressed in yeast, and as a consequence the reporter genes may be activated even by weak or transient interactions that might not be detectable by other physical methods such as co-immunoprecipitation. This system was used in this work to test direct interactions between all 49 possible pairs of the seven proteins/ peptide fragments. All these proteins were suspected to potentially interact with HMW2 based on circumdtantial evidence from other experiments.

Several candidates, suggested from pilot experiments of the two-hybrid screen with HMW2 fragment 3 (HMW2-3), were tested and some of their interactions were confirmed again by the pairwise test: interaction of HMW2-3 with the C-terminal fragment of HMW1 (MPN447) and with the MPN297 encoded 17-kDa protein. By applying the pairwise test, no interactions between HMW2-3 and two other candidates, P115 and the gene product of MPN298, were observed. This indicated that both proteins retrieved from the two-hybrid screen were probably false-positive results.

Two additional proteins, the C-terminal region of P1 and HMW2 fragement 4 (HMW2-4) were also tested in this two-hybrid pairwise experiment to prove their interaction with other proteins. P1 was observed by comparative protein analyses of *M. pneumoniae* WT and the mutant *M. pneumoniae* A3 (*hmw2*$^{-}$) to have a significantly higher turnover rate in the mutant A3. Although P1 did not to directly interact with HMW2-3 (or HMW2-4), but it was shown to interact with both HMW1 and, again, the gene product of MPN297, indicating an indirrect interaction of P1 with HMW2.

In this study, two proteins, the gene product of MPN297 and HMW2 fragment 4, were observed to interact with themselves, forming different kinds of homopolymers. These results support earlier observations that HMW2 showed a much higher molar mass by SDS-PAGE in the absence of reducing agents [Stevens and Krause, 1990; Krebs et al., 1995]

MPN447 (Cytadherence accessory protein (HMW1)

The cytadherence accessory protein HMW1 is one of the proteins proposed to be involved in formation of cytoskeleton-like structures in *M. pneumoniae*. It was shown to be located on the mycoplasma cell surface but lacks typical secretion signal, transmembrane sequences or significant homology to any proteins found beyond the mycoplasmas [Balish et al., 2001; Dirksen et al., 1996]. HMW1 has been localized to the attachment organelle [Seto et al., 2001, Stevens and Krause, 1991] as well as to the trailing filament [Stevens and Krause, 1991], depending on the experimental approach.

HMW1, as well as HMW2, HMW3, P65 and P200, have been found almost exclusively in the Triton X-100 insoluble fraction [Regula et al., 2001]. The loss of HMW1 in the mutant M6 (*hmw1*$^-$) is correlated specifically with HMW2 instability, the failure of HMW2 to localize to the attachment organelle, and the absence of an electron-dense core [Willby et al., 2004]. The C-terminal domain of HMW1 was reported to be essential for the proper localization of HMW1 to the attachment organelle [Willby et al., 2004], for its function in cell morphology [Hahn et al., 1998], for its accelerated turnover in the absence of HMW2 , and the ability to stabilize HMW2 [Willby et al., 2004].

The data reported above correlated with the results from the two-hybrid pilot experiments. If the HMW2 fragment 3 was used as bait and screened against random fragments of *M. pneumoniae* genomic DNA (pray), the C-terminal region of HMW1 was frequently identified as a positive clone. In the pairwise tests, the interaction of HMW1 C-terminal region with HMW2 fragment 3 was also confirmed. Furthermore, the C-terminal region of HMW1 was additionally found to interact with the C-terminal region of P1. This finding supported previous reports about interaction of these two proteins (more detail in "MPN141").

MPN297 (conserved hypothetical)

The gene product of MPN297 (H10_orf149) appeared to be a promising new protein candidate for the functional group of proposed structural proteins, not only because it is interacting with HMW2, but also because it was found in the Triton X-100 insoluble fraction [Regula et al., 2001] and has a predicted coiled-coil structures (Fig. 7.4, appendix). Protein fragments of the gene product of MPN297 were found as positive clones by screening HMW2 fragment 3 (bait) against the random fragments of *M. pneumoniae* genomic DNA (pray) in the two-hybrid pilot experiment.

In the pairwise test, using the entire protein, the interaction with HMW2 was confirmed. Interstingly, this protein was observed to interact with another important protein, the C-terminal region of the adhesin P1. Furthermore, it was shown to form homopolymers. This was also observed in Western blot analyses. Three different forms of the proposed 17-kDa protein of MPN297 were found in total protein extracts of *M. pneumoniae* WT, monomers, trimers and higher, not exactly defined multimeric forms.

MPN141 (Adhesin P1; ORF*5*, P1 operon)

The adhesin P1 of *M. pneumoniae*, a transmembrane protein with a molecular mass of 170 kDa, as well as the adhesin-related 30 kDa protein and the two cytadherence-associated proteins of 40 and 90 kDa cluster densely in the membrane of the attachment organelle [Dallo et al., 1990; Feldner et al., 1982; Franzoso et al., 1993; Layh-Schmitt and Herrmann, 1992, 1994]. P40, P9 and and the P1 molecules are retained in the Triton-X-100-insoluble fraction of th*M. pneumoniae* cells [Regula et al., 2001]. The participation of the P1 adhesin in gliding of *M. pneumoniae* was examined by Seto and coworker. They reported that anti-P1 antibodies reduced the gliding speed and removed the gliding cells from the glass over time in a concentration-dependent manner but had only a slight effect on nongliding cells, the authors concluded that the conformational changes of P1 adhesin and its displacement were involved in the gliding mechanism [Seto et al., 2005].

Although the influence of binding of the anti-P1 antibodies on other proteins for sterical reason was not considered. Layh-Schmitt and coworkers analyzed interactions of the P1 adhesin with other membrane proteins or with cytoskeleton-like proteins by cross-linking studies using the membrane permeable reagent paraformaldehyde. The cross-linked protein complex was isolated by immunoaffinity chromatography, and proteins complexed to the P1 protein were identified by immunoblot analysis and by mass spectrometry [Layh-Schmitt et al., 2000]. In addition to the P1 protein and a truncated form of the same protein, P30, P40, P90, P65, HMW1, HMW3, DnaK and E1*a* subunit of pyruvate dehydrogenase were found to be components of the isolated protein complex or complexes.

The interaction between P1 and HMW1 was confirmed by the pairwise tests in this work. The regions of P1 and HMW1 required for the physical interaction were determined. It was shown that the C-terminal part of both proteins interacted with each other. Furthermore, additional direct interactions of P1 with other proteins was detected by the pairwise test: the weakly/ transient interaction of P1 with the gene product of MPN297, which might not be detectable by other physical methods such as co-immunoprecipitation.

No direct interaction between HMW2 and P1 was observed in either the cross-linking experiments [Layh-Schmitt et al., 2000] or in two-hybrid experiments in this work. However, P1 was shown to interact with the same proteins which also interact with HMW2, indicating a indirect links between those two proteins.

HMW2 fragment 4

A speculative model for the structural role of HMW2 in the formation of the *M. pneumoniae* attachment organelle has been published [Balish et al., 2003]. This model described the possible role of HMW2-s together with HMW2 in the arrangement of the electron-dense core. It was suggested that HMW2 and HMW2-s might be arranged in parallel fashion. Monomeric HMW2 may cross over to interact with other monomers, resulting in a network. If dimer and trimer formations were important in specifying the degree of network interactions among HMW2 monomers, then HMW2-s might limit the cross-linking within the HMW2 network [Balish et al., 2003].

Physical associations of HMW2-s and part of HMW2 were determined by two-hybrid pairwise tests. Instead of the complete HMW2, the HMW2-4 fragment (amino acid position 1413-1818) was used in the pairwise test which included also HMW2-s (amino acid position 1617 – 1818). It was shown that the HMW2 fragment 4 could interact with itself, but not with the HMW2 fragment 3 indicating a specific defined binding region within this coiled-coil rich protein (HMW2 fragment 4). Concerning the proposed model, HMW2-s might specifically binds to the C-terminal region of HMW2 or even bind themselves, although no dimeric or multimeric forms of HMW2-s were found in Western blot analyses.

False positive results and disadvantages of the two-hybrid system

Although the two-hybrid system is presently one of the most powerful methods for detecting protein-protein interaction, this system still has several disadvantages. Based on the observation that most characterized proteins interact with 5–7 other proteins [Ito et al., 2000], about 30,000–40,000 interactions were estimated to take place in yeast [Snyder and Kumar, 2002]. These numbers are significantly higher than the 4500 interaction identified thus far. This is likely due to the fact that most of the studies done thus far have been carried out in pools of yeast transformants and therefor failed to detect all possible individual interactions [Zhu et al., 2003]. Therefore, the screens are not saturated and direct pairwise tests of all possible individual interactions have to be done. A second disadvantage of the two-hybrid system is that it identifies a large number of false positives, presumably by spurious interactions between proteins that do not normally occur *in vivo* [von Mering et al., 2002]. Approximately 50% of the interactions are estimated to be false positives [Zhu et al., 2003]. A third drawback of the conventional two-hybrid analysis is that the interaction occurs in the nucleus and uses a transcriptional readout. Consequently, the interaction of many membrane proteins and transcription factors cannot be measured.

The results of the pairwise tests in this work also showed that 50% of the interactions found in the two-hybrid screenings (pilot experiment) were false positives. The interactions of HMW2 fragment 3 with the C-terminal region HMW1 and with the gene product of MPN297 were confirmed, but the interactions of HMW2 with P115 and the gene product of MPN298 could not, and therefor they might be false postive results from the screening experiments.
However, P115 was found as a component of protein complexes isolated by means of the TAP-tagged HMW2. This disagrees with the negative result in the pairwise test, but supports the results of the two-hybrid screen pilot experiment. In these experiments, the initial interaction between P115 and HMW2-3 was found with a peptide fragment containing 173 aa of P115, but in this pairwise test the full-length P115 containg 983 aa was tested (Fig. 7.6, Appendices). This could lead to a negative result in this test, since large protein fragment should be avoided by two-hybrid experiment [Dr. Koegl, personal communication]. The two-hybrid system might not be able to accommodate such large fragement and the protein probably could not fold properly in yeast due to its large size. This could be one of the limitations in the two-hybrid system.
Limitation in developing a two-hybrid test for *M. pneumoniae* is the inability to express all proteins of *M. pneumoniae* in heterologous expression systems due to the unusual codon usage of the UGA codon. Although in *S. cerevisae* and other organisms this is a stop codon, in *M. pneumoniae* it codes for tryptophan. In the two-hybrid screening test, random, about 1200 bp long genomic DNA fragments of *M. pneumoniae* were prepared by sonifying the genomic DNA and then cloning individual fragments into the pray vector. That means, a fraction of the cloned *M. pneumoniae* fragments contains one or more UGA codons causing a premature translational stop of a potentially interacting peptide and will not be detected. The introduction of a UGA suppressor tRNA in the yeast strains could help, but this might create problems for the host, since the UGA (Trp) codon does not function as a terminal codon in all instances and promote readthrough and synthesis of elongated proteins.

Autoactivation

It must always be tested whether the fusion protein can initiate the transcription of the reporter gene by itself. If such a case happens, all other tests would be meaningless. It could be that such a protein is a transcription activator by itself. It has benn also described that short acidic domains are responsible for undesirable, nonspecific activation. Many proteins that autonomously activate reporter genes in the two-hybrid system have been found to contain such acidic domains. In fact, acidic residues are thought to be associated with activating regions of transcription factors [Ma and Ptashne, 1987; Ruden, 1992; Ruden et al., 1991].

Several examples for autoactivation were found in my studies, for instance, the C-terminal region of HMW1, which was strongly autoactive as bait construct and weakly autoactive as pray. Therefore, the pairwise test could be done only with the pray construct of the HMW1 C-terminal region. In addition, HMW2 and the gene product of MPN297, both in bait construct, were also weakly autoactive, while the P1 in the pray construct was found to be strongly autoactive.
Protein profiles indicate that the C-terminal region of HMW1 contains both coiled-coil domains and acidic domains (negatively charge region) (Fig. 7.3, appendix), both features increase the possibility for autoactivation of this part of HMW1. HMW2 fragment 3 does not contain significant acidic regions but there are extended coiled-coil domains present in the entire fragment (Fig. 7.2), being a possible reason for its weak autoactivity observed in the bait construct of this fragment. Although the gene product of MPN297 contains both coiled-coil and acidic domains (Fig. 7.4), it showed only a weak autoactivation when it baits construct was used. This might be due to its small size (149 aa), compared to the larger C-terminal region of HMW1 (399 aa) since insertions of large DNA fragments in two-hybird vectors could increase the possibility of autoactivation [Dr. Koegl, personal communication]. Autoactivation mostly occurred when proteins were used as bait [Dr. Koegl, personal communication]. However, the strong autoactivation in the pray construct observed for the C-terminal region of P1 might be caused by its acidic stretches within this fragment.
It is still unclear why a certain protein is autoactive in one construct but not in the another one. It should be noted, however, that not all acidic proteins have transcriptional activation function. According to observations from the pairwise tests, proteins containing acidic domain showed stronger autoactivity than those without such domains.

A spontaneous, nonspecific activation is often not very strong and therefor the possibility to get falsh-positive results can be reduced by strengthening the test conditions for the *HIS3* reporter gene. 3-Amino-1,2,4-Triazole (3AT), a competitive inhibitor of the *HIS3* gene product should help to reduce the activity of the *HIS3* gene and eliminate weak positive signals. This application is useful in library screenings to reduce the number of false-postive clones. But 3AT is a toxic substance, which could inhibit cell growth at concentrations above 30 mM, so that in case of a very strong autoactivation, additional selection markers should be applied. The use of two different reporter genes under the control of significantly different promoters automatically eliminates many false positive. In the case of a very strong autoactivation of the transcription of the reporter gene, suitable methods to use that protein in the two-hybrid experiment are not available. It would be

conceivable to remove the autoactivating domains from the protein by introducing deletions within the gene until no autoactivating function is left. However, one has to consider that such deletions could also eliminate the potentially interacting domains. If all these manipulations do not work, the choice of another test system with other binding and activator domains might solve the problem.

In the pairwise tests, I applied both, *HIS3* and *ADE2* reporter genes for identifying protein-protein interactions. The *GAL7* promoter of the *lacZ* gene appears to be activated in response to treatments commonly used for cell permeabilization, such as flash freezing. This causes background beta galactosidase activity that makes the use of filter assays unfeasible. However this is not a serious problem, since two simple and effective plate assays already exist using the *GAL2-ADE2* and *GALl-HIS3* reporters. The *GAL2-ADE2* reporter in this yeast strain was so efficient that at the end of proper pairwise testings, only true positive genes/ proteins are left. [James et al., 1996].

5.5 Components of the fraction from TAP-tag purification

The identified proteins (table 4.7) were retrieved by purification of the TAP-tagged HMW2. This indicated that those proteins could potentially interact with the HMW2 protein. The newly identified protein provide additional informations about the involvement of HMW2 in the organisation of cellular structures in *M. pneumoniae.*

HMW1 and the gene product of MPN297, which were found to interact with HMW2 in two-hybrid system, could be detected in the enriched protein complexes by Western blotting. This data supports their direct interaction with HMW2. Furthermore, P115 was also presented in this components, confirming its interaction with HMW2 derived from the initial two-hybrid screen pilot experiment. P1 and P90 were also found in very small amount in this components.

MPN426 (P115)

P115 is a member of the SMC (Structural maintenance of chromosomes) ubiquitous protein superfamily, members of which are present in almost all organisms so far analysed except for a few bacteria [Koshland and Strunnikov, 1996; Melby et al., 1998]. They function *in vivo* in chromosome condensation, chromosome segregation and DNA recombination repair in eukaryotes, and are able to introduce positive writhe into DNA *in vitro* [Graumann, 2001; Hirano 1999; Holmes and Cozzarelli, 2000; Strunnikov and Jessberger, 1999].

SMC proteins and the structurally homologous MukB protein are unusual ATPases that form antiparallel dimers, with long coiled-coil segments separating globular ends capable of binding DNA [Graumann, 2001; Harvey et al., 2002; Koshland and Strunnikov, 1996; Melby et al., 1998]. Recently, SMC proteins have been shown to be essential for chromosome condensation, segregation and cell cycle progression in bacteria [Britton et al., 1998; Graumann, 2000; Graumann, 2001; Jensen and Shapiro, 1999; Moriya et al., 1998].
In *M. pneumoniae*, P115 belongs to the same structural family with HMW2. It has the Leucine-Zipper motive and coiled-coil domains. Interestingly, it turned out by searching HMW2 for conserved domains (NCBI) [Marchler-Bauer and Bryant, 2004],that HMW2 also has conserved domains for SMC proteins (Fig. 7.2, appendices). Both fragment (HMW2 fragment 3 and P115 fragment identified from two-hybrid screen) contains ATPase domains. P115 proteins are also found in other mycoplasmas [Alexander, 1987; Notarnicola et al., 1991].

MPN015 (conserved hypothetical)

A non-redundant amino acid sequence database (National Center for Biotechnology Information, NIH) was searched for similarity of MPN015 to related proteins using the BLAST program [Altschul et al., 1990]. The output of the BLAST search showed both a similarity of the amino acid sequence and conserved domain (NCBI Conserved Domain Search; [Marchler-Bauer and Bryant, 2004]) of MPN015 to RimK, an RNA-binding protein [Koonin et al., 1994]. RimK is an enzyme that catalyzes in *E. coil* the addition of glutamic acid residues to the N-terminus of the ribosomal protein S6 [Kang et al., 1989]. The members of a protein family including RimK from *E.coli* are defined as ATP-dependent carboxylate-amine ligase activity, where acylphosphate intermediates are involved in the catalytic mechanism [Galperin and Koonin 1997]. Nothing is known about functional interaction of MPN 015 with HMW2 so far, future experiments like construction of mutants in MPN015 and their characterization have to be done.

MPN160 (conserved hypothetical; MG147 homolog)

No putative conserved domains were detected by the BLAST search of the amino acid sequence of MPN160. The gene product of MPN160 shows significant homology to MG147 of *M. genitalium* and poor similarity to Mfl543 of *Mesoplasma florum*. To further characterize this protein, additional experiments have to be done.

MPN392 (Pyruvate dehydrogenase E_1-beta subunit; PdhB)

Proteome maps of total protein extracts of *M. pneumoniae* showed that, PdhB gave a very strong protein signal [Ueberle et al., 2002]. It is also one of the main components of the Triton X-100 insoluble fraction [Regula et al., 2001]. In addition to its function as a pyruvate dehydrogenase, it was found as a surface exposed protein and acting as a fibronectin binding protein implying that PdhB has two completly different functions [Dallo et al., 2002]. In *B. subtilis*, PdhB was reported to effect the late stages of engulfment (or membrane fusion) during the sporulation [Gao et al., 2002].

The presence of HMW2 and PdhB, both in the Triton X-100 insoluble fraction and in the purified protein complex, provided evidence that PdhB might be one of the interacting partners of HMW2. To finally confirm this result, the physical interaction between HMW2 and PdhB has to be demonstrated by a second independent method (e.g. two-hybrid).

MPN430 (Glycerladehyde-3-phosphate dehydrogenase; Gap; GAPDH)

Glyceraldehyde-3-phosphate dehydrogenase is a key component of the glycolytic pathway. It is a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis. An interesting additional function of this protein is its ability of *M. genitalium* to bind human vaginal/cervical (V/C) mucin [Alvarez et al., 2003]. Antiserum raised against recombinant GAPDH blocked binding of intact biosynthetically labelled mycoplasmas to mucin to approximately 70% [Alvarez et al., 2003]. Whole cell radioimmunoprecipitation indicated that GAPDH was surface-accessible and surface localization of GAPDH was further verified by membrane fractionation and immunoelectron microscopy [Alvarez et al., 2003]. Their results indicated that 10% of total GAPDH is membrane localized [Alvarez et al., 2003].

The non-metabolic function of GAPDH (i.e. mucin binding) in *M. genitalium* is biologically appealing and not only provides insights into the organism's mechanisms of adherence and colonization but is also relevant to the genetic limitations and versatility of this microorganism. Impressively, the association of a certain percentage of GAPDH to the *M. genitalium* membrane surface confers a completely unrelated and unpredictable property. The important role of GAPDH from other organisms in targeted host cell parasitism has also been reported.

Pancholi and Fischetti [Pancholi and Fischetti,1992] described a surface located dehydrogenase (SDH) present in Group A streptococci with the ability to bind fibronectin, lysozyme, as well as the cytoskeletal proteins myosin and actin. SDH did not bind to the streptococcal M protein, tropomyosin, or the coiled-coil domain of myosin. This SDH was

later identified as GAPDH, and was found to be expressed on the surface of GBS (serotype III group B streptococci) [Seifert et al., 2003]. A similar phenomenon was reported in *Candida albicans* in which a phosphorylated form of GAPDH was surface located and possessed fibronectin binding ability [Gozalbo et al., 1998]. In eukaryotic cells, GAPDH is known to be associated with cytoskeletal structures and it binds specifically to both tubulins and colchicine [Gupta and Soltys, 1996]. In muscle cells, GAPDH-containing protein complex was suggested to be involved in reinforcement of established cytoskeletal structures [McDonald et al., 1998].

MPN573 (heat shock protein; GroEL)

Among all the protein spots indentified from the 2-D gel electrophoresis analyses of the total protein extract of *M. pneumoniae*, GroEL represented the fourth largest protein spot (6.5 % of the total spot volume) [Ueberle, 2001] indicating that it is one of the most abundant proteins in *M. pneumoniae*.

GroEL belongs to TCP-1/cpn60 chaperonin family. This family includes members from the HSP60 chaperone family and the TCP-1 (T-complex protein) family. Chaperonins are key components of the cell machinery and are involved in the productive folding of proteins. Chaperonins have been classified into two groups according to their sequence homologies: type I, whose better known member is GroEL, and type II comprising the eukaryotic cytosolic chaperonin (CCT) and the archaebacterial thermosome, among others [Carrascosa et al., 2001]. Two mechanisms have thus far been characterized for the assistance by chaperonins of the folding of other proteins. The first and best described is that of the prokaryotic chaperonin GroEL, which interacts with a large spectrum of proteins. A second mechanism has been described for the CCT, which interacts mainly with the cytoskeletal proteins actin and tubulin [Llorca et al., 2001]. Furthermore, GroEL requires a cofactor (GroES) that is not present in the type II chaperonins [Carrascosa et al., 2001].

Although *M. pneumoniae* has a cytoskeleton-like structure, it lacks sequence homolog to the CCT. Due to the reduced genome size of *M. pneumoniae,* GroEL might also take function like CCT and, playing a role as a universal chaperonin, interacting with 'cytoskeletal proteins' e.g. HMW2 of *M. pneumoniae*. The presence of GroEL in the purified protein complex of TAP-tagged HMW2 indicated the interaction of GroEL with HMW2. This finding supports the suggestion about possible additional function of GroEL as discussed above.

MPN665 (EF-Tu)

The elongations factor Tu (EF-Tu) is the second most prominent protein of the complete soluble protein extract of M. pneumoniae and of the Triton X-100 insoluble fraction [Regula et al., 2001]. EF-Tu binds an aminoacyl-tRNA (GTP-dependently) and inserts this at the correct anticodon-position into the nascent polypeptide chain. Hydrolysis of GTP to GDP changes the conformation of EF-Tu and releases the connection with the tRNA [Nyborg and Kjeldgaard, 1996]. EF-Tu is then present freely diffusable as a monomer protein in the cytosol.

Beside the well characterized feature of EF-Tu in the protein synthesis [Nyborg and Kjeldgaard, 1996; Yogev et al., 1990], structural characteristics of this protein were reported by Beck and coworkers. Purified EF-Tu polymerized *in vitro* to filaments and filamental bundles and binds DNase I in presence of KCl and $MgCl_2$ [Beck et al., 1978]. By applying a protocol for the isolation of actin, Neimark characterized a protein isolated from *Mycoplasma pneumoniae* [Neimark, 1977]. This protein had the same solubility as actin, a molecular mass of 45 kDa in the SDS-PAA gel, ATP- and Mg^{2+}-dependent filament formation, binding affinity to the heavy Meromyosin (specific cleavage product of myosin) and represents approximately 6% of the total protein by Coomassie Blue staining [Neimark, 1977]. This protein was first claimed to be a bacterial actin, but it turned out later that it was indeed EF-Tu. Similar characteristics were also described for EF-Tu of *E. coli* [Beck, 1978]. EF-Tu does not have any significant sequence similarity with actin. A gene with a sequence similarity to actin was not found in the annotation of the complete genome sequence of *M. pneumoniae* [Himmelreich et al., 1996; Dandekar et al., 2000].

Recently, a new aspect arose concerning the function of EF-Tu, since EF-Tu, together with PdhB, was found on the surface of *Mycoplasma pneumoniae* and showed binding affinity to fibronectin [Dallo et al., 2002].

By the isolation of protein complexes containing TAP-tagged HMW2, EF-Tu was found as a component indicating an (directly or indirectly) interaction between EF-Tu and HMW2. Considering the 'triple' functions of EF-Tu, one could speculate that HMW2 might somehow effect the host-pathogen interaction by it possible involvement in organizing the protein complex binding to fibronectin. The possibility also exists that EF-Tu forms cytoskeletal structures in cooperation with HMW2 or modulates the features of the cytoskeletal elements in *M. pneumoniae*, indepentdent of its function in translation. Whether the interaction between EF-Tu and HMW2 also plays a role in protein synthesis is still an open question. Mutants having an inactive *hmw2* gene could be used to test whether an interaction between HMW2 and EF-Tu is the prerequisit for binding of *M. pneumoniae* to fibronectin.

Proteins of the P1 operon: MPN140 (ORF4 gene product), MPN142 (P90)

Interestingly, proteins from the P1 operon [Inamine et al., 1988] were found in the purified protein complex of TAP-tagged HMW2. The 28 kDa encoded gene product of the ORF4 gene has still not been experimentally well characterized. Hence, the presence of this protein as a component of protein complex with the TAP-tagged HMW2 indicated that, in addition to P1 (from two-hybrid experiment), the gene product of ORF4 also link the cytadherence to cytoskeletal proteins in *M. pneumoniae*.

By the BLAST search [Dandekar et al., 2000; http://www.bork.embl-heidelberg.de/ Annot/ cgi/annot1.pl?min=014&max=014&toggle=0] MPN140 was shown to have sequence similarity to a novel family of predicted phosphoesterases (DHH family) including the Prune protein from *Drosophila melanogaster* and the bacterial RecJ exonuclease [Aravind and Koonin, 1998]. It was suggested that the gene product of ORF4, as a phosphoesterase, should functinally aid the attachment process by modification of selected proteins.

Recently it has been shown that the protein P40 and P90, cleavage products derived from the ORF6 gene (MPN142) of the P1 operon [Inamine et al., 1988; Sperker et al., 1991] are essential for the the correct integration of the adhesin protein P1 in the terminal organelle [Catrein et al., 2004; Waldo et al., 2005]. By cross-linking experiment, they were linked to other two proteins, P1 and P30, in the intact bacterial membrane indicating that these proteins are located as a complex in the tip structure of *M. pneumoniae* [Layh-Schmitt and Herrmann, 1994].

Summary of the co-purified proteins with TAP-tagged HMW2.

Presently, it can not be decided how the HMW2-containg proteins complexes look like. It is fair to assume that several different complexes were isolated and that the co-purified proteins do not have to bind directly with HMW2. Some may bind indirectly by a shared linking proteins. Other proteins, specially those which are very abundant, could be just contaminations. The protein complex containing HMW2 TAP-tagged was very insoluble and therefor appeared in the purified fraction only in very small amount that it could be detected by Western blotting using sensitive fluorescence like ECF method. To prove these preliminary results, isolation of protein complexes has to be repeated, now with those proteins tagged which were found in the HMW2 protein complexes.

The main problem is the low solubility of HMW2 and probably of all HMW2 containing complexes under native conditions. This does prevent the isolation of complexes in amounts sufficient to do extended protein biochemistry. Nevertheless, due to the increased sensitivity of the analytical methods e.g. mass spectrometry, even with small amounts od protein complex(es). The identification of their components can be done.

In addition, other independent methods should be applied to confirm specially the 'unsual interactions'. That are those with proteins-enzyme which were known to have functions in metabolic pathways. There are at least two alternative interpretations of these results: The enzymes have also a function as structural components and they interact for this reason with HMW2 or, HMW2-independent of its role in formation of cytoskeleton-like structures might be also involved in organizing those proteins into complexes which participate in the same metabolic pathway.

5.6 Localization of HMW2 in *M. pneumoniae*

The localization of HMW2 at the tip structure was proposed based on experiments with a fusion protein between HMW2 and GFP [Balish et al., 2003]. However, co-localization of the fluorescence signal and tip structure is not a convincing proof that HMW2 is the structural component of the rod, since additional structures have been observed in the tip organelle [Hegermann et al., 2002] and the resolution of fluorescence microscopy is by far to low to assign a fluorescene signal to one of the structures seen in thin sections with an electron microscope.

A more precise sublocalization of HMW2 was succesful with the new anti-HMW2 antiserum (Fig. 4.57). The immuno-gold labelling of ultrathin cryosections of *M. pneumoniae* with a new anti-HMW2 antiserum showed signals in the proximal region of the rod. This indicated that HMW2 is not exposed on the surface and is also not a part of the peripheral cytoskeleton [Hegermann, 2003]. The positive reaction near the proximal region of the rod suggests a participation of HMW2 in the structure of the proximal end of the rod or a participation in the formation of the attached circular structure.

Balish and coworker suggesteda model of the rod, in which HMW2 molecules, as main component of the rod, are arranged in parallel filaments [Balish et al., 2003]. Therefore, the rod is flexible and connected laterally with the cytosol oriented part of cell membran by linker. An organization of the rod mediated by parallel HMW2 dimers was proposed [Balish et al., 2003].

HMW1 - as a linker connected with the rod

It was previously shown that HMW2 stabilizes HMW1 [Balish et al., 2001]. The stabilization probably occures at the C-terminal region of HMW1 [Willby et al., 2004]. This region was also found to interact with HMW2 fragment 3 in the two-hybrid experiments. Furthermore, the two-hybrid pairwise tests also confirmed the interactions between the C-terminal region of HMW1 and the adhesin P1. The interaction between P1 and HMW1 was also revealed by cross-linking experiments [Layh-Schmitt et al., 2000].

Electron microscopic studies of cells carefully pretreated with Triton X-100 showed that P1 is localized at the tip of either cells with cytoplama membrane, or cells, from which the cytoplasma membrane was at least partially removed [Hegermann, 2003]. This protein seems to be inserted deeper in the membrane, possibly connected neighbouring with the peripheral cytoskelett. This is not unexpected, since specific computer programme predicts up to five transmembrane segments for P1. This would generate cytoplasmic-oriented P1 regions located between amino acids 1-14, 504-532 and 1124-1526. The last region was included in the P1 fragment used in the pairwise test (amino acid position 1156-1525), which interacted with HMW1.

By immune gold labelling, HMW1 could be located at the rod [Hegermann, 2003]. Based on these data, HMW1 was suggested to link between the rod and the peripheral cytoskelett [Hegermann, 2003]. The model of the cytoskeleton-like structure of *M. pneumoniae* described by Hegerman and other (Fig. 1.3), are based on the assumption that the rod consists of HMW2 and HMW1 functions as linker between the rod and the membrane [Hegermann et al., 2002].

Connection of P1-HMW1-HMW2

The interaction of HMW1 with the adhesin P1 has been shown previously by cross-linking experiments [Layh-Schmitt et al., 2000]. The suggested subcellular location of HMW1 as a connected ‘spoke’ linking with P1 is in agreement with the location of the P1 adhesion complex and its proposed integration into the membrane. The result of the two-hybrid pairwise tests suggest that the C-terminal region of HMW1 interact physically with both P1 and HMW2 fragment 3. These observations contradict the suggested model of the sublocation of HMW2 and HMW1.

It is still unclear is how could the C-terminal region of HMW1 interact simultaneously with P1 and HMW2, if HMW2 represented the ‘rod’ structure? If HMW2 indeed forms the long filament bundle in the middle of the ‘tip’ as observed in the electron microscope, how could the same region of HMW1 be connected to two proteins, which are probably not located in a

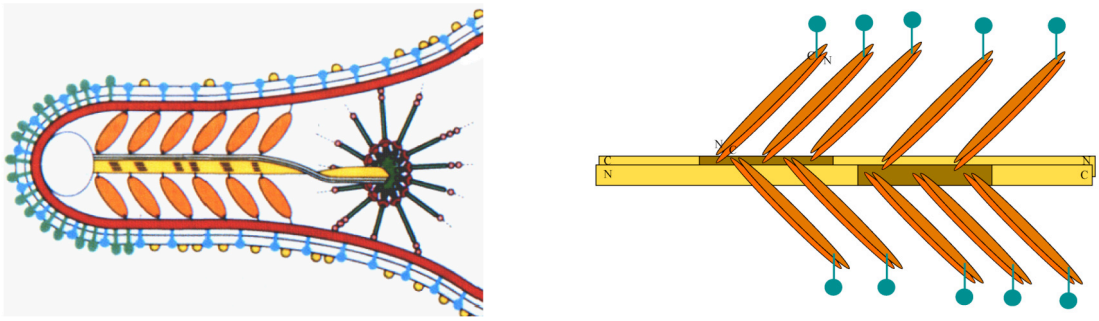
close distance, namely in the membrane and in the middle of the cell? One possible explanation is that HMW1 might form a head-to-tail structure, so that N- and C-termnial part of HMW1 are presented at both ends of the 'spoke' (Fig. 5.4). Based on the results from the two-hybrid analysis, the C-terminal region of HMW1 interacted only with the HMW2 fragment 3 and not with the HMW2 fragment 4, which suggests a certain binding specificity. If the 'spoke' are assembled from HMW1, which was observed to be distributed along the lateral site of the 'rod', then it is difficult to concieve a filamentous structure of HMW2.
Several models have been described trying to combine the results from different experimental approaches in a logical way. The main questions concern the protein composition of the rod structure in the attachment organelle. The main candidate for the structural component of the rod is presently HMW2. The structure could be assembled from HMW2 by forming fibers either head-head or head-to-tail, but there is still no convincing experimental evidence for this models.

5.6.3 Suggestions for localizing of HMW2 in *M. pneumoniae* and model of the possible organization of P1, HMW1 and HMW2

The protein A tag has been used in immunoelectron microscopy to localize various proteins fused with this tag [Rout et al., 2000]. In those experiments, affinity-purified rabbit IgG was used as primary and gold-labeled anti-rabbit IgG as secondary antibodies. Since localization of HMW2 with various antisera against different parts of HMW2 did not give a satisfying results, it seemed promissing to localize HMW2 indirectly by labelling the protein A domain of the TAP-tagged HMW2 with IgG. This would introduce an extended structure which is not coil-coiled like almost the entire HMW2. This new structure would improve the specific binding of IgG to this structure, and the possible false-positive results from the relative unspecific *in vivo* binding to of antibodies to coiled-coil structures would be eliminated.

To further define the physical interacting region of HMW1 and HMW2, the two-hybrid system provides a possibility for such investigation. Taking its advantage that domains of the interested protein can be individually tested. In the case of the two-hybrid pilot experiment, HMW2 fragemet 1 and 2, corresponding the N-terminal half of HMW2, showed autoactivation and could not be used in the screening experiments. This problem might be overcome by splitting each of both fragments into two smaller fragments, the smaller fragments might not be autoactive anymore and could be tested for their interaction

with the likewise splitted small domains of HMW1. This could provide us more details about all possible interacting region of those proteins, which could be useful to better describe or define the protein-localization and models of the cytoskeleton-like structure in *M. pneumoniae*.



A B

Fig. 5.4: A) Schematic illustration of the proposed model of the organization of cytoskeletal-like structure in M.pneumoniae [Hegermann et al., 2002].
B) Schematic illustration suggesting the possible organization of P1, HMW1 and HMW2 based on interactions known from literatures and this study.

= HMW2; = HMW2 fragment 3; = HMW1; = P1

5.7 Conclusions and perspectives

The mycoplasma cell might serve as a prokaryotic model organism for studying protein-protein interactions as well as structure-function relationships because its complete genome sequence is known, the number of genes and proteins (approx. 700) is relatively low, and its architecture (lack of cell wall) is simple.

With the aim to contribute to the understanding of the formation of cytoskeleton-like structures in *Mycoplasma pneumoniae*, the following experimental approaches to identify proteins interacting with HMW2 were taken: comparative Western blot analysis, comparative 2-D gel electrophoresis, two-hybrid pairwise tests and purifying of protein complexes containing TAP tagged HMW2. The most straightforward approach is the isolation of protein complexes, but this approach os hampered if the complex is not or poorly soluble. Important identified proteins interacting with HMW2 from all experimental approaches are summary in Fig. 5.5.

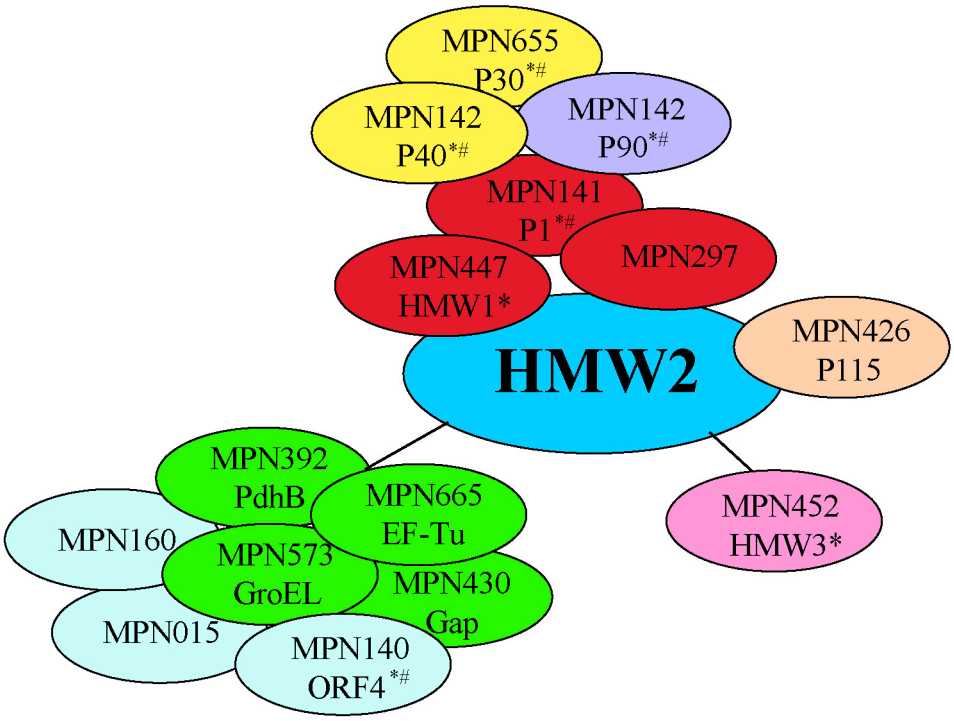


Fig. 5.5: Schematic illustration showing all identified proteins which are, based on experimental evidences, suggested to potentially interact directly or indirectly with HMW2.
= Triton X-100 insoluble fraction [Regula et al., 2001].
= result from 2-D gel analysis
= results from TAP tag purification
= results from TAP tag +Triton X-100 insoluble fraction
= results from the preliminary two-hybrid screen [Boonmee, unpublished] + TAP tag
= results from two-hybrid pairwise tests + TAP tag +Triton X-100 insoluble fraction
= results from TAP tag purification + Triton X-100 insoluble fraction.
* = results from comparative Western blot analyses. # = proteins from P1 operon [Inamine et al., 1988; Sperker et al., 1991]

The data presented here provide insights into the interactions 1) between the membrane and cytoskeletal proteins, 2) among cytoskeletal proteins and 3) between other cellular proteins in the cell and cytoskeletal proteins, of *M. pneumoniae*.

For a better understanding of the assembly of the attachment organelle of *M. pneumoniae*, further studies are needed to define: 1) assignment of specific proteins to observed structures, 2) the domains of individual proteins essential for the mycoplasma protein-protein interactions, 3) cellular process concerning mechanisms of the assembly of cytoskeletal structures, 4) regulations of their dynamic functions and 5) their possible functions in the cell.

6. Literatures

Abhayawardhane, Y. & Stewart, G. C. (1995). Bacillus subtilis possesses a second determinant with extensive sequence similarity to the Escherichia coli mreB morphogene. *J Bacteriol* 177, 765-773.

Alban, A., David, S. O., Bjorkesten, L., Andersson, C., Sloge, E., Lewis, S. & Currie, I. (2003). A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* 3, 36-44.

Alexander, A. G. (1987). Analysis of protein antigens of Mycoplasma hominis: detection of polypeptides involved in the human immune response. *Isr J Med Sci* 23, 608-612.

Allen, J. B., Walberg, M. W., Edwards, M. C. & Elledge, S. J. (1995). Finding prospective partners in the library: the two-hybrid system and phage display find a match. *Trends Biochem Sci* 20, 511-516.

Almagor, M., Yatziv, S. & Kahane, I. (1983). Inhibition of host cell catalase by Mycoplasma pneumoniae: a possible mechanism for cell injury. *Infect Immun* 41, 251-256.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* 215, 403-410.

Alvarez, R. A., Blaylock, M. W. & Baseman, J. B. (2003). Surface localized glyceraldehyde-3-phosphate dehydrogenase of Mycoplasma genitalium binds mucin. *Mol Microbiol* 48, 1417-1425.

Andersen, H., Christiansen, G. & Christiansen, C. (1984). Electrophoretic analysis of proteins from Mycoplasma capricolum and related serotypes using extracts from intact cells and from minicells containing cloned mycoplasma DNA. *J Gen Microbiol* 130, 1409-1418.

Aravind, L. & Koonin, E. V. (1998). A novel family of predicted phosphoesterases includes Drosophila prune protein and bacterial RecJ exonuclease. *Trends Biochem Sci* 23, 17-19.

Ausubel F.M. *et al.* (2003). Current protocols in molecular biology 4th edition, John Wiley & Sons Inc.

Bader, G. D., Betel, D. & Hogue, C. W. (2003). BIND: the Biomolecular Interaction Network Database. *Nucleic Acids Res* 31, 248-250.

Balish, M. F., Hahn, T. W., Popham, P. L. & Krause, D. C. (2001). Stability of Mycoplasma pneumoniae cytadherence-accessory protein HMW1 correlates with its association with the triton shell. *J Bacteriol* 183, 3680-3688.

Balish, M. F., Santurri, R. T., Ricci, A. M., Lee, K. K. & Krause, D. C. (2003). Localization of Mycoplasma pneumoniae cytadherence-associated protein HMW2 by fusion with green fluorescent protein: implications for attachment organelle structure. *Mol Microbiol* 47, 49-60.

Barettino, D., Feigenbutz, M., Valcarcel, R. & Stunnenberg, H. G. (1994). Improved method for PCR-mediated site-directed mutagenesis. *Nucleic Acids Res* 22, 541-542.

Baseman, J. B., Morrison-Plummer, J., Drouillard, D., Puleo-Scheppke, B., Tryon, V. V. & Holt, S. C. (1987). Identification of a 32-kilodalton protein of Mycoplasma pneumoniae associated with hemadsorption. *Isr J Med Sci* 23, 474-479.

Beck, B. D. (1979). Polymerization of the bacterial elongation factor for protein synthesis, EF-Tu. *Eur J Biochem* 97, 495-502.

Beck, B. D., Arscott, P. G. & Jacobson, A. (1978). Novel properties of bacterial elongation factor Tu. *Proc Natl Acad Sci U S A* 75, 1250-1254.

Biberfeld, G. & Biberfeld, P. (1970). Ultrastructural features of Mycoplasma pneumoniae. *J Bacteriol* 102, 855-861.

Blum, H., Beier, H. and Gross, H. J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels, *Electrophoresis*, 8 , 93-99.

Boatmen, E. S., (1979) Morphology and ultrastructure of the Mycoplasmatales, in The Mycoplasmas: Cell Biology (Vol. 1), Barile, M.F. and Razin, S., (Ed.) 63-102, Academic Press, New York.

Bohl, F., Kruse, C., Frank, A., Ferring, D. & Jansen, R. P. (2000). She2p, a novel RNA-binding protein tethers ASH1 mRNA to the Myo4p myosin motor via She3p. *Embo J* 19, 5514-5524.

Boonmee, A. (2000). Untersuchungen zur Protein-Protein Wechselwirkung in *Mycoplasma pneumoniae,* Diplomarbeit, Ruprecht-Karls-Universität Heidelberg.

Bork, P., Sander, C. & Valencia, A. (1992). An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. *Proc Natl Acad Sci U S A* 89, 7290-7294.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-254.

Bredt, W., Barile, M.F. und Razin, S. (1979). Motility in the Mycoplasmas. Akademic Press, New York.

Brent, R. & Ptashne, M. (1985). A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell* 43, 729-736.

Britton, R. A., Lin, D. C. & Grossman, A. D. (1998). Characterization of a prokaryotic SMC protein involved in chromosome partitioning. *Genes Dev* 12, 1254-1259.

Carballido-Lopez, R. & Errington, J. (2003). The bacterial cytoskeleton: in vivo dynamics of the actin-like protein Mbl of Bacillus subtilis. *Dev Cell* 4, 19-28.

Carrascosa, J. L., Llorca, O. & Valpuesta, J. M. (2001). Structural comparison of prokaryotic and eukaryotic chaperonins. *Micron* 32, 43-50.

Carstens, CP & Waesche, A. (1999). Codon bias-adjusted BL21 derivatives for protein expression. Strategies Newsletters (*Stratagene)* 12 (2), 49-51.

Catrein, I., Dumke, R., Weiner, J., 3rd, Jacobs, E. & Herrmann, R. (2004). Cross-complementation between the products of the genes P1 and ORF6 of Mycoplasma pneumoniae subtypes 1 and 2. *Microbiology* 150, 3989-4000.

Chanock, R. M., Hayflick, L. & Barile, M. F. (1962). Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. *Proc Natl Acad Sci U S A* 48, 41-49.

Chien, C. T., Bartel, P. L., Sternglanz, R. & Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci U S A* 88, 9578-9582.

Cremers, A. F., Bosch, L. & Mellema, J. E. (1981). Characterization of regular polymerization products of elongation factor EF-Tu from Escherichia coli by electron microscopy and image processing. *J Mol Biol* 153, 477-486.

Csank, C., Costanzo, M. C., Hirschman, J., Hodges, P., Kranz, J. E., Mangan, M., O'Neill, K., Robertson, L. S., Skrzypek, M. S., Brooks, J. & Garrels, J. I. (2002). Three yeast proteome databases: YPD, PombePD, and CalPD (MycoPathPD). *Methods Enzymol* 350, 347-373.

Cuatrecasas, P. & Wilchek, M. (1968). Single-step purification of avidine from egg white by affinity chromatography on biocytin-Sepharose columns. *Biochem Biophys Res Commun* 33, 235-239.

Dallo, S. F., Chavoya, A. & Baseman, J. B. (1990). Characterization of the gene for a 30-kilodalton adhesion-related protein of Mycoplasma pneumoniae. *Infect Immun* 58, 4163-4165.

Dallo, S. F., Kannan, T. R., Blaylock, M. W. & Baseman, J. B. (2002). Elongation factor Tu and E1 beta subunit of pyruvate dehydrogenase complex act as fibronectin binding proteins in Mycoplasma pneumoniae. *Mol Microbiol* 46, 1041-1051.

Dandekar, T., Huynen, M., Regula, J. T., Ueberle, B., Zimmermann, C. U., Andrade, M. A., Doerks, T., Sanchez-Pulido, L., *et al.* (2000). Re-annotating the Mycoplasma pneumoniae genome sequence: adding value, function and reading frames. *Nucleic Acids Res* 28, 3278-3288.

Dhandayuthapani, S., Rasmussen, W. G. & Baseman, J. B. (1999). Disruption of gene mg218 of Mycoplasma genitalium through homologous recombination leads to an adherence-deficient phenotype. *Proc Natl Acad Sci U S A* 96, 5227-5232.

Dirksen, L. B., Krebes, K. A. & Krause, D. C. (1994). Phosphorylation of cytadherence-accessory proteins in Mycoplasma pneumoniae. *J Bacteriol* 176, 7499-7505.

Dirksen, L. B., Proft, T., Hilbert, H., Plagens, H., Herrmann, R. & Krause, D. C. (1996). Sequence analysis and characterization of the hmw gene cluster of Mycoplasma pneumoniae. *Gene* 171, 19-25.

Dobzhansky, T., (1946). Genetics of natural populations. XIII. Recombination and variability in populations of Drosophila pseudoobscura. *Genetics* 31, 269-290.

Drewes, G. & Bouwmeester, T. (2003). Global approaches to protein-protein interactions. *Curr Opin Cell Biol* 15, 199-205.

Eaton, M.D., Meiklejohn, G., van Herick, W. (1944). Studies on the etiology of primary atypical pneumonia. A filterable agent transmissible to cotton rats, hamsters, and chicken embryos. *J Exp Med* 79, 649- 668.

Eisenberg, D., Marcotte, E. M., Xenarios, I. & Yeates, T. O. (2000). Protein function in the post-genomic era. *Nature* 405, 823-826.

Feldner, J., Gobel, U. & Bredt, W. (1982). Mycoplasma pneumoniae adhesin localized to tip structure by monoclonal antibody. *Nature* 298, 765-767.

Fields, S. & Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature* 340, 245-246.

Fields, S. & Sternglanz, R. (1994). The two-hybrid system: an assay for protein-protein interactions. *Trends Genet* 10, 286-292.

Fisseha, M., Gohlmann, H. W., Herrmann, R. & Krause, D. C. (1999). Identification and complementation of frameshift mutations associated with loss of cytadherence in Mycoplasma pneumoniae. *J Bacteriol* 181, 4404-4410.

Forrer, P. & Jaussi, R. (1998). High-level expression of soluble heterologous proteins in the cytoplasm of Escherichia coli by fusion to the bacteriophage lambda head protein D. *Gene* 224, 45-52.

Franzoso, G., Hu, P. C., Meloni, G. A. & Barile, M. F. (1993). The immunodominant 90-kilodalton protein is localized on the terminal tip structure of Mycoplasma pneumoniae. *Infect Immun* 61, 1523-1530.

Frederickson, R. M. (1998). Macromolecular matchmaking: advances in two-hybrid and related technologies. *Curr Opin Biotechnol* 9, 90-96.

Galperin, M. Y. & Koonin, E. V. (1997). A diverse superfamily of enzymes with ATP-dependent carboxylate-amine/thiol ligase activity. *Protein Sci* 6, 2639-2643.

Gao, H., Jiang, X., Pogliano, K. & Aronson, A. I. (2002). The E1beta and E2 subunits of the Bacillus subtilis pyruvate dehydrogenase complex are involved in regulation of sporulation. *J Bacteriol* 184, 2780-2788.

Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., *et al.* (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* 415, 141-147.

Gilman A.G., *et al.* (2002). Overview of the Alliance for Cellular Signaling. *Nature* 420, 703-6.

Gobel, U., Speth, V. & Bredt, W. (1981). Filamentous structures in adherent Mycoplasma pneumoniae cells treated with nonionic detergents. *J Cell Biol* 91, 537-543.

Görg, A., Boguth, G., Obermaier, C., Posch, A. & Weiss, W. (1995). Two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients in the first dimension (IPG-Dalt): the state of the art and the controversy of vertical versus horizontal systems. *Electrophoresis* 16, 1079-1086.

Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R. & Weiss, W. (2000). The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 21, 1037-1053.

Görg, A., Obermaier, C., Boguth, G. & Weiss, W. (1999). Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures. *Electrophoresis* 20, 712-717.

Görg, A., Postel, W. & Gunther, S. (1988). The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 9, 531-546.

Gozalbo, D., Gil-Navarro, I., Azorin, I., Renau-Piqueras, J., Martinez, J. P. & Gil, M. L. (1998). The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of Candida albicans is also a fibronectin and laminin binding protein. *Infect Immun* 66, 2052-2059.

Graumann, P. L. (2000). Bacillus subtilis SMC is required for proper arrangement of the chromosome and for efficient segregation of replication termini but not for bipolar movement of newly duplicated origin regions. *J Bacteriol* 182, 6463-6471.

Graumann, P. L. (2001). SMC proteins in bacteria: condensation motors for chromosome segregation? *Biochimie* 83, 53-59.

Guo, D., Hazbun, T. R., Xu, X. J., Ng, S. L., Fields, S. & Kuo, M. H. (2004). A tethered catalysis, two-hybrid system to identify protein-protein interactions requiring post-translational modifications. *Nat Biotechnol* 22, 888-892.

Gupta, A., Onda, M., Pastan, I., Adhya, S. & Chaudhary, V. K. (2003). High-density functional display of proteins on bacteriophage lambda. *J Mol Biol* 334, 241-254.

Gupta, R. S. & Soltys, B. J. (1996). Prokaryotic homolog of tubulin? Consideration of FtsZ and glyceraldehyde 3-phosphate dehydrogenase as probable candidates. *Biochem Mol Biol Int* 38, 1211-1221.

Hahn, T. W., Krebes, K. A. & Krause, D. C. (1996). Expression in Mycoplasma pneumoniae of the recombinant gene encoding the cytadherence-associated protein HMW1 and identification of HMW4 as a product. *Mol Microbiol* 19, 1085-1093.

Hahn, T. W., Mothershed, E. A., Waldo, R. H., 3rd & Krause, D. C. (1999). Construction and analysis of a modified Tn4001 conferring chloramphenicol resistance in Mycoplasma pneumoniae. *Plasmid* 41, 120-124.

Hahn, T. W., Willby, M. J. & Krause, D. C. (1998). HMW1 is required for cytadhesin P1 trafficking to the attachment organelle in Mycoplasma pneumoniae. *J Bacteriol* 180, 1270-1276.

Harry, J. L., Wilkins, M. R., Herbert, B. R., Packer, N. H., Gooley, A. A. & Williams, K. L. (2000). Proteomics: capacity versus utility. *Electrophoresis* 21, 1071-1081.

Hartman, P. E. & Roth, J. R. (1973). Mechanisms of suppression. *Adv Genet* 17, 1-105.

Harvey, S. H., Krien, M. J. & O'Connell, M. J. (2002). Structural maintenance of chromosomes (SMC) proteins, a family of conserved ATPases. *Genome Biol* 3, REVIEWS3003.

Hase, M. E., Kuznetsov, N. V. & Cordes, V. C. (2001). Amino acid substitutions of coiled-coil protein Tpr abrogate anchorage to the nuclear pore complex but not parallel, in-register homodimerization. *Mol Biol Cell* 12, 2433-2452.

Hayflick, L. (1965). Tissue cultures and mycoplasmas. *Tex Rep Biol Med* 23, Suppl 1:285+.

Hedreyda, C. T., Lee, K. K. & Krause, D. C. (1993). Transformation of Mycoplasma pneumoniae with Tn4001 by electroporation. *Plasmid* 30, 170-175.

Hegermann, J. (2003) Untersuchungen von Cytoskelett-Komponenten und Motilität bei *Mycoplasma pneumoniae,* Dissertation, Georg-August-Universität zu Göttingen

Hegermann, J., Herrmann, R. & Mayer, F. (2002). Cytoskeletal elements in the bacterium Mycoplasma pneumoniae. *Naturwissenschaften* 89, 453-458.

Helms, M. K. & Jameson, D. M. (1995). Polymerization of an Escherichia coli elongation factor Tu. *Arch Biochem Biophys* 321, 303-310.

Herrmann, H. & Wiche, G. (1983). Specific in situ phosphorylation of plectin in detergent-resistant cytoskeletons from cultured Chinese hamster ovary cells. *J Biol Chem* 258, 14610-14618.

Herskowitz, I. (1987). Functional inactivation of genes by dominant negative mutations. *Nature* 329, 219-222.

Heslot, H. & Gaillardin, C., eds. (1992). Molecular biology and genetic engineering of yeast (CRC Press, Inc.).

Heukeshoven, J. & Dernick, R. (1988). Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels. *Electrophoresis* 9, 28-32.

Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B. C. & Herrmann, R. (1996). Complete sequence analysis of the genome of the bacterium Mycoplasma pneumoniae. *Nucleic Acids Res* 24, 4420-4449.

Hirano, T. (1999). SMC-mediated chromosome mechanics: a conserved scheme from bacteria to vertebrates? *Genes Dev* 13, 11-19.

Hochuli, E., Dobeli, H. & Schacher, A. (1987). New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J Chromatogr* 411, 177-184.

Holmes, V. F. & Cozzarelli, N. R. (2000). Closing the ring: links between SMC proteins and chromosome partitioning, condensation, and supercoiling. *Proc Natl Acad Sci U S A* 97, 1322-1324.

Hope, I. A. & Struhl, K. (1986). Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* 46, 885-894.

Hu, P. C., Cole, R. M., Huang, Y. S., Graham, J. A., Gardner, D. E., Collier, A. M. & Clyde, W. A., Jr. (1982). Mycoplasma pneumoniae infection: role of a surface protein in the attachment organelle. *Science* 216, 313-315.

Hu, P. C., Collier, A. M. & Baseman, J. B. (1977). Surface parasitism by Mycoplasma pneumoniae of respiratory epithelium. *J Exp Med* 145, 1328-1343.

Inamine, J. M., Ho, K. C., Loechel, S. & Hu, P. C. (1990). 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.

Inamine, J. M., Loechel, S. & Hu, P. C. (1988). Analysis of the nucleotide sequence of the P1 operon of Mycoplasma pneumoniae. *Gene* 73, 175-183.

Ito, T., Tashiro, K., Muta, S., Ozawa, R., Chiba, T., Nishizawa, M., Yamamoto, K., Kuhara, S. & Sakaki, Y. (2000). Toward a protein-protein interaction map of the budding yeast: A comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. *Proc Natl Acad Sci U S A* 97, 1143-1147.

Jacobs, E. (1991). *Mycoplasma pneumoniae* virulence factors and the immune response. *Reviews in Medical Microbiology* 2, 83-90.

James, P., Halladay, J. & Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* 144, 1425-1436.

Jensen, R. B. & Shapiro, L. (1999). The Caulobacter crescentus smc gene is required for cell cycle progression and chromosome segregation. *Proc Natl Acad Sci U S A* 96, 10661-10666.

Johnston, C. C., Jr. & Slemenda, C. W. (1990). Non-invasive methods for quantitating bone loss of aging. *Exp Gerontol* 25, 297-301.

Jones, L. J., Carballido-Lopez, R. & Errington, J. (2001). Control of cell shape in bacteria: helical, actin-like filaments in Bacillus subtilis. *Cell* 104, 913-922.

Jordan, J. L., Berry, K. M., Balish, M. F. & Krause, D. C. (2001). Stability and subcellular localization of cytadherence-associated protein P65 in Mycoplasma pneumoniae. *J Bacteriol* 183, 7387-7391.

Kahane, I., Tucker, S. & Baseman, J. B. (1985). Detection of Mycoplasma pneumoniae adhesin (P1) in the nonhemadsorbing population of virulent Mycoplasma pneumoniae. *Infect Immun* 49, 457-458.

Kane, J. F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in Escherichia coli. *Curr Opin Biotechnol* 6, 494-500.

Kanehisa, M. & Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 28, 27-30.

Kang, W. K., Icho, T., Isono, S., Kitakawa, M. & Isono, K. (1989). Characterization of the gene rimK responsible for the addition of glutamic acid residues to the C-terminus of ribosomal protein S6 in Escherichia coli K12. *Mol Gen Genet* 217, 281-288.

Kannan, T. R. & Baseman, J. B. (2000). Expression of UGA-containing Mycoplasma genes in Bacillus subtilis. *J Bacteriol* 182, 2664-2667.

Karow, M. L., Rogers, E. J., Lovett, P. S. & Piggot, P. J. (1998). Suppression of TGA mutations in the Bacillus subtilis spoIIR gene by prfB mutations. *J Bacteriol* 180, 4166-4170.

Karp, P. D., Riley, M., Paley, S. M. & Pellegrini-Toole, A. (2002). The MetaCyc Database. *Nucleic Acids Res* 30, 59-61.

Keegan, L., Gill, G. & Ptashne, M. (1986). Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. *Science* 231, 699-704.

Koonin, E. V., Bork, P. & Sander, C. (1994). A novel RNA-binding motif in omnipotent suppressors of translation termination, ribosomal proteins and a ribosome modification enzyme? *Nucleic Acids Res* 22, 2166-2167.

Koshland, D. & Strunnikov, A. (1996). Mitotic chromosome condensation. *Annu Rev Cell Dev Biol* 12, 305-333.

Krause, D. C. (1996). Mycoplasma pneumoniae cytadherence: unravelling the tie that binds. *Mol Microbiol* 20, 247-253.

Krause, D. C. & Balish, M. F. (2001). Structure, function, and assembly of the terminal organelle of Mycoplasma pneumoniae. *FEMS Microbiol Lett* 198, 1-7.

Krause, D. C. & Balish, M. F. (2004). Cellular engineering in a minimal microbe: structure and assembly of the terminal organelle of Mycoplasma pneumoniae. *Mol Microbiol* 51, 917-924.

Krause, D. C. & Baseman, J. B. (1982). Mycoplasma pneumoniae proteins that selectively bind to host cells. *Infect Immun* 37, 382-386.

Krause, D. C., Leith, D. K., Wilson, R. M. & Baseman, J. B. (1982). Identification of Mycoplasma pneumoniae proteins associated with hemadsorption and virulence. *Infect Immun* 35, 809-817.

Krause, D. C., Proft, T., Hedreyda, C. T., Hilbert, H., Plagens, H. & Herrmann, R. (1997). Transposon mutagenesis reinforces the correlation between Mycoplasma pneumoniae cytoskeletal protein HMW2 and cytadherence. *J Bacteriol* 179, 2668-2677.

Krebes, K. A., Dirksen, L. B. & Krause, D. C. (1995). Phosphorylation of Mycoplasma pneumoniae cytadherence-accessory proteins in cell extracts. *J Bacteriol* 177, 4571-4574.

Krivan, H. C., Olson, L. D., Barile, M. F., Ginsburg, V. & Roberts, D. D. (1989). Adhesion of Mycoplasma pneumoniae to sulfated glycolipids and inhibition by dextran sulfate. *J Biol Chem* 264, 9283-9288.

Kurland, C. G., Hughes, D. and Ehrenberg, M. (1996). Limitations of Translational Accuracy, In Neidhardt, F. C. (ed.), *Escherichia coli* and *Salmonella.* ASM Press, Washington, D.C., Vol. 1, pp. 979-1004.

Lan, S., Wang, H., Jiang, H., Mao, H., Liu, X., Zhang, X., Hu, Y., Xiang, L. & Yuan, Z. (2003). Direct interaction between alpha-actinin and hepatitis C virus NS5B. *FEBS Lett* 554, 289-294.

Layh-Schmitt, G. & Harkenthal, M. (1999). The 40- and 90-kDa membrane proteins (ORF6 gene product) of Mycoplasma pneumoniae are responsible for the tip structure formation and P1 (adhesin) association with the Triton shell. *FEMS Microbiol Lett* 174, 143-149.

Layh-Schmitt, G. & Herrmann, R. (1992). Localization and biochemical characterization of the ORF6 gene product of the Mycoplasma pneumoniae P1 operon. *Infect Immun* 60, 2906-2913.

Layh-Schmitt, G. & Herrmann, R. (1994). Spatial arrangement of gene products of the P1 operon in the membrane of Mycoplasma pneumoniae. *Infect Immun* 62, 974-979.

Layh-Schmitt, G., Hilbert, H. & Pirkl, E. (1995). A spontaneous hemadsorption-negative mutant of Mycoplasma pneumoniae exhibits a truncated adhesin-related 30-kilodalton protein and lacks the cytadherence-accessory protein HMW1. *J Bacteriol* 177, 843-846.

Layh-Schmitt, G., Podtelejnikov, A. & Mann, M. (2000). Proteins complexed to the P1 adhesin of Mycoplasma pneumoniae. *Microbiology* 146 (Pt 3), 741-747.

Lee, J., Choi, S. I., Jang, J. S., Jang, K., Moon, J. W., Bae, C. S., Yang, D. S. & Seong, B. L. (1999). Novel secretion system of recombinant Saccharomyces cerevisiae using an N-terminus residue of human IL-1 beta as secretion enhancer. *Biotechnol Prog* 15, 884-890.

Liu, Y. & Eisenberg, D. (2002). 3D domain swapping: as domains continue to swap. *Protein Sci* 11, 1285-1299.

Llorca, O., Martin-Benito, J., Gomez-Puertas, P., Ritco-Vonsovici, M., Willison, K. R., Carrascosa, J. L. & Valpuesta, J. M. (2001). Analysis of the interaction between the eukaryotic chaperonin CCT and its substrates actin and tubulin. *J Struct Biol* 135, 205-218.

Looman, A. C., Bodlaender, J., Comstock, L. J., Eaton, D., Jhurani, P., de Boer, H. A. & van Knippenberg, P. H. (1987). Influence of the codon following the AUG initiation codon on the expression of a modified lacZ gene in Escherichia coli. *Embo J* 6, 2489-2492.

Lovett, P. S., Ambulos, N. P., Jr., Mulbry, W., Noguchi, N. & Rogers, E. J. (1991). UGA can be decoded as tryptophan at low efficiency in Bacillus subtilis. *J Bacteriol* 173, 1810-1812.

Löwe, J. & Amos, L. A. (1998). Crystal structure of the bacterial cell-division protein FtsZ. *Nature* 391, 203-206.

Löwe, J., van den Ent, F. & Amos, L. A. (2004). Molecules of the bacterial cytoskeleton. *Annu Rev Biophys Biomol Struct* 33, 177-198.

Lu, C., Reedy, M. & Erickson, H. P. (2000). Straight and curved conformations of FtsZ are regulated by GTP hydrolysis. *J Bacteriol* 182, 164-170.

Lutkenhaus, J. (1990). Regulation of cell division in E. coli. *Trends Genet* 6, 22-25.

Lutkenhaus, J. (2003). Another cytoskeleton in the closet. *Cell* 115, 648-650.

Lutkenhaus, J. & Addinall, S. G. (1997). Bacterial cell division and the Z ring. *Annu Rev Biochem* 66, 93-116.

Lutz, R. & Bujard, H. (1997). Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucleic Acids Res* 25, 1203-1210.

Ma, J. & Ptashne, M. (1987). A new class of yeast transcriptional activators. *Cell* 51, 113-119.

Ma, J. & Ptashne, M. (1988). Converting a eukaryotic transcriptional inhibitor into an activator. *Cell* 55, 443-446.

Marchler-Bauer, A. & Bryant, S. H. (2004). CD-Search: protein domain annotations on the fly. *Nucleic Acids Res* 32, W327-331.

Mayer, F. (2003). Cytoskeletons in prokaryotes. *Cell Biol Int* 27, 429-438.

Mayer, F. & Gottschalk, G. (2003). The bacterial cytoskeleton and its putative role in membrane vesicle formation observed in a Gram-positive bacterium producing starch-degrading enzymes. *J Mol Microbiol Biotechnol* 6, 127-132.

McDonald, K. A., Muschler, J. & Horwitz, A. F. (1998). Immunopurification of a sarcomeric junctional protein complex containing GAPDH. *Exp Cell Res* 243, 305-318.

Meeks-Wagner, D. & Hartwell, L. H. (1986). Normal stoichiometry of histone dimer sets is necessary for high fidelity of mitotic chromosome transmission. *Cell* 44, 43-52.

Melby, T. E., Ciampaglio, C. N., Briscoe, G. & Erickson, H. P. (1998). The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge. *J Cell Biol* 142, 1595-1604.

Mendelsohn, A. R. & Brent, R. (1994). Applications of interaction traps/two-hybrid systems to biotechnology research. *Curr Opin Biotechnol* 5, 482-486.

Meng, K. E. & Pfister, R. M. (1980). Intracellular structures of Mycoplasma pneumoniae revealed after membrane removal. *J Bacteriol* 144, 390-399.

Mewes, H. W., Frishman, D., Guldener, U., Mannhaupt, G., Mayer, K., Mokrejs, M., Morgenstern, B., Munsterkotter, M., Rudd, S. & Weil, B. (2002). MIPS: a database for genomes and protein sequences. *Nucleic Acids Res* 30, 31-34.

Moller-Jensen, J., Jensen, R. B., Lowe, J. & Gerdes, K. (2002). Prokaryotic DNA segregation by an actin-like filament. *Embo J* 21, 3119-3127.

Moriya, S., Tsujikawa, E., Hassan, A. K., Asai, K., Kodama, T. & Ogasawara, N. (1998). A Bacillus subtilis gene-encoding protein homologous to eukaryotic SMC motor protein is necessary for chromosome partition. *Mol Microbiol* 29, 179-187.

Mouches, C., Candresse, T., Barroso, G., Saillard, C., Wroblewski, H. & Bove, J. M. (1985). Gene for spiralin, the major membrane protein of the helical mollicute Spiroplasma citri: cloning and expression in Escherichia coli. *J Bacteriol* 164, 1094-1099.

Mura, C., Katz, J. E., Clarke, S. G. & Eisenberg, D. (2003). Structure and function of an archaeal homolog of survival protein E (SurEalpha): an acid phosphatase with purine nucleotide specificity. *J Mol Biol* 326, 1559-1575.

Nanninga, N. (2001). Cytokinesis in prokaryotes and eukaryotes: common principles and different solutions. *Microbiol Mol Biol Rev* 65, 319-333 ; third page, table of contents.

Neimark, H. C. (1977). Extraction of an actin-like protein from the prokaryote Mycoplasma pneumoniae. *Proc Natl Acad Sci U S A* 74, 4041-4045.

Neuhoff, V., Arold, N., Taube, D. & Ehrhardt, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9, 255-262.

Nocard & Roux (1990). The microbe of pleuropneumonia. 1896. *Rev Infect Dis* 12, 354-358.

Nogales, E., Downing, K. H., Amos, L. A. & Lowe, J. (1998). Tubulin and FtsZ form a distinct family of GTPases. *Nat Struct Biol* 5, 451-458.

Norris, V., Turnock, G. & Sigee, D. (1996). The Escherichia coli enzoskeleton. *Mol Microbiol* 19, 197-204.

Notarnicola, S. M., McIntosh, M. A. & Wise, K. S. (1990). Multiple translational products from a Mycoplasma hyorhinis gene expressed in Escherichia coli. *J Bacteriol* 172, 2986-2995.

Nyborg, J. & Kjeldgaard, M. (1996). Elongation in bacterial protein biosynthesis. *Curr Opin Biotechnol* 7, 369-375.

Ogle, K. F., Lee, K. K. & Krause, D. C. (1991). Cloning and analysis of the gene encoding the cytadherence phase-variable protein HMW3 from Mycoplasma pneumoniae. *Gene* 97, 69-75.

Ogle, K. F., Lee, K. K. & Krause, D. C. (1992). Nucleotide sequence analysis reveals novel features of the phase-variable cytadherence accessory protein HMW3 of Mycoplasma pneumoniae. *Infect Immun* 60, 1633-1641.

Pancholi, V. & Fischetti, V. A. (1992). A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. *J Exp Med* 176, 415-426.

Parker, K. C. & Wiley, D. C. (1989). Overexpression of native human beta 2-microglobulin in Escherichia coli and its purification. *Gene* 83, 117-124.

Peri, S., Navarro, J. D., Amanchy, R., Kristiansen, T. Z., Jonnalagadda, C. K., Surendranath, V., Niranjan, V., Muthusamy, B., *et al.* (2003). Development of human protein reference database as an initial platform for approaching systems biology in humans. *Genome Res* 13, 2363-2371.

Person, M. D., Brown, K. C., Mahrus, S., Craik, C. S. & Burlingame, A. L. (2001). Novel inter-protein cross-link identified in the GGH-ecotin D137Y dimer. *Protein Sci* 10, 1549-1562.

Phizicky, E., Bastiaens, P. I., Zhu, H., Snyder, M. & Fields, S. (2003). Protein analysis on a proteomic scale. *Nature* 422, 208-215.

Phizicky, E. M. & Fields, S. (1995). Protein-protein interactions: methods for detection and analysis. *Microbiol Rev* 59, 94-123.

Popham, P. L., Hahn, T. W., Krebes, K. A. & Krause, D. C. (1997). Loss of HMW1 and HMW3 in noncytadhering mutants of Mycoplasma pneumoniae occurs post-translationally. *Proc Natl Acad Sci U S A* 94, 13979-13984.

Proft, T. (1994). Identifizierung und Charakterisierung von Genprodukten bei Mycoplasma pneumoniae, Inaugural-Dissertation. Ruprecht-Karls-Universität Heidelberg.

Proft, T. & Herrmann, R. (1994). Identification and characterization of hitherto unknown Mycoplasma pneumoniae proteins. *Mol Microbiol* 13, 337-348.

Proft, T., Hilbert, H., Layh-Schmitt, G. & Herrmann, R. (1995). The proline-rich P65 protein of Mycoplasma pneumoniae is a component of the Triton X-100-insoluble fraction and exhibits size polymorphism in the strains M129 and FH. *J Bacteriol* 177, 3370-3378.

Proft, T., Hilbert, H., Plagens, H. & Herrmann, R. (1996). The P200 protein of Mycoplasma pneumoniae shows common features with the cytadherence-associated proteins HMW1 and HMW3. *Gene* 171, 79-82.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M. & Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* 24, 218-229.

Puig, O., Rutz, B., Luukkonen, B. G., Kandels-Lewis, S., Bragado-Nilsson, E. & Seraphin, B. (1998). New constructs and strategies for efficient PCR-based gene manipulations in yeast. *Yeast* 14, 1139-1146.

Radestock, U. & Bredt, W. (1977). Motility of Mycoplasma pneumoniae. *J Bacteriol* 129, 1495-1501.

Razin, S. (1985). Molecular biology and genetics of mycoplasmas (Mollicutes). *Microbiol Rev* 49, 419-455.

Razin, S. (1992). Peculiar properties of mycoplasmas: the smallest self-replicating prokaryotes. *FEMS Microbiol Lett* 79, 423-431.

Razin, S. & Jacobs, E. (1992). Mycoplasma adhesion. *J Gen Microbiol* 138, 407-422.

Razin, S., Yogev, D. & Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev* 62, 1094-1156.

Regula, J. T. (1999). Auf dem Weg zum Proteom von *Mycoplasma pneumoniae,* Inaugural-Dissertation. Ruprecht-Karls-Universität Heidelberg.

Regula, J. T., Boguth, G., Gorg, A., Hegermann, J., Mayer, F., Frank, R. & Herrmann, R. (2001). 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.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. & Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* 17, 1030-1032.

Rine, J. (1991). Gene overexpression in studies of Saccharomyces cerevisiae. *Methods Enzymol* 194, 239-251.

Roberts, D. D., Olson, L. D., Barile, M. F., Ginsburg, V. & Krivan, H. C. (1989). Sialic acid-dependent adhesion of Mycoplasma pneumoniae to purified glycoproteins. *J Biol Chem* 264, 9289-9293.

Romero-Arroyo, C. E., Jordan, J., Peacock, S. J., Willby, M. J., Farmer, M. A. & Krause, D. C. (1999). Mycoplasma pneumoniae protein P30 is required for cytadherence and associated with proper cell development. *J Bacteriol* 181, 1079-1087.

Roth, J. R. (1970). UGA nonsense mutations in Salmonella typhimurium. *J Bacteriol* 102, 467-475.

Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y. & Chait, B. T. (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism. *J Cell Biol* 148, 635-651.

Ruden, D. M. (1992). Activating regions of yeast transcription factors must have both acidic and hydrophobic amino acids. *Chromosoma* 101, 342-348.

Ruden, D. M., Ma, J., Li, Y., Wood, K. & Ptashne, M. (1991). Generating yeast transcriptional activators containing no yeast protein sequences. *Nature* 350, 250-252.

Salgado, H., Santos-Zavaleta, A., Gama-Castro, S., Millan-Zarate, D., Diaz-Peredo, E., Sanchez-Solano, F., Perez-Rueda, E., Bonavides-Martinez, C. & Collado-Vides, J. (2001). RegulonDB (version 3.2): transcriptional regulation and operon organization in Escherichia coli K-12. *Nucleic Acids Res* 29, 72-74.

Sambrock, J., Fritsch, E. F. and Maniatis, T. (2001). Molecular cloning - a laboratory manual, Third edition, Cold Spring Harbour Laboratory Press, New York.

Schaltmann, K. & Pongs, O. (1980). A simple procedure for blotting of proteins to study antibody specificity and antigen structure. *Hoppe Seylers Z Physiol Chem* 361, 207-210.

Schenk, P. M., Baumann, S., Mattes, R. & Steinbiss, H. H. (1995). Improved high-level expression system for eukaryotic genes in Escherichia coli using T7 RNA polymerase and rare ArgtRNAs. *Biotechniques* 19, 196-198, 200.

Schwarz, E. C., Geissler, H. & Soldati, T. (1999). A potentially exhaustive screening strategy reveals two novel divergent myosins in Dictyostelium. *Cell Biochem Biophys* 30, 413-435.

Seed, B. (1995). Developments in expression cloning. *Curr Opin Biotechnol* 6, 567-573.

Seifert, K. N., McArthur, W. P., Bleiweis, A. S. & Brady, L. J. (2003). Characterization of group B streptococcal glyceraldehyde-3-phosphate dehydrogenase: surface localization, enzymatic activity, and protein-protein interactions. *Can J Microbiol* 49, 350-356.

Seol, J. H., Baek, S. H., Kang, M. S., Ha, D. B. & Chung, C. H. (1995). Distinctive roles of the two ATP-binding sites in ClpA, the ATPase component of protease Ti in Escherichia coli. *J Biol Chem* 270, 8087-8092.

Seol, J. H., Woo, K. M., Kang, M. S., Ha, D. B. & Chung, C. H. (1995). Requirement of ATP hydrolysis for assembly of ClpA/ClpP complex, the ATP-dependent protease Ti in Escherichia coli. *Biochem Biophys Res Commun* 217, 41-51.

Seol, J. H., Yoo, S. J., Kim, K. I., Kang, M. S., Ha, D. B. & Chung, C. H. (1994). The 65-kDa protein derived from the internal translational initiation site of the clpA gene inhibits the ATP-dependent protease Ti in Escherichia coli. *J Biol Chem* 269, 29468-29473.

Seto, S., Kenri, T., Tomiyama, T. & Miyata, M. (2005). Involvement of P1 adhesin in gliding motility of Mycoplasma pneumoniae as revealed by the inhibitory effects of antibody under optimized gliding conditions. *J Bacteriol* 187, 1875-1877.

Seto, S., Layh-Schmitt, G., Kenri, T. & Miyata, M. (2001). Visualization of the attachment organelle and cytadherence proteins of Mycoplasma pneumoniae by immunofluorescence microscopy. *J Bacteriol* 183, 1621-1630.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228, 1315-1317.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem* 150, 76-85.

Snyder, M. & Kumar, A. (2002). Yeast genomics: past, present, and future promise. *Funct Integr Genomics* 2, 135-137.

Sperker, B., Hu, P. & Herrmann, R. (1991). Identification of gene products of the P1 operon of Mycoplasma pneumoniae. *Mol Microbiol* 5, 299-306.

Starger, J. M. & Goldman, R. D. (1977). Isolation and preliminary characterization of 10-nm filaments from baby hamster kidney (BHK-21) cells. *Proc Natl Acad Sci U S A* 74, 2422-2426.

Steinert, P. M., Zimmerman, S. B., Starger, J. M. & Goldman, R. D. (1978). Ten-nanometer filaments of hamster BHK-21 cells and epidermal keratin filaments have similar structures. *Proc Natl Acad Sci U S A* 75, 6098-6101.

Stevens, M. K. & Krause, D. C. (1990). Disulfide-linked protein associated with Mycoplasma pneumoniae cytadherence phase variation. *Infect Immun* 58, 3430-3433.

Stevens, M. K. & Krause, D. C. (1991). Localization of the Mycoplasma pneumoniae cytadherence-accessory proteins HMW1 and HMW4 in the cytoskeletonlike Triton shell. *J Bacteriol* 173, 1041-1050.

Stevens, M. K. & Krause, D. C. (1992). Mycoplasma pneumoniae cytadherence phase-variable protein HMW3 is a component of the attachment organelle. *J Bacteriol* 174, 4265-4274.

Strunnikov, A. V. & Jessberger, R. (1999). Structural maintenance of chromosomes (SMC) proteins: conserved molecular properties for multiple biological functions. *Eur J Biochem* 263, 6-13.

Su, C. J., Tryon, V. V. & Baseman, J. B. (1987). Cloning and sequence analysis of cytadhesin P1 gene from Mycoplasma pneumoniae. *Infect Immun* 55, 3023-3029.

Sudbery, P. E. (1996). The expression of recombinant proteins in yeasts. *Curr Opin Biotechnol* 7, 517-524.

Taylor-Robinson, D. (1996). Infections due to species of Mycoplasma and Ureaplasma: an update. *Clin Infect Dis* 23, 671-682; quiz 683-674.

Trachtenberg, S. & Gilad, R. (2001). A bacterial linear motor: cellular and molecular organization of the contractile cytoskeleton of the helical bacterium Spiroplasma melliferum BC3. *Mol Microbiol* 41, 827-848.

Ueberle, B. (2001). Proteomanalyse von *Mycoplasma pneumoniae,* Inaugural-Dissertation. Ruprecht-Karls-Universität Heidelberg.

Ueberle, B., Frank, R. & Herrmann, R. (2002). The proteome of the bacterium Mycoplasma pneumoniae: comparing predicted open reading frames to identified gene products. *Proteomics* 2, 754-764.

van den Ent, F., Amos, L. A. & Lowe, J. (2001). Prokaryotic origin of the actin cytoskeleton. *Nature* 413, 39-44.

van den Ent, F., Moller-Jensen, J., Amos, L. A., Gerdes, K. & Lowe, J. (2002). F-actin-like filaments formed by plasmid segregation protein ParM. *Embo J* 21, 6935-6943.

Varshavsky, A. (1997). The N-end rule pathway of protein degradation. *Genes Cells* 2, 13-28.

Voges, D., Watzele, M., Nemetz, C., Wizemann, S. & Buchberger, B. (2004). Analyzing and enhancing mRNA translational efficiency in an Escherichia coli in vitro expression system. *Biochem Biophys Res Commun* 318, 601-614.

von Mering, C., Krause, R., Snel, B., Cornell, M., Oliver, S. G., Fields, S. & Bork, P. (2002). Comparative assessment of large-scale data sets of protein-protein interactions. *Nature* 417, 399-403.

Wachi, M., Doi, M., Tamaki, S., Park, W., Nakajima-Iijima, S. & Matsuhashi, M. (1987). Mutant isolation and molecular cloning of mre genes, which determine cell shape, sensitivity to mecillinam, and amount of penicillin-binding proteins in Escherichia coli. *J Bacteriol* 169, 4935-4940.

Wakagi, T., Oshima, T., Imamura, H. & Matsuzawa, H. (1998). Cloning of the gene for inorganic pyrophosphatase from a thermoacidophilic archaeon, Sulfolobus sp. strain 7, and overproduction of the enzyme by coexpression of tRNA for arginine rare codon. *Biosci Biotechnol Biochem* 62, 2408-2414.

Waldo, R. H., 3rd, Jordan, J. L. & Krause, D. C. (2005). Identification and complementation of a mutation associated with loss of Mycoplasma pneumoniae virulence-specific proteins B and C. *J Bacteriol* 187, 747-751.

Weiner, J., 3rd, Herrmann, R. & Browning, G. F. (2000). Transcription in Mycoplasma pneumoniae. *Nucleic Acids Res* 28, 4488-4496.

Wenzel, R. & Herrmann, R. (1989). Cloning of the complete Mycoplasma pneumoniae genome. *Nucleic Acids Res* 17, 7029-7043.

Wenzel, R., Pirkl, E. & Herrmann, R. (1992). Construction of an EcoRI restriction map of Mycoplasma pneumoniae and localization of selected genes. *J Bacteriol* 174, 7289-7296.

Wessel, D. & Flugge, U. I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem* 138, 141-143.

Willby, M. J., Balish, M. F., Ross, S. M., Lee, K. K., Jordan, J. L. & Krause, D. C. (2004). HMW1 is required for stability and localization of HMW2 to the attachment organelle of Mycoplasma pneumoniae. *J Bacteriol* 186, 8221-8228.

Willby, M. J. & Krause, D. C. (2002). Characterization of a Mycoplasma pneumoniae hmw3 mutant: implications for attachment organelle assembly. *J Bacteriol* 184, 3061-3068.

Williamson, D. L. (1974). Unusual fibrils from the spirochete-like sex ratio organism. *J Bacteriol* 117, 904-906.

Williamson, D. L., Renaudin, J. & Bove, J. M. (1991). Nucleotide sequence of the Spiroplasma citri fibril protein gene. *J Bacteriol* 173, 4353-4362.

Wilson, M. H. & Collier, A. M. (1976). Ultrastructural study of Mycoplasma pneumoniae in organ culture. *J Bacteriol* 125, 332-339.

Wingender, E., Chen, X., Fricke, E., Geffers, R., Hehl, R., Liebich, I., Krull, M., Matys, V., *et al.* (2001). The TRANSFAC system on gene expression regulation. *Nucleic Acids Res* 29, 281-283.

Xenarios, I., Salwinski, L., Duan, X. J., Higney, P., Kim, S. M. & Eisenberg, D. (2002). DIP, the Database of Interacting Proteins: a research tool for studying cellular networks of protein interactions. *Nucleic Acids Res* 30, 303-305.

Yogev, D., Sela, S., Bercovier, H. & Razin, S. (1990). Nucleotide sequence and codon usage of the elongation factor Tu(EF-Tu) gene from Mycoplasma pneumoniae. *Mol Microbiol* 4, 1303-1310.

Young, K. D. (2003). Bacterial shape. *Mol Microbiol* 49, 571-580.

Zal, T. & Gascoigne, N. R. (2004). Using live FRET imaging to reveal early protein-protein interactions during T cell activation. *Curr Opin Immunol* 16, 418-427.

Zhu, H., Bilgin, M. & Snyder, M. (2003). Proteomics. *Annu Rev Biochem* 72, 783-812.

Zimmerman, C. U. (2005). Global transcription analysis of *Mycoplasma pneumoniae* during two environmental conditions: oxidative stress and infection. Dissertation, the Ruperto-Carola University of Heidelberg.

Zoller, M. J. & Smith, M. (1987). Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *Methods Enzymol* 154, 329-350.

7 Appendices

7.1 Figures

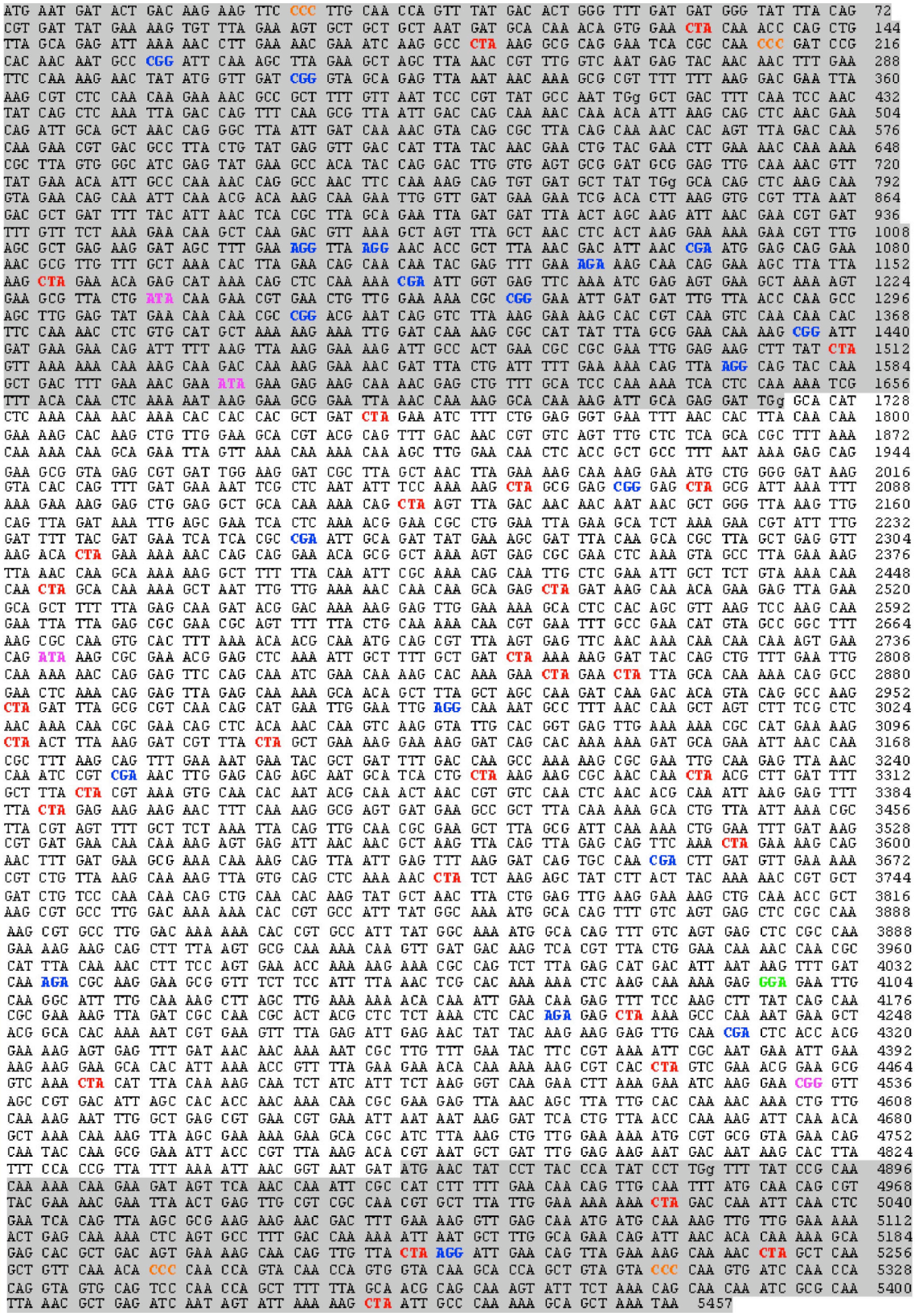


Fig. 7.1: DNA sequence of the gene *hmw2*. Rare codons coding for different amino acids are indicated with color: arginine (AGG, AGA, CGA, CGG), leucine (CTA), isoleucine (ATA), proline (CCC) and glycin (GGA). Truncated parts of *hmw2* which were shown to be well-expressed in *E.coli* are marked with gray

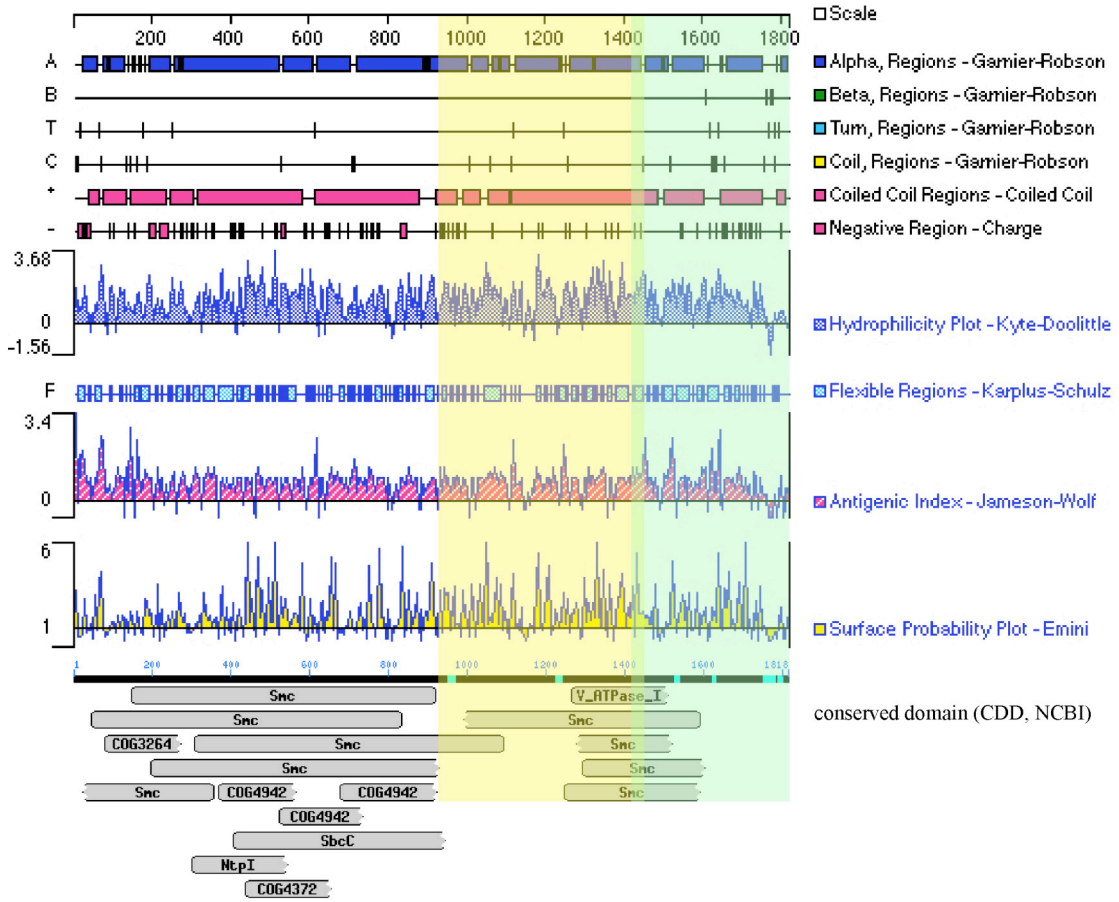


Fig. 7.2: Total profile of the protein HMW2 (MPN310). The colored regions indicate protein fragments used in the two-hybrid pairwise tests (yellow = HMW2 fragment 3, green = HMW2 fragment 4). Number above the profile gives amino acid positions.

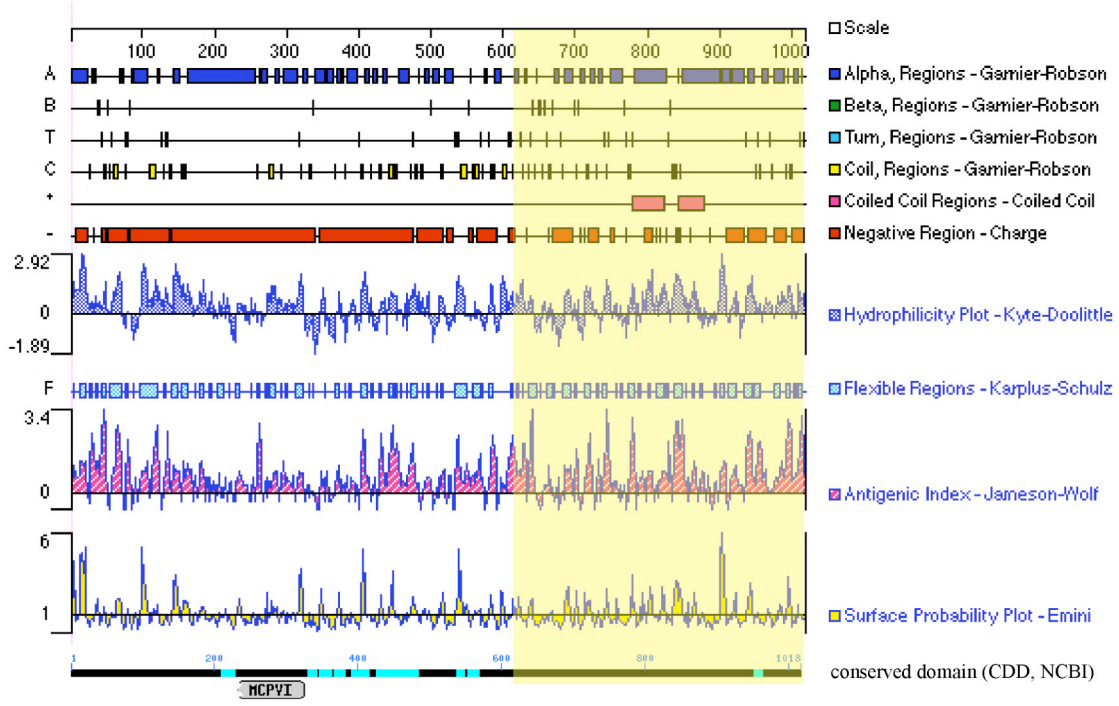


Fig. 7.3: Total profile of the protein HMW1 (MPN447). The yellow region indicates protein fragments used in the two-hybrid pairwise tests. Number above the profile gives amino acid positions.

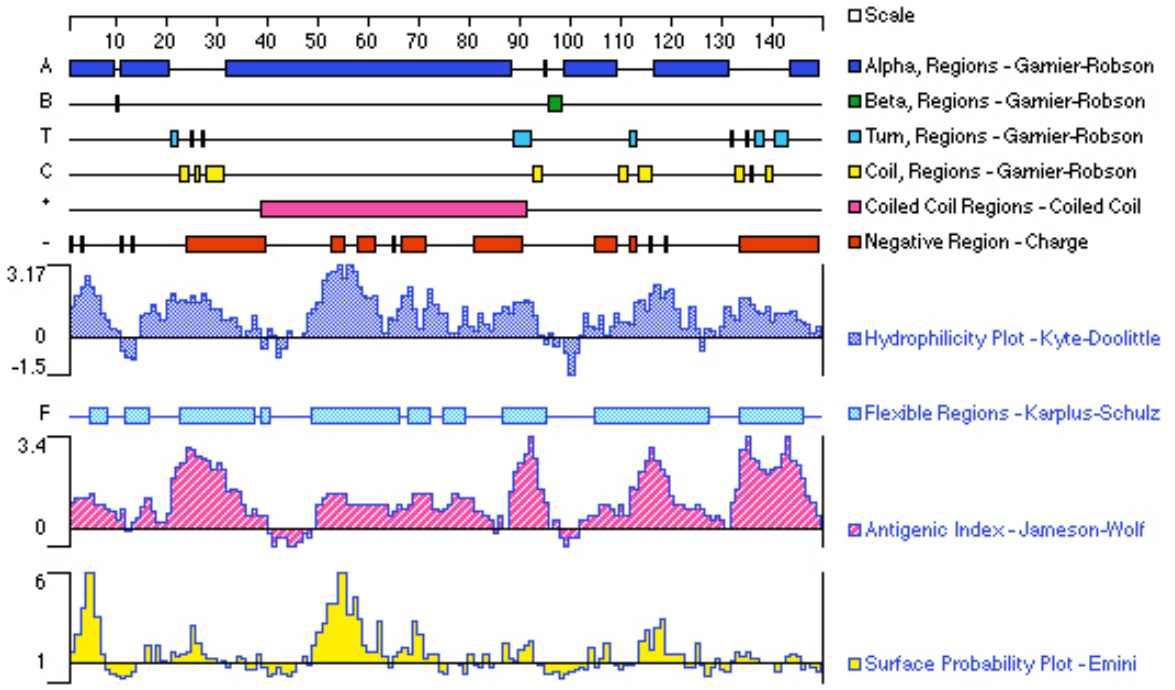


Fig. 7.4: Total profile of the protein encoded from MPN297. The entired protein sequence was used in the two-hybrid pairwise tests. Number above the profile gives amino acid positions.

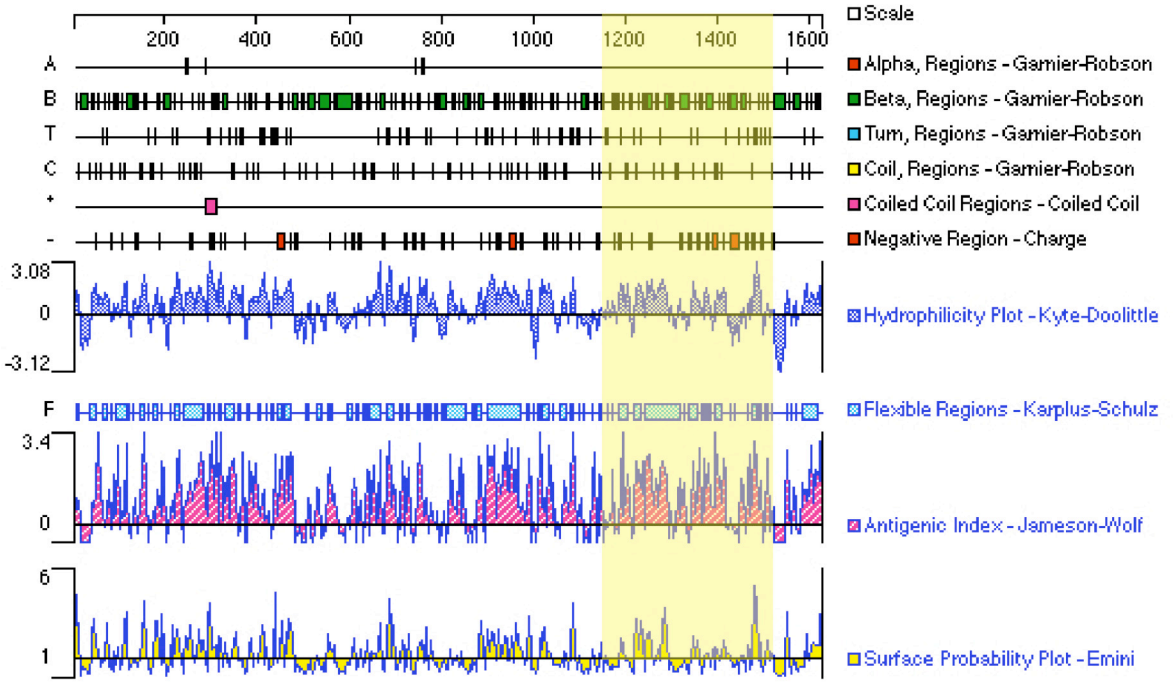


Fig. 7.5: Total profile of the adhesin P1(MPN141). The yellow region are protein fragments used in the two-hybrid pairwise tests. Number above the profile gives amino acid positions.

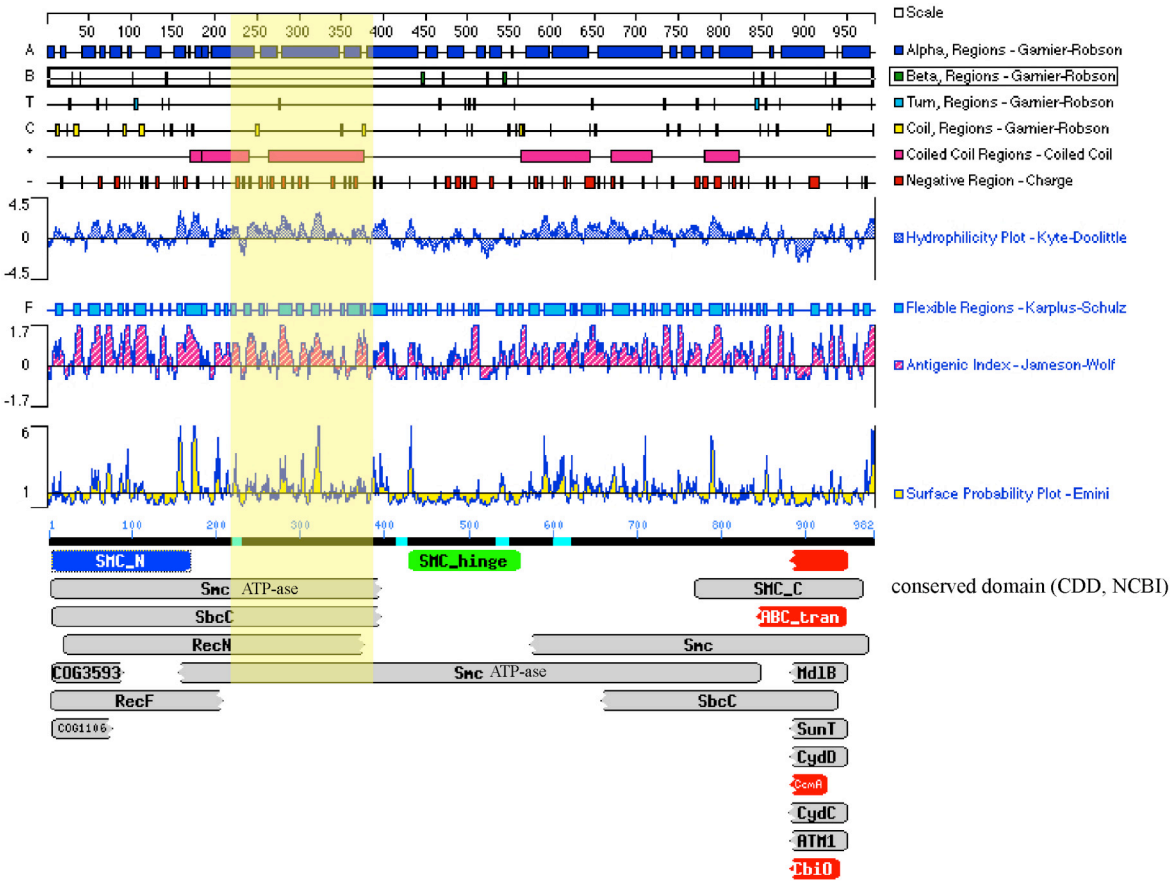


Fig. 7.6: Total profile of the adhesin P115 (MPN426). The yellow region are protein fragments found from the two-hybrid screen pilot experiment. Number above the profile gives amino acid positions. The whole protein was used in the pairwise test.

7.2 Tables

Table 7.1: The codon usage of *E.coli* and *M. pneumoniae* [Himmelreich, 1997; Zhang et al., 1991] compared with codon presented in *hmw2* gene.

Codon	Amino acid	Frequency in *hmw2* gene /1000	Frequency in *E. coli* /1000	Frequency in *M. pneumoniae* /1000
AAU	Asn	18.2	17.6	25.0
AAC	Asn	42.4	21.7	36.9
AAA	Lys	59.4	33.6	46.3
AAG	Lys	62.7	10.2	39.1
ACU	Thr	6.1	8.9	19.3
ACC	Thr	6.1	23.4	21.9
ACA	Thr	11.6	7.0	10.4
ACG	Thr	8.3	14.4	7.9
AGU	Ser	13.8	8.7	21.0
AGC	Ser	11.0	16.0	10.6
AGA	Arg	1.7	2.1	4.0
AGG	Arg	2.8	1.2	2.8
AUU	Ile	29.7	30.4	46.1
AUC	Ile	6.6	25.0	14.4
AUA	Ile	9.4	4.3	5.4
AUG	Met	6.1	27.8	15.6
CAU	His	6.6	12.9	6.2
CAC	His	15.4	9.7	11.9
CAA	Gln	94.1	15.5	37.9
CAG	Gln	37.4	28.8	15.6
CCU	Pro	1.1	7.0	8.3
CCC	Pro	2.2	5.5	9.1
CCA	Pro	4.4	8.5	10.9
CCG	Pro	1.7	23.3	6.6
CGU	Arg	22.6	21.0	9.7
CGC	Arg	22.6	22.0	10.7
CGA	Arg	3.3	3.5	2.5
CGG	Arg	56.1	5.4	5.0
CUU	Leu	7.7	11.0	10.1
CUC	Leu	16.5	11.0	12.2
CUA	Leu	16.5	3.9	10.6
CUG	Leu	134.8	52.8	9.5
GAU	Asp	31.9	32.2	30.3
GAC	Asp	16.0	19.1	19.2
GAA	Glu	82.0	39.6	41.9
GAG	Glu	34.1	17.8	14.7
GCU	Ala	31.4	15.3	25.2
GCC	Ala	13.2	25.5	16.5
GCA	Ala	19.8	20.3	13.8
GCG	Ala	13.2	33.7	11.1
GGU	Gly	2.8	24.9	27.9
GGC	Gly	2.8	29.4	11.8

Table 7.1; (continued)

Codon	Amino acid	Frequency in *hmw2* gene /1000	Frequency in *E. coli* /1000	Frequency in *M. pneumoniae* /1000
GGA	Gly	0.6	7.9	6.4
GGG	Gly	8.3	11.0	8.9
UAU	Tyr	12.1	16.3	14.3
UAC	Tyr	8.8	12.3	17.9
UAA	STOP	0.6	2.0	2.1
UAG	STOP	—	0.2	0.8
GUU	Val	11.0	18.4	21.2
GUC	Val	5.0	15.2	11.0
GUA	Val	8.8	10.9	13.8
GUG	Val	6.1	26.2	11.8
UCU	Ser	6.1	8.5	8.2
UCC	Ser	5.5	8.6	9.6
UCA	Ser	6.6	7.1	8.7
UCG	Ser	2.8	8.9	6.4
UGU	Cys	1.1	5.2	5.4
UGC	Cys	0.6	6.4	2.1
UGA	STOP[1]/Try[2]	2.2[1]/ — [2]	0.9	6.0
UGG	Try	1[1]/ 2.8[2]	15.3	5.8
UUU	Phe	31.4	22.4	43.1
UUC	Phe	5.0	16.6	12.7
UUA	Leu	52.9	13.9	39.3
UUG	Leu	27.0	13.7	21.5

1 = number of the original codon; 2 = number of the codons after mutagenesis. Red = codon frequency more than in *E.coli* and *M. p.* Red bold = codon frequency with significant different to *E. coli* and *M.p.*

Table 7.2: The 8 least used codons in *E. coli* [Zhang et al., 1991] and *M. pneumoniae* [Himmelreich, 1997].

E. coli	*M. pneumoniae*	amino acid
AGA	AGA	arginine
AGG	AGG	arginine
AUA	AUA	isoleucine
CUA		leucine
CGA	CGA	arginine
CGG	CGG	arginine
CCC		proline
UCG		serine
	UGA	tryptophan
	UGC	cysteine
	UGG	tryptophan

7.3 Presentations

Oral presentations:

Boonmee, A., Koegl, M., Loeser E., Schaller W., Herrmann R. (2001). Protein-Protein Wechselwirkung in *Mycoplasma pneumoniae*. 3. Deutsches Mycoplasmen-Symposium, Lübeck.

Boonmee, A., Herrmann R., Untersuchungen zur Protein-Protein Wechselwirkung in *Mycoplasma pneumoniae* am Beispiel von HMW2 (2003). 4. Deutsches Mycoplasmen-Symposium, 26-27.06.2003, Düsseldorf.

Poster presentations:

Boonmee, A., Koegl, M., Loeser E., Schaller W., Herrmann R. (2002). Protein-protein interaction in *Mycoplasma pneumoniae*, Building protein-protein interaction netwerks in Heidelberg, 18.06.2002, Heidelberg.

Boonmee, A., Koegl, M., Loeser E., Schaller W., Herrmann R. (2002). Protein-protein interaction in *Mycoplasma pneumoniae*, 14th Congress of the International Organization for Mycoplasmology (IOM), 7-12.07.2002 Vienna, Austria.

Boonmee, A., Koegl, M., Loeser E., Schaller W., Herrmann R. (2003). Analysis of protein-protein interaction in formation of a bacterial cytoskeleton in *Mycoplasma pneumoniae*, 55. Tagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM), 28.09-01.10-2003, Dresden.

Boonmee, A., Herrmann R. (2004). Analysis of protein-protein interaction in formation of a bacterial cytoskeleton in *Mycoplasma pneumoniae,* 15th Congress of the International Organization for Mycoplasmology (IOM), 11-16.07.2004, Athens, USA.