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DISSERTATION

Mycoplasma gallisepticum – Host Interactions: Identification, Localization and Role of Cytadherence Proteins

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Content

Abbreviations

A	adenin
A.	*Acholeplasma*
aa	amino acid
Abs	antibodies
Amp	ampicillin
AO	attachment organelle
AS	air sack
ATCC	American Type Culture Collection
ATP	adenosin triphosphate
bp	base pairs
BCA	bicinchoninic acid
BSA	bovine serum albumin
C	cytosin
Cam	chloramphenicol
CFU	colony forming units
COG	conserved orthologue group
CRD	chronic respiratory disease
ddH_2O	double-destilled H2O
DIF	double immunofluorescence
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotid triphosphate
dsDNA	double stranded DNA
E	endonuclease
E.	*Escherischia*
EDTA	ethylene diamine tetraacetic acid
EtOH	ethanol
Fig.	figure
FITC	fluoresceinisothiocyanate
G	guanin
Gm	gentamicin
HA	hemadsorption
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histidine
Ig	immunoglobuline
INV	invasive
IPTG	isopropyl-beta-D-thiogalactopyranoside
IS	inverted sequences
kb	kilobase pairs
kDa	kilodalton
LB	lysogeny broth
LR-PCR	long-range PCR
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
M.	*Mycoplasma*
MAb	monoclonal antibody
MBP	maltose binding protein
MEM	minimum essential medium Eagle
MG	*Mycoplasma gallisepticum*
mRNA	messenger RNA
NEB	New England Biolabs
nt	nucleotide
OD	optical density

PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAP	RNA polymerase
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription PCR
S.	*Spiroplasma*
SD	standard deviation
SDS	sodium dodecyl sulfate
SOB	super optimal broth
SOC	super optimal broth with catabolite repression
ssDNA	single stranded DNA
T	thymin
TBS	Tris-buffered saline
Tet	tetracycline
tetR	casette consiting of the *tetM* under *tetPO*
Tn	transposon
tRNA	transfer RNA
Trp	trpyptophan amino acid
U	unit
X-gal	5-bromo-4-chloro-3-indolyl b-D-galactopyranoside
2D	two dimensional

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14th International congress of the International Organization for Mycoplasmology (IOM), Vienna, Austria, July 7th-12th, 2002

- Identification of Variable Products of *Mycoplasma gallisepticum* Strain R Involved in Binding Erythrocytes
  I. *__Markova__, F. Winner, G. Vogl, K. Siebert-Gulle, S. Geary, R. Rosengarten, C. Citti

- Characterization of a *Mycoplasma gallisepticum* Mutant Obtained by Transposition Mutagenesis and Deficient for Hemadsorption
  P. Much, F. Winner, **I.** *__Markova__, K. Siebert-Gulle, G. Vogl, R. Rosengarten, C. Citti

55th Congress of the German Society for Hygiene and Microbilogy (DGHM), Dresden, Germany, September 29th – October 1st, 2003

- Factors Mediating *Mycoplasma gallisepticum* Host Colonization and Virulence
  I. *__Markova__, L. Stipkovits, R. Rosengarten, C. Citti

15th International Congress of the International Organization for Mycoplasmology (IOM), Athens, Georgia, July 11th – 16th, 2004

- *In vivo* and *in vitro* Approaches to Investigate *Mycoplasma gallisepticum* Factors Involved in Colonization and Systemic Infection of Chickens
  C. Citti, F. Winner, L. Stipkovits, **I. Indikova**, R. Rosengarten

- Role of *Mycoplasma gallisepticum* Cytadherence Proteins in Local and Systemic Infection of Chickens
  I. Indikova, L. Stipkovits, P. Much, R. Rosengarten, C. Citti
  (Poster nominated for Harry Morton Student award)

109th General Meeting of The American Society for Microbiology (ASM), Philadelphia, PA, USA, May 17th – 21st, 2009

- The Cytadherence - Related Molecule CrmA of *Mycoplasma gallisepticum* is Involved in Cellular Morphology, Motility, and Cytadherence, but not in Cell Invasion
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1. SUMMARY

Mycoplasma gallisepticum (MG) is an important avian pathogen that causes respiratory diseases in chickens and turkeys imposing severe commercial losses on the poultry industry worldwide. Cytadherence of MG which is the prerequisite for a successful infection is mediated by a terminal tip structure composed of several proteins with GapA being recognized as the major cytadherence protein which shares homology with the major adhesion of the human pathogen *M. pneumoniae,* P1. Interestingly, the well-described MG prototype strain R_{low} and its highly passaged derivative R_{high} differ not only in virulence but also in the expression of GapA. While the virulent strain R_{low} expresses GapA and is pathogenic for chickens, the attenuated strain R_{high} lacks GapA.

In order to elucidate the role GapA in cytadherence, the ability of MG to bind erythrocytes *in vitro* (hemadsorption [HA]) was examined. Virulent strain R_{low} displayed HA(+), HA(-) and mixed phenotypes (sectored colonies), and R_{high} was found to express exclusively the HA(-) phenotype. The absence of GapA in R_{low} –derived HA(-) clones resulted from mutation in the *gapA* gene, which differed from the mutation previously described for R_{high} and was found to switch with a high frequency. Importantly, the ability of hemadsorbtion correlated with the presence of GapA and CrmA, which was shown by generation of the *crmA*-negative mutant mHAD3 using transposon mutagenesis. The disruption of the *crmA* gene resulted in a decreased expression of GapA. Further analysis using RT-PCR revealed that both genes, *gapA* and *crmA*, are present on a single polycistronic RNA.

In order to investigate the role of the cytadherence-related proteins GapA and CrmA in cell invasion and host colonization, strains R_{low}, R_{high} and several clones displaying the HA(-) phenotype were subjected to the double immunofluorescence assay and the gentamicin invasion assay. It was demonstrated that GapA and CrmA are not required for cell invasion *in vitro*, although they are necessary for translocation through a polarized cell monolayer, as clones lacking GapA and CrmA, though able to invade non-phagocytic cells, were unable to translocate. To simulate the situation in the host, chickens were infected by aerosol, and after necropsy they were scored for the severity of air sac lesions, as well as for the re-isolation of MG from different inner organs. Although the re-isolation from the respiratory tract was not significantly different, only clones displaying the GapA/CrmA-positive phenotype were capable of successful infection *in vivo*.

For further elucidation of the role of the two cytadhesins, GapA and CrmA, in MG virulence and host colonization, chickens were infected by aerosol with the HA(-) mutants RCL2 (nonsense mutation in *gapA*) and mHAD3 (*crmA* disrupted by a transposon) and after necropsy scored for the severity of air sac lesions, as well as for the re-isolation of MG from different inner organs. Data indicated that the mutant mHAD3 had lost its virulence properties similarly to strain R_{high}. Surprisingly, however, the mutant RCL2 displayed a higher virulence than R_{high}, although a lower one than R_{low}. Furthermore, a correlation between the frequency of re-isolation from air sacks and inner organs was observed. The more often MG was re-isolated from the air sacks, as higher was the frequency of re-isolation for inner organs.

To reveal the function of various cytadherence-related genes of MG clustered in the "*mgc*-locus", a project was started to develop a targeted gene disruption. Suicide vectors carrying the subcloned target gene disrupted by the tetracycline resistance gene, *tetM*, were created and used for transformation of MG. Unfortunately, no transformants were obtained by following this direct approach. When a fragment of MG's origin of replication (*oriC´*) was added to the vector for disruption of *gapA*, such a modified vector gave rise to several transformants. Analyses of the clones revealed that indeed a homologous recombination event took place, however, not in the *gapA* locus but in the *oriC* region of the MG genome. Although not leading to the desired knock-out mutants, this vector allows the future development of specifically integrating expression vectors.

To define the role of each gene product of an "extended" *mgc* operon in the complex cytadherence process of MG and their localization within the cell, the development of specific antibodies was initiated. The selected genes, namely *mgc2*, *crmB* and *crmC*, were subjected to a site-directed mutagenesis and cloned into *E. coli* expression vectors. Purified fusion

proteins were used for the immunization of rabbits. The hyperimmune sera showed reactivity with MG proteins of the approximately expected sizes. With the exception of the Mgc2-specific antiserum that detected only a single band in all the clones tested, additional proteins were detected by CrmB- and CrmC-specific antisera. The characteristics of the proteins detected remains to be elucidated by other techniques, such as MALDI-TOF analysis or 2D-gel electrophoresis.

2. ZUSAMMENFASSUNG

Mycoplasma gallisepticum ist ein aviärer Krankheitserreger, der bei Vögeln respiratorische Erkrankungen oft chronischer Natur verursacht, und auch zu systemischen Infektionen führen kann. Eine entscheidende Rolle für den Infektionsverlauf spielt hierbei das Zytoadhärenzprotein GapA, welches dem Zytoadhärenzprotein P1 des humanen Krankheitserregers *M. pneumoniae* strukturell und funktionell sehr ähnlich ist. Interessanterweise unterscheiden sich die beiden *M. gallisepticum* Laborstämme R_{low} und R_{high} nicht nur in ihrer Virulenz, sondern auch in Bezug auf ihre GapA-Expression. Während der virulente Stamm R_{low} GapA exprimiert und für Hühner pathogen ist, exprimiert der vielfach passagierte Laborstamm R_{high} kein GapA und ist auch nicht mehr virulent.
Um die Bedeutung von GapA für die Virulenz zu untersuchen, wurde die Fähigkeit von *M. gallisepticum* getestet, Erythrozyten *in vitro* zu binden (Hämadsorption [HA]). Der virulente Stamm R_{low} zeigte hierbei einen HA(+)-, HA(-)- bzw. einen gemischten Phänotyp. Im Gegensatz dazu zeigte der Stamm R_{high} ausschließlich einen HA(-)-Phänotyp. Die Abwesenheit von GapA in HA(-) R_{low}, oder auch in HA(-)-Derivaten wird durch eine Mutation im Gen *gapA* verursacht, welche sich von einer zuvor beschriebenen anderen Mutation im Gen *gapA* von R_{high} unterscheidet und mit einer hohen Frequenz auftritt. Im Wesentlichen korrelierte die Fähigkeit, Erythrozyten zu binden mit der Anwesenheit von GapA und CrmA. Dies wurde durch die Erzeugung der CrmA-negativen Mutante mHAD3 mittels Transposon-Mutagenese nachgewiesen. Das Zerstören des *crmA*-Gens führte hierbei zu einer reduzierten GapA-Expression. Durch weitere Analysen mittels RT-PCR konnte gezeigt werden, dass die beiden Gene *gapA* und *crmA* auf einer polycistronischen mRNA liegen.
Um die Rolle von GapA und CrmA währen der Zellinvasion und der Wirtskolonisierung zu untersuchen, wurden die Stämme R_{low}, R_{high} und Derivate mit einem HA(-)-Phänotyp einem Gentamicin-Invasionsversuch unterzogen, sowie mittels Doppelter Immunfluoreszenzmikroskopie untersucht.
In in vitro Experimenten wurde bereits nachgewiesen, dass weder GapA noch CrmA für eine Zellinvasion benötig werden. Dennoch sind diese Proteine für eine Translokation durch eine polarisierte Zell-Einzelschicht (Zell-Monolayer) von Bedeutung. Klone, welche weder GapA noch CrmA exprimieren und daher in nicht-phagozytotische Zellen eindringen können, sind nicht in der Lage, eine polarisierte Zell-Einzelschicht zu durchdringen. In einem in vivo Experiment wurden Hühner mittels Aerosolen, bestehend aus Rlow, Rhigh und gemischten bakteriellen Kulturen, infiziert. Die infizierten Tiere wurden dann hinsichtlich des Schweregrades von Luftsackläsionen und der Anwesenheit von *M. gallisepticum* in verschiedenen inneren Organen untersucht. Unabhängig der, für die Infektion eingesetzten, bakteriellen Stämme konnte *M. gallisepticum* aus den Atemwegen der infizierten Tieren re-isoliert werden. Viel wichtiger ist jedoch die Tatsache, dass nur Klone mit einem GapA/CrmA-positiven Phänotyp in der Lage waren, unterschiedliche innere Organe zu besiedeln.
Um die Rolle der beiden Zytoadhäsine GapA und CrmA in Bezug auf die Virulenz und Wirtskolonisierung von *M. gallisepticum* weiter zu untersuchen, wurden Hühner mit den HA(-)-Mutanten RCL2 (*gapA* mit einem vorzeitigen Stoppcodon) sowie mHAD3 (*crmA* zerstört durch ein Transposon) infiziert und wie bereits beschrieben untersucht. Die Daten zeigten, dass die Mutante mHAD3 ihre Virulenz, ähnlich wie der Stamm R_{high}, verloren hat.

Die Mutante RCL2 zeigte überraschenderweise eine höhere Virulenz als R_{high}, aber dennoch eine geringere Virulenz als R_{low}. Zusätzlich wurde eine Korrelation zwischen der Häufigkeit der Reisolierung aus den Luftsäcken und den inneren Organen festgestellt. Je öfters *M. gallisepticum* aus Luftsäcken isoliert werden konnte, desto höher war auch die Frequenz der Reisolierung aus den inneren Organen.
Die Funktion einiger Zytoadhärenz-verwandten Gene von *M. gallisepticum*, welche im „mgc Lokus“ als Cluster vorliegen, wurde mittels gezielter Gendisruption untersucht. Hierzu wurden Suizidvektoren, welche Teile der zu disruptierenden Ziel-Gene trugen, erstellt, und in die Ziel-Genfragmente wurde eine Tetracyclinresistenzkassette (*tetPO/tetM*) inseriert. Unglücklicherweise konnten nach Transformation von *M. gallisepticum* mit diesen Suizidvektoren keine Transformanten erhalten werden. Durch das zusätzliche Hinzufügen eines Fragments des Replikationsursprungs (oriC) von *M. gallisepticum* war dies aber schlussendlich möglich. Eine Analyse der Transformanten zeigte, dass die Integration der Tetracyclinresistenz via homologe Rekombination nicht im gewünschten Zielgen *gapA*, sondern in der oriC-Region des *M. gallisepticum* -Genoms stattgefunden hat. Obwohl keine gewünschten “knock-out“-Mutanten hergestellt werden konnten, erlaubt dieser Vektor in der Zukunft die Entwicklung von spezifisch integrierenden Expressions-Vektoren.
Um die Funktion der Genprodukte eines „erweiterten“ mgc-Operons im komplexen Prozess der Zytoadhärenz von *M. gallisepticum*, sowie deren Lokalisation in der Bakterienzelle zu untersuchen, wurden spezifische Antikörper entwickelt. Die ausgewählten Gene *mgc2*, *crmB* und *crmC* wurden in *E. coli*-Expressionsvektoren kloniert und ortsspezifisch mutiert, um dem andersartigen Codon usage von Mycoplasmen Rechnung zu tragen. Fusionsproteine wurden hergestellt, gereinigt, und für die Immunisierung von Kaninchen verwendet. Die gewonnenen Sera zeigten Reaktivität mit *M. gallisepticum* Proteinen der erwarteten Größen. Im Gegensatz zum Mgc2-spezifischen Antiserum, welches nur eine einzelne Proteinbande detektierte, wurden mit den CrmB- und CrmC-spezifischen Antiseren zusätzliche Proteine detektiert. Die Art dieser Proteine, und damit die Spezifität der Seren, muss nun mit andern Techniken, wie zum Beispiel der MALDI-TOF-Analyse oder einer 2D-Gelelektrophorese bestimmt werden.

3. INTRODUCTION

3.1. Mycoplasmas

Mycoplasmas are the smallest prokaryotes known capable of self-replication. They are distinguished from other bacteria by the absence of a cell wall. Taxonomically, the lack of the cell wall is used to separate the class *Mollicutes*, which comprises of mycoplasmas and other small procaryotic organisms bounded by a single trilaminar cell membrane (spiroplasmas, acholeplasmas, ureaplasmas and others). *Mollicutes* are wide-spread in nature occurring as saprophytes or obligate parasites of humans, mammals, reptiles, fish, arthropods, and plants (Razin, 1992). Many of them are pathogenic and play a proven primary role in certain infectious diseases. The number of established mollicute species is continuously increasing and it is widely agreed that the species defined to date are only a minor part of all mollicutes living in nature. Helpful tools for species and strains identification are, besides serology, molecular-biological techniques such as ribotyping, comparison of other conserved gene sequences, and restriction fragment length polymorphism (RFLP). Genetic evidence indicates that mycoplasmas arose by degenerative evolution from Gram-positive eubacteria with DNA of a low GC content. Ribosomal transfer – RNA analysis suggests a relationship to bacteria of the genus *Clostridium*. The mycoplasma genome consists of a circular dsDNA molecule with a size ranging between 600 kb to over 2.200 kb. The genome sizes are variable not only within the same genus but even among strains of the same species (Carle et al., 1995; Huang, Robertson, and Stemke, 1995; Robertson et al., 1990). This is due to the frequent occurrence of repetitive elements, which are often subject of chromosomal rearrangements (see below) (Dybvig and Voelker, 1996). Hence, mycoplasmas are organisms with the smallest genome size among all known self-replicating organisms. Despite of the marked reduction of mycoplasma genomes, the gene density remained the same. Interestingly, some promoter regions were detected within the C-terminal coding sequence of the upstream gene. But such overlaps appear to be few and short and hence do

not appear to have a significant effect on genome thickness in mollicutes (Bork et al., 1995; Peterson et al., 1995).

The fast development in genome-sequencing methodology has brought new light into the mycoplasmatology. The first mycoplasmas, whose genome was completely sequenced, were the closely related human pathogens *Mycoplasma genitalium* (Fraser et al., 1995) and *Mycoplasma pneumoniae* (Himmelreich et al., 1996). The comparison of *M. genitalium* and *M. pneumoniae* sequences to that of *Haemophilus influenzae* (Fleischmann et al., 1995) suggested the set of genes, which are essential for a minimal cell (Mushegian and Koonin, 1996). Most striking are the results concerning genes of biosynthetic pathways. *M. genitalium* and *M. pneumoniae* have lost all the genes involved in amino acid biosynthesis during their evolution, and thus require the full spectrum of essential amino acids from the host or from the artificial culture medium. Significant savings in genetic information resulted also from the loss of genes involved in cell wall biosynthesis. Furthermore, mycoplasma energy and protein metabolism has undergone reductions as well. Mycoplasmas lack many systems such as tricarboxylic acid cycle, quinones and cytochromes, the electron transport system is reduced, and ATP is produced by substrate-level phosphorylation (Razin, Yogev, and Naot, 1998). Most mycoplasmas have also an unique requirement for cholesterol and lipids for membrane synthesis. They also lack the enzymatic pathways for the synthesis of purines and pyrimidines. As a result of these reductions, mycoplasmas require complex culture media such as for example beef-heart infusion, broth supplemented with horse serum, yeast extract and nucleic acids for cultivation. When grown on solid media, mycoplasmas slowly form a dome-shaped colony on the surface of agar. The central part of the colony grows down into the agar, producing a denser central core. When viewed from above the colony resembles a fried egg in appearance. The colonies are very small and therefore require dissecting microscopes for their visualisation. The growth speed is slower in comparison to other prokaryotes. A doubling time of one to six hours means that up to three weeks may be necessary before colony formation becomes visible.

On the contrary to all genome reductions, some repetitive elements were found in the mycoplasma genome. These segments of genetic information enable homologous recombination and genomic rearrangements that may play a role in the antigenic variation of the mycoplasmal cell surface and this subsequently helps the parasite to evade the host immune response. Repetitive elements consisting of short segments of the cytadhesin operon are distributed over the whole genome of *M. genitalium* and *M. pneumoniae* (Su, Chavoya, and Baseman, 1988). Other examples are the *vlhA* genes in *M. gallisepticum* (MG), encoding for immunogenic haemagglutinins, which occupy about 16% of the MG genome (Baseggio et al., 1996; Glew et al., 1995; Markham et al., 1994). Recombination between the *vlhA* genes generates changes in antigenic determinants and hence helps to evade the host immune response.

The obligatory parasitic lifestyle requires special associated properties – mycoplasma cells possess surface components enabling their attachment to the host cells. Intimate contact of the mycoplasmas with their host is required to furnish nutrients and specific growth factors, especially nucleic acid precursors, which mycoplasmas are unable to synthetize. In some cases, including *M. genitalium* and *M. pneumoniae* and MG, the mycoplasmas developed special attachment organelles (AO), best studied in *M. pneumoniae* (Hahn, Willby, and Krause, 1998; Krause, 1998; Krause and Balish, 2001; Seto et al., 2001; Willby et al., 2004). The AO is a terminal structure, a tapered membrane protrusion involved in several processes essential to the cell *in vivo* (Balish, 2002; Krause and Balish, 2001). Mediation of attachment to host cell (cytadherence) is the best-characterized function (Krause, 1998). The AO renders mycoplasma cells asymmetric and functions as a leading end for gliding motility of *M. pneumoniae* (Henderson and Jensen, 2006). This organelle also may have a role in initiating cell division (Miyata and Seto, 1999; Seto et al., 2001). The AO and the polar filamentous cell shape of *M. pneumoniae* are thought to be stabilized by intracellular cytoskeleton-like structures, which have been observed in electron micrographs of *M. pneumoniae* (Shimizu and Miyata, 2002). The most remarkable architectural feature of the cytoskeleton-like structures is the electron-dense core, a rod-like structure that exists at the

center of the AO. The rod-like structure has a knob at the distal end (terminal button) (Balish, 2005).

Lacking a cell wall and intracytoplasmic membranes, the mollicutes have only one type of membrane, the plasma membrane. Proteins constitute over two-thirds of the mycoplasma membrane mass, with the rest being membrane lipids. Furthermore, membrane lipoproteins are among the most dominant antigens in mollicutes, and a majority of the mycoplasma cell surface antigens known to undergo antigenic and/or size variation are lipoproteins. In *M. hyorhinis*, elongated surface lipoproteins also may protect mycoplasma cells from growth-inhibiting antibodies (Citti, Kim, and Wise, 1997). The unusually large number of lipoproteins in mollicutes may be attributed to the absence of a periplasmic space in the wall-less mollicutes. Mollicutes possess typical eubacterial signal peptides that direct the newly synthesized proteins into a secretory pathway for transport across the cell membrane (Yogev et al., 1991b).

3.2. Mycoplasma pathogenicity

Mycoplasmas usually exhibit a rather strict host and tissue specificity, probably reflecting their exact nutitional requirement and obligate parasitic mode of life. *M. pneumoniae* is found preferentially in the respiratory tract and *M. genitalium* is found primarily in the urogenital tract, although exceptions are possible (Goulet et al., 1995).

The primary habitats of human and animal mycoplasmas are the mucous surfaces of the respiratory and urogenital tracts, the eyes, alimentary canal, mammary glands, and joints. The obligatory anaerobic anaeroplasmas have so far been found in the bovine and ovine rumen only (Razin, Yogev, and Naot, 1998). Infections with pathogenic mycoplasmas usually follow a chronic course with low mortality.

Adhesion of mollicutes to host cells is a prerequisite for colonization and for infection (Razin and Jacobs, 1992). The loss of adhesion capacity by mutation results in a loss of infectivity, and reversion to the cytadhering phenotype is accompanied by regaining infectivity and virulence (Krause, Leith, and Baseman, 1983; Romero-Arroyo et al., 1999). The critical role

of cytadherence in virulence is reflected by the inability of noncytadhering mycoplasma strains to cause disease in experimentally infected animals (Much et al., 2002; Papazisi et al., 2002). The cytadherence process appears to be multifactorial involving a number of accessory membrane proteins (Baseman et al., 1982). These accessory proteins act in concert with cytoskeletal elements to facilitate the lateral movement and concentration of the adhesin molecules at the attachment tip organelle. The best studied example is the cascade of accessory proteins properly localizing the main cytadhesin P1 of *M. pneumoniae* (Krause and Balish, 2001).

To meet the challenges imposed by host defence mechanisms and changing environments, microorganism populations possess mechanisms and strategies allowing them to sense environmental changes and to rapidly respond and adapt to the new surroundings. Such mechanisms may include mimicry of host antigens, survival within professional phagocytes, and generation of phenotypic plasticity. The latter has been defined as the ability of a single genotype to produce more than one alternative form of morphology, physiological state, and/or behaviour in response to environmental conditions. One of the most common examples for phenotypic plasticity is antigenic variation. The term “antigenic variation” or “phenotypic switching” refers to the ability of a microbial species to alter the antigenic character of its surface components including flagella, pili, outer membrane proteins, and capsules (Barbour and Restrepo, 2000; Finlay and Falkow, 1997; Henderson, Owen, and Nataro, 1999). The above mentioned cell components are the major targets of host antibody response; therefore, the ability of a microorganism to rapidly change the immunogenicity of these structures and consequently to vary the surface antigenic repertoire allows to effectively avoid recognition of the immune system. One of the mechanisms for surface antigenic variation is based on the ability of a microbial population to spontaneously and randomly generate distinct cell populations with different antigenic phenotypes. The frequency of occurrence of such antigenic variants is strikingly high (10^{-4} to 10^{-2}) per cell per generation (Citti, 2005; Wise, 1993; Yogev et al., 1991a) compared to 10^{-6} to 10^{-8} for other normally occurring mutations. The molecular switching events leading to the generation of

these heterotypes are reversible, and the escape variants produced through random genetic variation must inherit the ability to produce, at high frequency, a wide range of antigenic phenotypes. It may also provide the pathogen, during the course of infection, the flexibility within the host to reach and adapt to different niches where distinctive receptors may be required for colonization.

The discovery made by comparative genomics that the minute mycoplasmas possess an impressive capability of maintaining a surface architecture that is antigenically and functionally versatile has placed the mycoplasmas in the “elite” group of bacterial pathogens and parasites distinguished by remarkable antigenic variability (Robertson and Meyer, 1992). The extreme variability of mycoplasma cell surface composition among clonal populations (Citti, 2005; Citti and Rosengarten, 1997; Dybvig and Voelker, 1996; Razin, Yogev, and Naot, 1998; Rosengarten et al., 2000; Wise, 1993) is based on two types of variation, both of which occur spontaneously at a high frequency: (i) phase-variation, known as ON and OFF switching, where a certain component undergoes variation in expression, (ii) size variation, which affects the structure of these components, in most cases by altering the length of their carboxyl-terminal region and (iii) epitope masking/demasking. A given cell component may be subject to either of these types of variation, or to both in an independent manner. The variable cell surface components that have been described to date in mycoplasmas are proteins and are products of either gene families or single genes (Citti, 2005).

A well-established test for determining whether a particular surface antigen undergoes high-frequency phenotypic switching is the colony immunoblot technique. Immunostaining with monoclonal or polyclonal antibodies allows the identification of colonies exhibiting variation in the expression of surface proteins (Rosengarten and Wise, 1990). One of the most conspicious ways this heterogeneity takes shape in *in vitro* studies is by colony sectoring (Athamna et al., 1997; Rosengarten and Wise, 1990). A sector is defined as an immunologically distinct region within a single colony in which a change in protein expression has occurred.

3.3. *Mycoplasma gallisepticum*

MG is an avian pathogen belonging to the phylogenetic cluster of *M. pneumoniae*. It causes chronic respiratory disease (CRD) in chickens (Jordan, 1979; Stipkovits and Kempf, 1996) and infectious sinusitis in turkeys (Davidson et al., 1982), and is therefore imposing a major problem for the poultry industry worldwide. Infection with this bacterium is spread by aerosol exposure or via egg transmission. Outbreaks spread rapidly through flocks, establish chronic infections, and are difficult to control with antimicrobial therapy. The disease causes substantial economic losses from decreased egg production and hatchability and condemnation of the infected flocks. Like many other members of the mycoplasma group, this avian pathogen colonizes its host via the mucosal surfaces of the respiratory tract and must adhere to the epithelial cells to withstand clearance by the host. This intimate contact is mediated by a unipolar terminal organelle that is similar to the tip structure of the two human pathogens, *M. genitalium* and *M. pneumoniae*. The chronic nature of the mycoplasma infection demonstrates a failure of the host immune system to deal effectively with these organisms. Antigenic variation of surface proteins allows MG to evade the host's immune response through the generation of escape variants (Glew et al., 2000; Gorton, Goh, and Geary, 1995; Levisohn, Rosengarten, and Yogev, 1995). Intracellular invasion and survival within eucaryotic cells by MG may contribute to this organism's resistance to the host's immune response and antimicrobial therapy (Vogl et al., 2008; Winner, Rosengarten, and Citti, 2000).

MG colonizes the respiratory system of chickens but has also been isolated from the inner organs, brain and eyes of several avian species (Fischer et al., 1997; Much et al., 2002). These finding would suggest that MG has the capability to translocate across the respiratory mucosal barrier and disseminate throughout the body. The virulence factors that promote MG infection and induce disease are not well understood and are most likely influenced by the host and environment. Early studies revealed that MG strains differ markedly in their pathogenicity for chickens (Levisohn, 1985), and that *in vitro* passages in culture medium of a particular MG strain affect its virulence (Levisohn, 1985). More specifically, the evaluation

of the pathogenic effects of MG on the respiratory tract by infection of chickens revealed that a low (R_{low}) as well as a high laboratory passage (R_{high}) of the MG prototype strain R (Lin and Kleven, 1984) both colonize the trachea, while only R_{low} induces air sac lesions. Recently, it was also shown that these two passages also differ in their ability to invade non-phagocytic eucaryotic cells *in vitro*: while R_{low} was capable to enter and survive within the host cell, the ability to establish intracellular residence of R_{high} was decreased (Winner, Rosengarten, and Citti, 2000). Experimental aerosol infection of chickens demonstrated the attenuated virulence of R_{high}, which was isolated from the upper respiratory tract only and was unable to induce air sacculitis and systemic infection. On the contrary, the low passage R_{low} was colonizing the whole respiratory tract, inducing air sac lesions and spreading throughout the body by crossing the mucosal barrier and entering the bloodstream. Re-isolations revealed the presence of R_{low} in inner organs – i. e. spleen, heart, kidney and brain (Much et al., 2002).

The molecular basis of the diminished cytadherence and attenuated virulence of R_{high} was elucidated by a study (Papazisi et al., 2000), which showed the lack of at least two proteins, GapA and CrmA. The cytadhesin gene *gapA* (Goh et al., 1998), also referred to as *mgc1* (Keeler et al., 1996), is not expressed in R_{high} due to a insertion of single adenine in the beginning of the *gapA* gene thereby causing a frameshift mutation. As a consequence, a stop codon is created soon after resulting in premature termination of translation (Papazisi et al., 2000). Lack of the cytadhesin-related molecule, CrmA, was described as a consequence of the premature termination of translation of GapA. (Papazisi et al., 2000). A reversible mutational event occurring at a high frequency within the coding region of *gapA* has a polar effect on the expression of the *crmA* gene, which is located downstream as a part of the same transcriptional unit (Winner et al., 2003). Expression of these two components has been correlated with binding to erythrocytes (Winner et al., 2003) and to efficient attachment to cultured MRC-5 human fetal lung fibroblasts (Papazisi et al., 2002). As mentioned above, also R_{high} was re-isolated from the respiratory tract of chickens after experimental infection suggesting that other factors are involved in initiating a local infection (Much et al., 2002).

Several other putative cytadhesion-related molecules have been characterised in MG, including PvpA, which independently undergoes both phase and size variation and which is localised in the AO (Boguslavsky et al., 2000; Rosengarten et al., 2000).

3.4. Literature cited

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4. PAPERS AND MANUSCRIPTS

4.1. Variation in expression of the GapA and CrmA cytadherence protein *in vitro*

Paper (see attached)

*Winner F., **I. * ^Markova**, P. Much, A. Lugmair, K. Siebert-Gulle, G. Vogl, R. Rosengarten, and C. Citti. 2003. Phenotypic switching in *Mycoplasma gallisepticum* hemadsorption is governed by a high frequency, reversible point mutation. Infect. Immun. 71: 1265-1273.

*F. Winner and I. Markova contributed equally to this study.
^ maiden name

4.2. Role of the GapA and CrmA cytadherence proteins in cell invasion and translocation of cell monolayers

Manuscript (see attached)

Winner, F., P. Much, L. Stipkovits, **I. Indikova**, K. Siebert-Gulle, R. Rosengarten, and C. Citti. *In vitro* translocation of *Mycoplasma gallisepticum* through cell monolayers with tight junctions.

4.3. Role of the GapA and CrmA cytadherence proteins *in vivo*

Manuscript (see attached)

Indikova, I., P. Much, L. Stipkovits, K. Siebert-Gulle, M. Szostak. R. Rosengarten, and C. Citti. Role of *Mycoplasma gallisepticum* GapA and CrmA cythadhesins in promoting virulence and in colonization of the natural chicken host.

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Phenotypic Switching in *Mycoplasma gallisepticum* Hemadsorption Is Governed by a High-Frequency, Reversible Point Mutation

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***Mycoplasma gallisepticum* is a flask-shaped organism that commonly induces chronic respiratory disease in chickens and infectious sinusitis in turkeys. Phenotypic switching in *M. gallisepticum* hemadsorption (HA) was found to correlate with phase variation of the GapA cytadhesin concurrently with that of the CrmA protein, which exhibits cytadhesin-related features and is encoded by a gene located downstream of the *gapA* gene as part of the same transcription unit. In clones derived from strain R_{low}, detailed genetic analyses further revealed that on-off switching in GapA expression is governed by a reversible base substitution occurring at the beginning of the *gapA* structural gene. In HA^- variants, this event generates a stop codon that results in the premature termination of GapA translation and consequently affects the expression of CrmA. Sequences flanking the mutation spot do not feature any repeated motifs that could account for error-prone mutation via DNA slippage and the exact mechanism underlying this high-frequency mutational event remains to be elucidated. An HA^- mutant deficient in producing CrmA, mHAD3, was obtained by disrupting the *crmA* gene by using transposition mutagenesis. Despite a fully functional *gapA* gene, the amount of GapA detected in this mutant was considerably lower than in HA^+ clonal variants, suggesting that, in absence of CrmA, GapA might be subjected to a higher turnover.**

Mycoplasma gallisepticum is a round flask-shaped organism commonly inducing chronic respiratory disease in chickens (14, 26, 32) and infectious sinusitis in turkeys (7). Like a large number of other mycoplasmas, this avian pathogen colonizes its host via the mucosal surfaces of the respiratory tract and must adhere to the epithelial cells to withstand clearance by the host. This intimate contact is mediated by a bleb-like structure (27, 28), a unipolar terminal organelle that is similar to the tip structure of the two human pathogens, *M. pneumoniae* and *M. genitalium* also involved in adhesion to host cells. Both mycoplasma species were shown to enter epithelial cells (2, 13), and recent in vitro assays have revealed that *M. gallisepticum* is likewise capable of establishing intracellular residence in nonphagocytic eukaryotic cells (29). During infection of highly immunocompetent hosts, the ability to enter and survive within host cells may provide these mycoplasmas with a survival strategy that relies first on adhesion. Cytadhesins and related components have been extensively studied in *M. pneumoniae* (17), and the data emerging from similar studies in *M. gallisepticum* suggest the occurrence of a family of cytadhesin genes conserved among pathogenic mycoplasmas that colonize widely divergent hosts. The identification and the characterization of *M. gallisepticum* surface-exposed components with adhesive properties are therefore of major importance in understanding the factors involved in promoting successful infection.

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In recent years, a large collection of data has underlined the versatility of the mycoplasma surface architecture, which is mediated via spontaneous high-frequency variation in the expression and structure of surface proteins (6, 24). In *M. gallisepticum*, systems generating phase variation of cytadhesins or cytadhesin-related molecules have been identified. These include the *pMGA* genes encoding a family of hemagglutinins (21, 22) that are subjected to phase variation (9, 23) and the single-copy *pvpA* gene (3), encoding a potential cytadhesin-related molecule that is localized at the tip structure of the organism and undergoes variation in size and expression independently (33). Binding of erythrocytes to *M. gallisepticum* strain A5969 was shown to occur via several surface-exposed proteins that undergo high-frequency variation in expression, although the exact nature of these products could not be clearly established (1). Three clustered genes have also been identified in the *M. gallisepticum* genome as encoding for products with homology to adhesin-related molecules of *M. pneumoniae*. These are, from 5′ to 3′, (i) *mgc2*, which encodes a 32-kDa product with homology to the P30 of *M. pneumoniae* (12); (ii) *mgc1* (15), also referred to as *gapA* (8), which encodes a 105-kDa protein and presents homology to the *M. pneumoniae* P1 adhesin; and (iii) *mgc3* (34), also referred as *crmA*, which encodes a 116-kDa product with homology to *M. pneumoniae* open reading frame 6 (ORF6) and is cotranscribed with *gapA* (24). Whether any of these three cytadhesin-related products is subject to phase variation and is involved in hemadsorption (HA) has still to be assessed; nevertheless, the presence of multiple adhesin genes in *M. gallisepticum* might emphasize the multifactorial nature of the cytadherence process. Interestingly, Yoshida et al. (34) showed that the product encoded by *mgc3* contained epitopes that could induce antibodies capable of inhibiting growth and metabolic activities of

M. gallisepticum strain R, suggesting that the function of the CrmA product might not be strictly restricted to adherence.

Recently, it was shown that low (R_{low}) and a high (R_{high}) laboratory passages of the prototype strain R (19), which markedly differ in their pathogenicities (18), also differ in the expression of the GapA and the CrmA proteins (24). More specifically, these proteins are expressed in the virulent R_{low}, whereas they are both lacking in the avirulent R_{high}.

In the present study, we have revisited the capability of *M. gallisepticum* to bind erythrocytes by using R_{low} and R_{high} and assessed the nature of the products involved in HA of strain R. Results showed that in R_{low} the GapA and CrmA products concomitantly undergo phase variation and are responsible for the binding of erythrocytes to *M. gallisepticum* cells in the HA assay. The genetic mechanism underlying this variation is a nonsense mutation that is occurring in the *gapA* gene and affects the expression of *gapA* and that of the *crmA* gene located downstream. In contrast to other previously reported high-frequency mutations generating phase variation in mycoplasmas or in other bacteria, the sequence surrounding the hotspot for mutation has no particular genetic feature such as repeated elements or homopolymeric nucleotide tracts that could promote error prone mutations by DNA slippage.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *M. gallisepticum* laboratory passages R_{low} and R_{high} used in the present study were kindly provided by S. Levisohn, Kimron Veterinary Institute, Bet Dagan, Israel. R_{low} and R_{high} correspond to the prototype strain R propagated 10 and 160 times in artificial medium, respectively (19). R_{low}P3 was previously described (29) and corresponds to three passages of R_{low} in HeLa cells. Mycoplasma cultures were grown at 37°C in modified Hayflick medium (30) containing 20% (vol/vol) heat-inactivated horse serum (Invitrogen Life Technologies, San Diego, Calif.) to mid-exponential phase, as indicated by the metabolic color change of the medium.

Clonal variants were obtained from R_{low} as follows. An optimal concentration of *M. gallisepticum* R_{low} cells was seeded onto modified Hayflick containing 1% (wt/vol) Noble agar and grown for 5 to 7 days at 37°C. Colonies that did or did not bind erythrocytes were picked, expanded in 1 ml of liquid medium, and plated onto solid medium at appropriate dilutions. Five to ten isolated colonies of the second generation were picked and grown in 1 ml of culture. An aliquot of each culture was then seeded onto agar plates, and the resulting colonies were subjected to the HA assay to assess the purity of the clones, whereas the remaining culture was frozen at −20°C for further analysis. Isolated colonies presenting $GapA^+$ or $GapA^-$ phenotypes were selected, grown in 1 ml of liquid broth, and stored at −80°C for further analysis.

Competent *Escherichia coli* DH10B (Invitrogen) was used as host to clone recombinant products and grown at 37°C in Luria-Bertani broth supplemented with 100 μg of ampicillin per ml for plasmid preparation.

Colony immunoblotting and HA assay. Colony immunoblotting was performed as previously described (5) with the antibodies and under the conditions described below for Western blot analyses. The HA assay was conducted directly on agar plate. After partial lifting of the mycoplasma colonies onto nitrocellulose membranes, the colonies were overlaid with 15 ml of fresh sheep blood washed and resuspended in phosphate-buffered saline (PBS) solution (2.7 mM KCl, 1.2 mM KH_2PO_4, 138 mM NaCl, 8.1 mM $Na_2HPO_4 \cdot 7H_2O$; pH 7.4) to a final concentration of 0.5% (vol/vol). After incubation at 37°C for 30 min, the suspension was then carefully discarded, and unbound erythrocytes were gently removed by a wash with PBS. Mycoplasma colony immunostaining and binding of the erythrocytes were observed by using an SMZ-U stereomicroscope (Nikon Corp., Tokyo, Japan).

SDS-PAGE and Western blot analysis. Protein profile analysis of the strains, clones, and mutant used in the present study was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie staining or Western blotting as previously described (5), by using the whole-cell extract or fractions obtained by Triton X-114 (Sigma) partitioning as described elsewhere (31). Antibodies used for immunostaining were previously reported and correspond (i) to rabbit anti-GapA (24) diluted 1:8,000 and (ii) to monoclonal antibody (MAb) 1E5 (33) diluted 1:50. Detection of antibody binding was achieved by using peroxidase-conjugated swine antiserum to rabbit immunoglobulin (Dako, Copenhagen, Denmark) or to mouse immunoglobulin M (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.). Antibodies were diluted in Tris-buffered saline solution (150 mM NacCl, 10 mM Tris base) containing 0.05% (vol/vol) Tween 20.

Transposition mutagenesis of *M. gallisepticum* and selection of the mHAD3 mutant. The plasmid pISM2062 used below for transformation was kindly provided by C. Minion (Iowa States University, Ames, Iowa) and carries Tn*4001*, in which a *Bam*HI restriction site has been previously inserted in the left inverted sequence (16). Prior to transformation, this plasmid was modified for other studies not reported here by inserting into the *Bam*HI restriction site random tag-oligonucleotide sequences generated as previously described by Hensel et al. (11) by using the oligonucleotide 5′-CTA**GGTACC**TACAACCTCAAGCTT $(NK)_{20}$AAGCTTGGTTAGAATG**GGTACC**ATG-3′ (the *Bam*HI restriction sites are indicated in boldface). The resulting tagged transposon and corresponding plasmids were designated in the present study as Tn*4001* mod and pISM2062-tag, respectively.

A culture of *M. gallisepticum* R_{low}P3 containing ca. 10^9 CFU was centrifuged and washed three times with electroporation buffer (8 mM HEPES [pH 7.4], 272 mM sucrose). The cells were then resuspended in 100 μl of electroporation buffer, incubated on ice for 10 min with 10 μg of the pISM2026-tag (see below), and subjected to electroporation (2.5 kV, 100 Ω, 25 μF). After electroporation, the cells were resuspended in 1 ml of chilled Hayflick medium and incubated on ice for 10 min and at 37°C for 90 min. Gentamicin was then added to the cell culture to a final concentration of 100 μg/ml, and aliquots of 25 to 100 μl were plated onto solid Hayflick medium containing 50 μg of gentamicin/ml. After incubation at 37°C for 8 days, 2,200 colonies were picked and individually grown in 96-well microtiter plates containing 200 μl of Hayflick medium per well. Using a replicator (Sigma Chemical Co., St. Louis, Mo.), the 2,200 cultures were transferred onto solid agar plates and were then subjected to the HA assay after 7 days of growth at 37°C as described above. HA^- cultures were then grown in 1 ml of Hayflick medium and seeded onto agar plates at high density, and the resulting colonies were subjected to the HA assay to define whether mutants were stable for the HA^- phenotype. One mutant designated mHAD3 was selected for analysis described in the present study.

DNA manipulations. Standard methods were used for DNA manipulations, including agarose gel electrophoresis, restriction endonuclease digestion, ligation, chemical transformation, and electroporation as described elsewhere or according to the manufacturer's instructions. Southern hybridizations were performed according to the Genius System *User's Guide for Membrane Hybridization*, version 3.0 (Roche Molecular Biochemicals, Mannheim, Germany). In-gel purification of DNA fragments was performed by using Quantum Prep Freeze'N'Squeeze DNA gel extraction spin column (Bio-Rad, Hercules, Calif.).

Cloning and sequencing analysis. Chromosomal DNA of the RCL2 clonal variant was digested to completion with *Xba*I. The resulting fragments were inserted into *Xba*I-restricted, dephosphorylated pUC18 vector, and the ligation mixture was used to transform competent *E. coli* DH10B cells by electroporation. Recombinant clones were detected by hybridization of colonies by using a digoxigenin-labeled *gapA* probe generated by PCR as described below. One recombinant plasmid (pRCL2) containing a 4.6-kbp *Xba*I insert that hybridized with the *gapA* probe (see Fig. 4B) was selected, and the region containing the *gapA* sequence was determined by primer walking.

For the cloning and sequencing of regions flanking the integrated Tn*4001* of mHAD3, 50 μg of genomic DNA was digested to completion with the *Xba*I enzyme. DNA fragments of ca. 7.3 kbp that hybridized with the transposon-specific probe were gel purified and precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold ethanol. An aliquot containing ~5 ng was incubated with T4 DNA ligase (Roche) overnight at 16°C and subjected to long-range PCR (LR-PCR) by using the expand long template PCR system (Roche) and primer pIS256rev. The LR-PCR cycling conditions were as follows: 2 min at 94°C; 10 cycles of 30 s at 94°C, 30 s at 66°C, and 4 min at 68°C; 20 cycles of 30 s at 94°C, 30 s at 66°C, and 4.5 min at 68°C, with cycle elongation of 20 s per cycle; and finally 7 min at 68°C. The resulting LR-PCR fragments were cloned into the pGEM-T Easy (Promega, Madison, Wis.) vector, and one recombinant clone, selected by the size of its DNA insert, was sequenced. The *gapA* gene of the mutant mHAD3 was obtained by LR-PCR with genomic DNA template and the primers GAPA0 and GAPA6, cloned into pGEM-T Easy, and sequenced. The cycling conditions for the LR-PCR were as follows: 2 min at 94°C; 10 cycles of 30 s at 94°C, 30 s at 56°C, and 4 min at 68°C; 20 cycles of 30 s at 94°C, 30 s at 56°C, and 4 min at 68°C, with cycle elongation of 20 s per cycle; and finally 7 min at 68°C.

DNA sequencing was performed at VBC-Genomics Bioscience Research-

TABLE 1. Oligonucleotide primers used in this study

Oligonucleotide	Nucleotide sequence (5′ to 3′)[a]	Description and localization[b] (nt position)	Source or reference[c]
GAPA0	GGCAGGACCAAGAGCTGG	Forward primer located upstream of the *gapA* gene (183–200)	This study
GAPA1	GGATTAGCAGTTTCTGGAGC	Forward primer located at the beginning of the *gapA* gene (457–476)	This study
GAPA2	TGTTCTTGTTGAACCGCTGC	Reverse primer located at the beginning of the *gapA* gene (881–861)	This study
GAPA4	TTCGGAAAATCCCTTTGCAGTAG	Forward primer located in the *gapA* gene (1275–1297)	This study
GAPA5	TAGAGGAGTAGTTGTTTGAGTTTC	Reverse primer located in the *gapA* gene (1491–1467)	This study
GAPA6	CTTGCAGAACCAAGAGCTCC	Reverse primer located at the beginning of the *crmA* gene (3854–3835)	This study
TufG15	TTCGATCGTAGTAAACCTCACG	Forward primer located in the *tuf* gene (107–128)	9
TufC26	GACGATTTTGAGTTGCGTATTC	Reverse primer located in the *tuf* gene (296–317)	9
Tn1	ACATGAATTACACGAGGGC	Forward primer located in the Tn*4001* (2440–2458)	This study
Tn2	GTTCTTCTTCTGACATAGTAG	Reverse primer located in the Tn*4001* (2840–2820)	This study
IS256rev	GGTCATGTAAAAGTCCTCCTGGG	Primer located in the IS256 of Tn*4001*tag in mHAD3 (340–318 and 4694–4715)	This study
IF	GCCGGATTGATTTGTATG	Forward primer located in the *gapA* gene (644–661)	24
IR	CAGAAGTAGAAGCAGTAGGA	Reverse primer located in the *gapA* gene (1105–1086)	24
JF	TAAGAAGACTCCACAAATGCT	Forward primer located in the *gapA* gene (2718–2738)	24
JR	TAGCATCTAGCGTTCTTGCTTG	Reverse primer located in the *crmA* gene (3928–3907)	24

[a] Oligonucleotides were designed based on previously published sequences.
[b] Nucleotide positions are indicated with regard to the sequence previously published (AF214004).
[c] References are indicated when primers have been previously used by others in similar studies.

GmbH, Vienna, Austria, with IRD 700 or IRD 800 dye-labeled sequencing primers, dideoxy PCR, and a Li-COR DNA 4200 sequencer.

PCR assays. PCR assays were performed by using 1 to 3 U of *Taq* DNA polymerase (Promega) in 1× buffer supplied by the manufacturer, 1.5 to 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate (dNTP; Promega), a 1 nM concentration of each primer listed in Table 1, and ca. 100 ng of chromosomal DNA as a template. The same conditions were used to generate digoxigenin labeling by PCR except that the dNTP mix contained a digoxigenin-11-dUTP (Roche)/dTTP ratio of 1:19.

Termocycling conditions were as follows: (i) for GAPA1/GAP2, 1 cycle at 95°C for 1 min; 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and (ii) for GAPA4/GAPA5, 1 cycle at 95°C for 3 min; 30 cycles at 95°C for 1 min, 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min, and a final extension cycle at 72°C for 5 min.

The PCR probe was incubated with the membrane in Church buffer (0.5 M Na_2HPO_4 [pH 7.2], 7% [wt/vol] SDS, 1% [wt/vol] bovine serum albumin, 1 mM Na_2EDTA) at 50°C overnight, washed two times for 5 min at room temperature in 2× SSC containing 0.1% (wt/vol) SDS, and then washed two times for 20 min at 50°C in 0.1× SSC containing 0.1% (wt/vol) SDS.

RT-PCR assays. Total RNA was extracted from 10 ml of mycoplasma broth culture as described elsewhere (5). RNA samples were incubated for 30 min at 37°C with 2 U of RGQ1 DNase (Promega) in a final volume of 20 μl of 1× RGQ1 buffer. After inactivation of the enzyme at 65°C for 10 min, RNAs were subjected to one-tube reverse transcription-PCR (RT-PCR) by using the Access RT-PCR System (Promega) and the primers TufG15 and TufC26, together with the primer pairs IF-IR or JF-JR. RT-PCR was performed as recommended by the manufacturer in a 50-μl final volume containing (i) 1 μl of RNA template (ca. 70 ng) obtained after DNase digestion; (ii) 48 μl of a master mix containing 5 U of *Tfl* DNA polymerase, 0.2 mM dNTPs, 1.3 mM $MgSO_4$, and 1 × buffer; and (iii) 50 pmol of each primer, in the presence or absence of 1 μl of avian myeloblastosis virus reverse transcriptase at 5 U/μl. Thermocycling was performed in a Perkin-Elmer DNA Thermo Cycler under the following cycling conditions: 1 cycle at 48°C for 45 min; 1 cycle at 95°C for 3 min; 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final extension cycle at 72°C for 5 min.

RESULTS

Difference in HA between R_{low} and R_{high}. The HA capability of *M. gallisepticum* was first assessed by using low (R_{low}) and high (R_{high}) laboratory passages of strain R (19), which have been shown to differ in their pathogenicity potentials (18). Binding of erythrocytes to the mycoplasma cells was monitored directly on colonies and revealed that R_{low} generates HA^+, HA^-, and sectored colonies (Fig. 1A), indicating that in R_{low}, as in strain A5969 (1), surface components undergoing high-frequency phase variation are involved in HA. In contrast, R_{high} appeared to exhibit exclusively the HA^- phenotype (Fig. 1B) even when a high number of colonies were tested. This suggests that in R_{high}, the mutation(s) affecting the expression of the component(s) involved in HA is irreversible or reverses with a low frequency.

Correlation between binding of erythrocytes and expression of GapA and CrmA. Recently, the expression of two proteins displaying homology to known cytadhesins of *M. pneumoniae*, namely, GapA and CrmA, was detected in R_{low} but not in R_{high} (24). To assess whether these two products are directly or indirectly involved in the binding of erythrocytes, single clones derived from R_{low} and presenting the HA^- or HA^+ phenotype were picked, and their protein content was analyzed by SDS-PAGE after Triton X-114 partitioning of the whole-cell extract. The results showed that all HA^- clones tested so far lacked two proteins of ca. 116 and 105 kDa that partitioned into the insoluble fraction. This is illustrated in Fig. 2A for three clonal variants derived from R_{low}, namely, RCL1, RCL2, and RCL3. In the HA^+ clones, RCL1 (lane 1) and RCL3 (lane 2), as well as in the parental strain R_{low} (lane 4), the two products were detected, whereas they were both missing in the HA^- RCL2 clone (lane 3) and in R_{high} (lane 6). Indeed, Western blot analysis revealed that rabbit anti-GapA antibodies (24) bind the 105-kDa protein, and this was exclusively detected in RCL1, RCL3, and R_{low}. The 116-kDa protein expressed in HA^+ clones most likely corresponds to the CrmA protein since (i) it displayed a migration in SDS-PAGE similar to that of the CrmA product detected in R_{low} (24), (ii) it

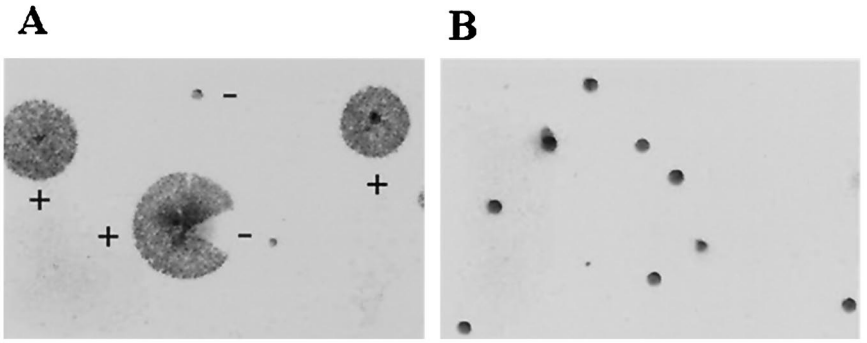

FIG. 1. Binding of erythrocytes to *M. gallisepticum* colonies. Mycoplasma colonies were partially transferred onto nitrocellulose and incubated with erythrocytes as described in Materials and Methods. Hemadsorbing colonies (+) and nonhemadsorbing colonies (−) were observed under a stereomicroscope (magnification, ×50). (A) Colonies derived from R_{low}. Note that, although erythrocytes binding to mycoplasma cells delineate the periphery of the colony, only the center of the nonhemasorbing colonies is visible after lifting. (B) Colonies derived from R_{high}.

partitioned into the insoluble fraction (24), and (iii) disruption of the *crmA* gene by transposition results in a HA^- mutant lacking the 116-kDa product (see below). These data suggested that the GapA and/or the CrmA proteins are involved in the binding erythrocytes and that they are undergoing high-frequency variation in expression. To confirm this hypothesis, colonies of clonal variants derived from R_{low} were partly transferred onto nitrocellulose and incubated with rabbit anti-GapA antibodies. The remainder of the colonies was then incubated with erythrocytes and results showed a perfect correlation between the binding of the anti-GapA antibodies and that of erythrocytes (Fig. 3A to D). The presence of multiple, corresponding HA^+ $GapA^+$ sectors within a single colony of the HA^- clonal variant, RCL2, confirmed that the GapA product is undergoing high-frequency variation in expression and correlates with the HA phenotype (Fig. 3C and D). The same experiment was performed with MAb 1E5, which binds to the surface exposed PvpA protein that was previously shown to undergo phase variation (33) and to share common motifs with the P30 cytadhesin accessory protein of *M. pneumoniae* (3). The results illustrated in Fig. 3E and F indicate that the variability in expression of PvpA does not correlate with that of the product(s) involved in HA since colonies presenting the HA^+ phenotype were not all immunostained with the MAb 1E5. This was confirmed by Western blot analysis (data not shown). We further demonstrated that the HA^- phenotype of RCL2 is spontaneously reversible by generating a clonal lineage from RCL2 that is composed of successive generations with alternating HA phenotypes, i.e., HA^- (RCL2) → HA^+ (RCL2-2) → HA^- (RCL2-2-2). SDS-PAGE and Western blot analyses confirmed that all HA^+ revertant clones expressed GapA and CrmA. Switching in the HA phenotype appeared to differ among clones and occurred at a frequency ranging from 5×10^{-2} to 2×10^{-4} per cell per generation.

Coordinated on-off switching of GapA and CrmA expression and its genetic basis. All of the clones tested so far that exhibited the HA^+ or HA^- phenotype showed a coordinated on-and-off switching of the two products, GapA and CrmA. Recent work has shown that the *gapA* gene of R_{high} contains a frameshift mutation at nucleotide (nt) 769 that results in the premature termination of the GapA synthesis and in the absence of mRNA corresponding to the *crmA* gene located immediately downstream as part of the same transcription unit (24). In order to define whether an identical mutational event is responsible for the lack of GapA expression in HA^- clonal variants derived from R_{low}, a 4.6-kbp genomic *Xba*I-DNA fragment that contained 83% of the *gapA* gene of RCL2 (see Fig. 4B) and hybridized with a *gapA*-specific probe was cloned into

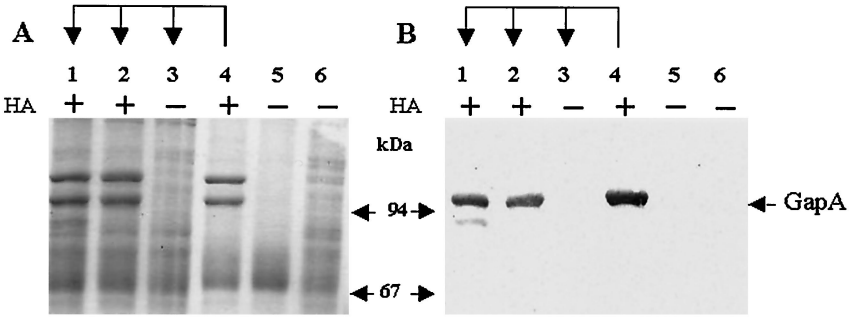

FIG. 2. Identification of proteins involved in HA of *M. gallisepticum* strain R. Whole organisms were subjected to Triton X-114 phase fractionation, and insoluble proteins were analyzed by SDS-PAGE, followed by Coomassie blue staining (A) or Western blot analysis (B), with a rabbit polyclonal antibodies raised against GapA (24). Lanes 1 to 6 correspond to proteins from clones RCL1 (lane 1), RCL3 (lane 2), and RCL2 (lane 3) or from strain R_{low} (lane 4), the mHAD3 mutant (lane 5), or strain R_{high} (lane 6). Arrows above panel A indicate that RCL1, RCL2, and RCL3 all derived from R_{low}. "+" and "−" indicate whether the organisms were shown to hemadsorb (HA). Except for clone RCL3, all selected clones expressed the PvpA protein. Molecular mass markers (at 94 and 67 kDa) and the protein band corresponding to GapA are indicated.

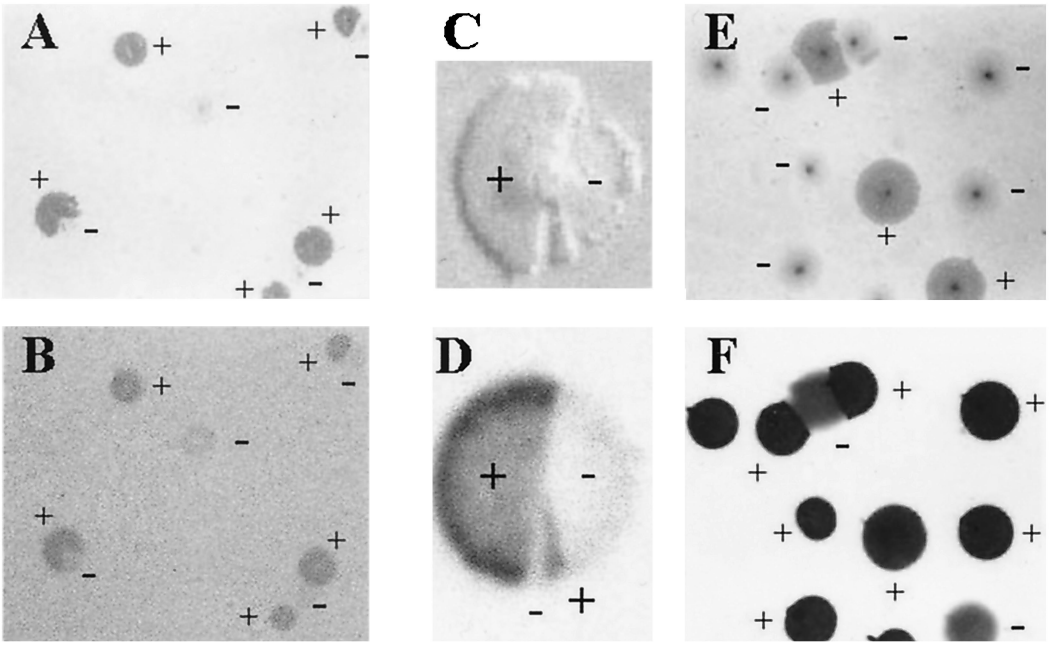

FIG. 3. Correlation between HA of erythrocytes to *M. gallisepticum* colonies and surface expression of GapA. Colonies derived from R_{low} (A, B, E, and F) or from RCL2 (C and D) were partially transferred onto nitrocellulose and then incubated with erythrocytes (A, C, and E), although the corresponding membranes were immunostained with a rabbit anti-GapA polyclonal antibody (B and D) or with MAb 1E5 that binds the PvpA surface protein (F). "+" and "−" indicate hemadsorbing and nonhemadsorbing colonies or positive and negative immunostaining, respectively.

the plasmid pUC18 and sequenced. Sequencing data revealed that in the HA^- variant RCL2 a mutation had occurred at nt 1393 that differed from the one previously described in R_{high} (24). As shown in Fig. 4B, the mutation in RCL2 corresponds to a nucleotide change (C into T) and generates a stop codon and an *Mse*I restriction site that are not present in the wild-type *gapA* gene. Using the GAPA4 and GAPA5 primers, a region of 217 bp encompassing the point mutation was amplified by PCR from the genomic DNA of R_{low} and from RCL2, respectively (Fig. 4B). Comparison of the *Mse*I restriction profiles of the resulting PCR products confirmed the presence of an additional *Mse*I site in the *gapA* gene of RCL2 (Fig. 5, lane 3b) and demonstrated that the mutation detected in the sequenced *Xba*I DNA fragment did not occur in *E. coli*. Indeed, the same experiment performed with PCR products generated from a set of clonal variants derived from R_{low} (Fig. 6) revealed that all HA^- clones (i) displayed identical *Mse*I restriction profiles and (ii) present an additional *Mse*I restriction site at the same position (Fig. 5, lanes 3b, 4b, and 7b) compared to the profiles obtained with their HA^+ siblings or progeny (Fig. 5, lanes 2b, 5b, and 6b). Finally, sequencing of the 217-bp fragment amplified from the RCL1 (HA^+) and RCL4 (HA^-) genomic DNA, respectively, by the GAPA4 and GAPA5 primers showed identical sequence except for the presence of the nonsense mutation in the RCL4 variant (data not shown).

Disruption of the *M. gallisepticum crmA* gene results in a mutant deficient in HA. A library of mutants derived from R_{low} was generated by random transposition by using the Tn*4001*mod (4). Mutants were then screened for their capability to bind erythrocytes on colonies and one, namely, mHAD3, was selected for its stable HA^- phenotype. As illustrated in Fig. 2, the protein profile of mHAD3 revealed the absence of both the GapA and the 116-kDa products (lane 5) that are detected in the HA^+ variants (lanes 1 and 2) and in the original R_{low} population (lane 4). Further Western blot analyses revealed that mHAD3 did indeed express GapA; however, its detection required at least five times the amount of proteins used for the detection of GapA in R_{low} or in the HA^+ clonal variant RCL1 (data not shown). Comparison of the Triton X-114 phase fraction of the HA^- RCL2 variant and mHAD3 mutant with that of the HA^+ RCL1 and RCL3 clones, indicate that neither of the mutations occurring in *gapA* or in *crmA* seems to affect the partitioning of GapA or CrmA (data not shown). Southern blot analysis showed that the transposon occurred as a single copy in the mHAD3 genome and that it is carried by an *Xba*I DNA fragment of ca. 7.3 kbp. Cloning and sequencing of the regions flanking the transposon revealed that the insertion has taken place within the *crmA* gene, 1,548 nt downstream of the ATG start codon (Fig. 4A). Using two primers GAPA0 and GAPA6, a fragment of 2,629 nt that encompasses the entire *gapA* gene of mHAD3 (Fig. 4A) was amplified by LR-PCR, cloned, and sequenced. Sequencing analyses showed that the *gapA* gene of mHAD3 encodes a fully functional ORF and that it is identical to its counterpart sequenced in RCL2, except for the nonsense mutation detected in RCL2 (Fig. 4B). Comparison of the mHAD3 *gapA* gene sequence with that previously published for strain R (15, 8, 24) revealed the presence of six nucleotide changes in mHAD3 that do not affect the *gapA* ORF. Overall, these results suggested that disruption of the *crmA* gene, which is located downstream of the *gapA* gene as part of the same transcription unit, influences the level of expression of GapA and confirmed

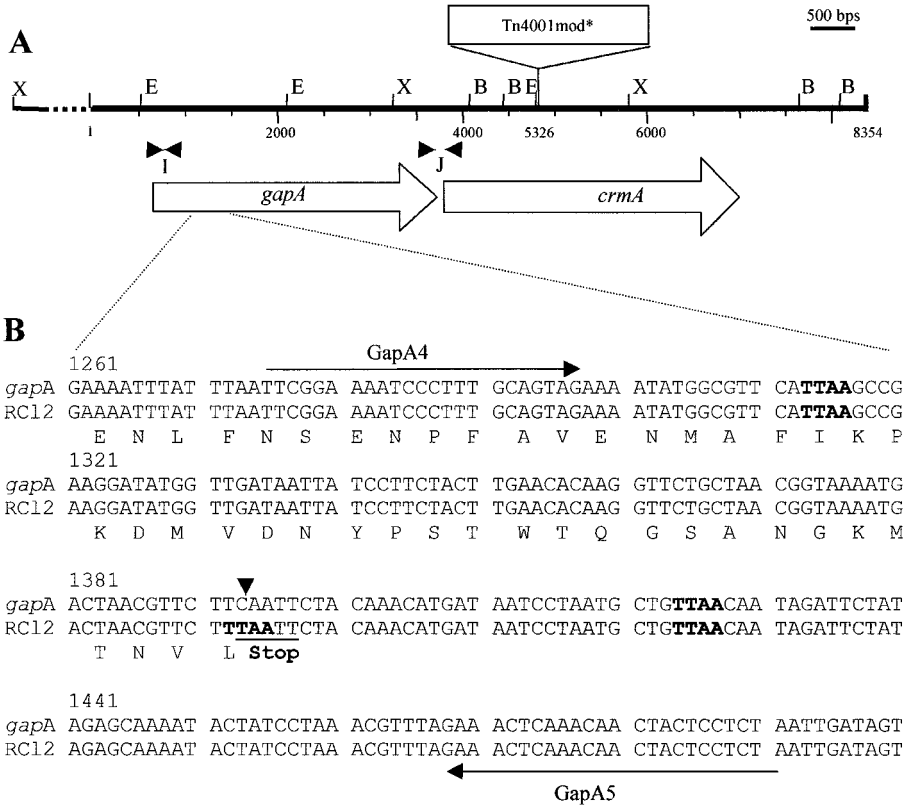

FIG. 4. (A) Schematic representation of the *gapA* and *crmA* gene organization based on the sequence published by Papazisi et al. (24) and localization of the transposon Tn*4001*mod in the mHAD3 mutant. Arrows below the solid line represent the primers used in the present study. (B) The sequence alignment represents the wild-type *gapA* gene sequenced in mHAD3 (upper line) and the corresponding portion sequenced from RCL2 (lower line). The arrowhead indicates the nucleotide that differs in between the two sequences. Boldface letters indicate the presence of *Mse*I restriction sites. Underlined nucleotides indicate the position of the nonsense mutation. Numbering was based on the entire sequence deposited in GenBank (AF214004) by Papazisi et al. (24), which is composed of 8,354 nt and contains the *gapA* and the *crmA* genes at position indicated in panel A. Arrows above or below the sequence indicate the positions of GapA4 and GapA5 primers used for amplifying the region in which the mutation occurred.

that the binding of erythrocytes to *M. gallisepticum* is linked to these two products.

Analysis of *gapA* and *crmA* transcription. The presence of the polycistronic mRNA corresponding to the *gapA* and *crmA* genes in the total RNA of RCL1 (HA^+), RCL2 (HA^-), and mHAD3 (HA^-) was assessed by RT-PCR. This was performed by using a multiplex RT-PCR assay in which the primer couples (i) I (IF and IR) corresponding to a 5′-end region of the *gapA* gene (24) or (ii) J (JF and JR) encompassing the end of *gapA* and the beginning of *crmA* (24) were used in combination with a pair of primers (TufG15 and TufC26) shown to detect the *tuf* mRNA (9).

Using one primer combination or the other, RT-PCR assays performed with the total RNA extracted from the HA^+ RCL1 variant resulted in the detection of two PCR products (Fig. 6): one of 250 bp corresponding to the *tuf* mRNA and a second of 460 or 350 bp corresponding to the *gapA* (lane 1a) or to the *gapA-crmA* (lane 6a) mRNAs, respectively. Indeed, RT-PCR assays performed with any of the RNA templates all generated the 250-bp product corresponding to the *tuf* mRNA, which was not detected in duplicate samples assayed without reverse transcriptase (Fig. 6, lanes 1b, 2b, 3b, 6b, 7b, and 8b). This result showed the presence of intact mRNA in the total RNA preparations and the absence of residual DNA that could generate false-positive results. A PCR product of 460 bp, corresponding to the *gapA* mRNA, was also obtained in the presence of primer I with all templates independently of the HA phenotype (Fig. 6, lanes 1a, 2a, and 3a). However, its amount relative to that corresponding to the *tuf* mRNA appeared to be lower for RCL2 (Fig. 6, lane 2a) than for RCL1 (lane 1a) or mHAD3 (lane 3a), suggesting that in RCL2 the *gapA* mRNA might be less abundant or less stable. Results obtained with primers J indicated that, in the RCL2 clone (lane 7a), mRNA corresponding to *crmA* is lacking or present at a concentration too low to be detected by our assay since a PCR product corresponding to the 350-base region of the *gapA-crmA* mRNA was only detected in RCL1 (lane 6a) and in mHAD3 (lane 8a). Interestingly, in the HA^- mHAD3 mutant, the detection level of mRNAs with any primer combination (lanes 3a and 8a) was comparable to that of the HA^+ RCL1 variant (lanes 1a and 6a), suggesting that the low amount of GapA product detected in this mutant was not due to the absence or to a limiting amount of the corresponding transcript.

DISCUSSION

This study demonstrates that in *M. gallisepticum* strain R the GapA cytadhesin (8), also described as MGC1 in strain S6

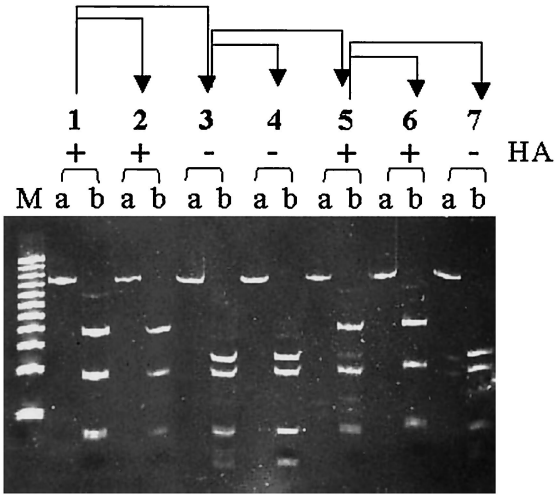

FIG. 5. Detection of an additional *Mse*I restriction site in HA$^-$ clones. The DNA region that encompassed the mutation detected in clone RCL2 was amplified in HA$^+$ and HA$^-$ variants and subjected to *Mse*I digestion. Undigested (lanes a) and digested (lanes b) PCR fragments were analyzed by electrophoresis in a 10% polyacrylamide gel. The predominant HA phenotype of R_{low} or of the clones is indicated above the gel by "+" and "−." The pedigree of the clones is represented by arrows above the panel. The samples correspond to R_{low} (lane 1), RCL1 (lane 2), RCL2 (lane 3), RCL2-1 (lane 4), RCL2-2 (lane 5), RCL2-2-1 (lane 6), and RCL2-2-2 (lane 7). M, DNA size marker.

(15), undergoes phase variation in expression, providing this avian pathogen with variable adhesive properties while it propagates. Whether this phenomenon that was observed in vitro also occurs in vivo is not yet known; however, variation in attachment of the avian pathogen to host cells may promote consecutive colonization of several hosts or of various niches within a single host. Phenotypic switching of the capacity of *M. gallisepticum* to bind erythrocytes was found to correlate with GapA phase variation concurrently with that of a second product, CrmA, which exhibits cytadhesin-related features and is

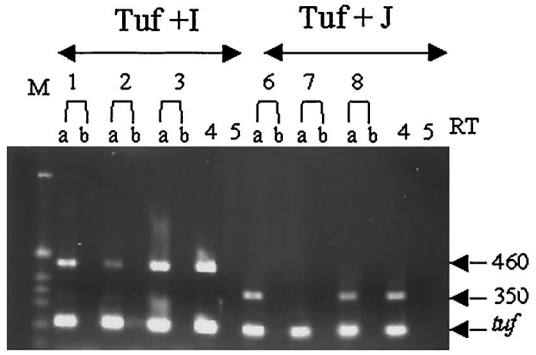

FIG. 6. Analyses of the *gapA* and *crmA* mRNA. A multiplex RT-PCR was applied to total RNA extracted from clones RCL1 (lanes 1 and 6), and RCL2 (lanes 2 and 7), and mutant mHAD3 (lanes 3 and 8) by using the Tuf primers in combination with the I or the J primers as indicated above the gel. Assays were performed in presence (a) or absence (b) of reverse transcriptase (RT). Controls include assays performed in absence of RT with the genomic DNA of RCL2 (lane 4) or with no template (lane 5). M, DNA size marker.

encoded by a gene located downstream of the *gapA* gene as part of the same transcription unit (24). Detailed genetic analyses revealed that the mutational event underlying the GapA on-off switching in clones derived from R_{low} is a point mutation that consequently affects the expression of CrmA. Papazisi et al. (24) have shown that the failure of R_{high} to express GapA and CrmA is due to a frameshift mutation at the 5′end of the *gapA* gene that generates a nonsense mutation leading to the premature termination of GapA translation. As in R_{high}, the absence of GapA expression in HA$^-$ variants derived from R_{low} is due to a mutation occurring at the beginning of the *gapA* gene, but downstream of the sequence mutated in R_{high}. In mycoplasmas, the on-and-off expression state of a large number of genes encoding surface components is governed by reversible mutations occurring by slipped-strand mispairing (6, 25). One common feature of these mutational events is that they are taking place in so-called mutator regions characterized by a short tract composed of a single nucleotide or directly repeated trinucleotides. For instance, on-off switching of the pMGA-hemagglutinin family of *M. gallisepticum* is driven by spontaneous insertion or deletion of trinucleotide unit(s) occurring within a $(GAA)_n$ motif whose length governs the transcription of a given pMGA gene (10, 20). A short tract of five-repeated GAA motif was recently identified at the beginning of the structural gene of the phase-variable, cytadhesin-related PvpA protein of *M. gallisepticum*. In PvpA-negative variants, a base substitution (G into T) precisely affects the guanine of the fourth GAA motif (3) and results in a premature termination of the translation. In their report the authors suggest that this mutation is either irreversible or occurs at a very low frequency, since attempts to obtain a PvpA positive revertant failed. Data collected in our study also revealed that GapA-negative variants are spontaneously generated after a base substitution that creates a nonsense mutation at the beginning of the *gapA* structural gene. Remarkably, this mutation is reversible, since GapA positive progeny clones could be isolated that displayed the wild-type gene feature. In contrast to the previous variable genes identified in *M. gallisepticum*, no GAA repeated motif was observed in the *gapA* gene sequence flanking the mutated base. The only feature displayed by this region is the presence of three TTC trinucleotides (or GAA on the cDNA strand), two of which are directly repeated and separated from the third one by two adenosine residues [5′-TTCTT(C/T)AATTC-3′]. The high-frequency mutational event governing the GapA on-and-off switching is a reversible base substitution whose occurrence cannot be explained by slipped-strand mispairing. Based on the sequence data obtained in the present study and previously by two other independent groups (8, 15, 24), there is no evidence for the presence of more than three TTC motifs in this region. If, indeed, GAA (or TTC on cDNA strand) repeated motifs are preferential targets for mutation in *M. gallisepticum* then the question arises whether the length of the TTC repeated motif observed in the *gapA* gene is sufficient to promote such an event. Since no DNA rearrangement was observed around the single-copy gene encoding GapA between GapA-negative and -positive clonal variants (data not shown), the exact molecular mechanism promoting the mutational event governing the GapA on-and-off switching in expression has yet to be elucidated.

In the HA$^-$ variant, RCL2, the nonsense mutation identified

in the *gapA* gene is likely to result in a premature disassembling of the translational apparatus that might result in a high turnover of the untranslated mRNA and/or in an early termination of the transcription. This might explain the lack of mRNA corresponding to the end of *gapA* and to the *crmA* gene and subsequently to the absence of a CrmA product in the RCL2 variant. From the data collected in the present study, it is unlikely that the CrmA product is directly subjected to phase variation in expression independently of the GapA variation; however, this possibility cannot be ruled out. Disruption of the *crmA* gene by transposition mutagenesis resulted in a mutant, mHAD3, which failed to hemadsorb, suggesting the involvement of the CrmA product in adherence of erythrocytes to mycoplasma cells. Despite a fully functional and efficiently transcribed *gapA* ORF, the amount of GapA detected in this mutant was considerably lower than in HA^+ clonal variants and raised the question of whether the HA^- phenotype displayed by mHAD3 is directly linked to the lack of CrmA. One hypothesis that would explain such observation is that GapA may be subjected to an accelerated turnover in the absence of CrmA. In *M. pneumoniae*, the cell shape of which is reminiscent of that of *M. gallisepticum*, the complex sequence of events leading to the assembly of the attachment organelle has begun to emerge (17). In this human pathogen, several cytadhesin accessory proteins are required for the proper localization of the main adhesin P1 at the tip structure of the organism. During this process, the absence of a key component required by other molecular players downstream of the cascade results in their accelerated turnover and in the loss of *M. pneumoniae* cytadherent properties (17). *M. gallisepticum* proteins involved in bleb formation have not been identified yet, but it is most likely that they will interact after a sequence of events in which both GapA and CrmA might be involved. So far, only two products with homology to known cytadhesins of *M. pneumoniae* have been shown to localize at the terminal structure of the avian mycoplasma. These are the MGC2 (12), the corresponding gene of which is localized upstream of *gapA*, and the phase-variable PvpA molecule, whose gene is located at a different locus (3, 33). The results presented in our study showed that the binding of erythrocytes to *M. gallisepticum* is independent of the PvpA expression status, whereas the role of MGC2 in this process has yet to be investigated. Finally, our hypothesis that both GapA and CrmA are required in HA is supported by the finding of Papizisi et al. (24) showing that introduction of the wild-type *gapA* gene alone into the HA^- R_{high} failed to restore adherence to MCR-5 cells.

Data emerging from *M. gallisepticum* studies indicate that several cytadhesin or related components have yet to be identified, defined, and characterized, a step which is crucial to understand the exact contribution of each of these molecules in promoting and maintaining a successful infection in the avian host. As well, the understanding of the role of cell invasion in vivo requires the identification of the molecular players that allow the avian mycoplasma to enter nonphagocytic eukaryotic cells, which is directly linked to adhesion.

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4.2. Role of the GapA and CrmA cytadherence proteins in cell invasion and translocation of cell monolayers

In vitro Translocation of *Mycoplasma gallisepticum* through Cell Monolayers with Tight Junctions

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Abstract

In this study it was shown that *M. gallisepticum* is able to cross CaCo-2 cell monolayers with tight junctions and that this event relies on particular phenotypes. While the virulent, cell-invasive [INV+] R_{low} population was able to cross the CaCo-2 epithelial barrier, the avirulent, cell-invasion deficient [INV-] R_{high} was not, a finding that correlates with previous *in vivo* data showing the dissemination of R_{low} from the respiratory tract to the inner organs of the chicken host, while R_{high} only generates local infection. In contrast, testing of the R_{high}P10 population (derived from R_{high} by 10 times passaging in cell cultures) revealed an intermediate translocation efficiency, a result which also correlates with our previous findings showing that R_{high}P10 is enriched with cell-invasive organisms and better disseminates throughout the host than R_{high}. Taken together, these data suggest that *M. gallisepticum* cell invasion appears to be involved in translocation through tight cell monolayers, a phenomenon that could participate in colonization of various host organs, if occurring *in vivo*. Although *M. gallisepticum* cell invasion surprisingly appeared to be independent of the presence of the GapA and CrmA cytadhesion-related products (corresponding to the hemadsorption-positive [HA+] phenotype), results obtained with the translocation assay suggest that these two proteins might play a role for crossing epithelium with tight cell junctions via transcytosis, as both the [INV+] and the [HA+] phenotypes are required.

Introduction

Organisms belonging to the genus *Mycoplasma* have often been portrayed as “simple” or “primitive” bacteria due to their lack of cell wall, the small size of their genome and the paucity of their metabolic pathways. Despite these apparent handicaps, several *Mycoplasma* species are successful pathogens of man and animals in which they cause mild to severe diseases often characterized by their morbidity and their chronic nature (Minion 2002, Ishihara et al. 2004, Waites and Talkington 2004). While data of entire mycoplasma genomes have shed lights on important aspects of mycoplasmas evolution and cell biology (Razin et al. 1998), very little has been gathered regarding the genetic information contributing to mycoplasma-host interactions and virulence. Indeed, filling this gap might be more difficult to achieve than expected, as these so-called simple organisms seem to have elaborated complex strategies to colonize and survive within their immunocompetent hosts, some of which involved spontaneous, high-frequency variation of their surface components (Citti and Rosengarten 1997, Razin et al. 1998, Rosengarten et al. 2000, Citti et al. 2005). Defining the exact nature of mycoplasma virulence factors requires the concomitant development of methods for targeted mutagenesis and *in vivo* and *in vitro* modeling.

In recent years, a better understanding of the strategies developed by *M. gallisepticum* to establish successful infection in its natural chicken host has started to emerge (Markham et

al. 1998, Papazisi et al. 2000, Winner et al. 2000, Yoshida et al. 2000, Papazisi et al. 2002, Winner et al. 2003) and offers a basis for appreciating the overall approaches taken by pathogenic mycoplasmas to subvert infected animals or human. Interestingly, *M. gallisepticum* and the human pathogens *M. genitalium*, *M. pneumoniae* and *M. penetrans* belong to the *Mycoplasma pneumoniae* phylogenetic group (Weisburg et al. 1989) and are among the few *Mycoplasma* species described so far as facultative intracellular organisms (Lo et al. 1993, Jensen et al. 1994**,** Baseman et al. 1995, Winner and al. 2000). Attachment of *M. gallisepticum* to host cells is thought to occur via a terminal organelle (Uppal and Chu 1977, Tajima et al.1979) also displayed by *M. genitalium* (Mernaugh et al. 1993) and by *M. pneumoniae* (Feldner et al. 1982, Krause and Balish 2001) and to involve surface proteins presenting similarities to cytadhesins described in *M. pneumoniae* (Papazisi et al. 2000, Boguslavsky et al. 2000). Like many other pathogenic mycoplasmas, *M. gallisepticum* displays a predilection for the mucosal surface of the respiratory tract of its natural host, and this surface colonization typically results in chronic respiratory disease. From this niche, this avian pathogen is also able to disseminate throughout the body to colonize several inner organs (Much et al. 2002), an event that requires the mycoplasma to cross the mucosal epithelial barrier. Recent sequencing of the *M. gallisepticum* genome revealed that the keys to this complex infection pattern resides within a total of 742 predicted coding DNA sequence, 39% of which do not fit into the current database of conserved orthologous groups (COGs; Papazisi et al. 2003).

Health and economic damages caused to the poultry industry by *M. gallisepticum* infections (Mohammed, et al.. 1987, Stipkovits and Kempf. 1996) have stimulated a number of studies related to the factors involved in virulence of this pathogen. Several of these have attempted to identify the molecular players responsible for the difference in pathogenicity observed between a low laboratory passage (R_{low}) and a high laboratory passage (R_{high}) in artificial culture media of the prototype strain R representing, respectively, virulent (R_{low}) and avirulent (R_{high}) populations, (Levisohn et al. 1986, Much et al. 2002). *In vivo*, R_{low} was shown to induce local and systemic infections accompanied by air sac lesions while colonization of chickens by R_{high} appeared to be restricted to the respiratory tract resulting in very mild to no damage (Much et al. 2002). Two *M. gallisepticum* cytadhesin related components identified in R_{low}, namely GapA and CrmA, are lacking in R_{high} (Papazisi et al. 2000). Restoration of the GapA and CrmA expression in R_{high} by introducing the corresponding genes of the wildtype did not result in tracheal lesions in infected birds but generated significant air sac lesions with an overall score lower than in chickens infected with R_{low}, suggesting that other factors might be responsible for *M. gallisepticum* pathogenicity (Papazisi et al. 2002). Interestingly, *in vitro* analyses have demonstrated that R_{low} is capable of establishing intracellular residence in non-phagocytic eukaryotic cells, while R_{high} remains extracellular (Winner et al. 2000). Once internalised, *M. gallisepticum* is capable to escape the intracellular space and leave the cell. These results, however, were

based on infection of conventional semi-confluent monolayers which did not mimic the polarized arrangement of mucosal cells *in vivo*. This raised the question of whether *M. gallisepticum* cell invasion could provide a basis for systemic infection by allowing the organism to cross epithelial layers with tight junctions. These intercellular junctions represent a rate-limiting barrier against passive flux of small and large solutes but also against bacteria which must disassemble the junctional complexes or enter the eukaryotic cells to resurface on the other side in order to translocate through the polarized epithelium (McCormick 2003).

The present study has assessed the capacity of *M. gallisepticum* to cross a cell monolayer with tight junctions using a classical *in vitro* translocation assay system. Results showed that *M. gallisepticum* translocates through a tight monolayer and further suggested that this process is occurring via transcytosis. It was also shown that translocation might be depending on the presence of the GapA and CrmA cytadhesion-related products which surprisingly did not appear to be essential for cell invasion. The role of *M. gallisepticum* cell adhesion and cell invasion in translocation through epithelial barriers is further addressed and discussed.

MATERIALS AND METHODS

Mycoplasma strains and growth conditions. *M. gallisepticum* laboratory passages R_{low} and R_{high} used in these and earlier studies were kindly provided by S. Levisohn, Kimron Veterinary Institute, Bet Dagan, Israel. R_{low} and R_{high} correspond to the prototype strain R propagated by 10 and 160 passages in artificial culture medium, respectively (Lin and Kleven 1984). R_{high}P10 was previously described and derived from R_{high} after 10 passages in the presence of HeLa cells (Winner et al. 2000). The RCL2 clonal variant was isolated from R_{low} and shown to display the hemadsorption negative phenotype [HA-] and to lack the GapA and the CrmA products (Winner et al. 2003). Mycoplasmas were grown at 37℃ in modified Hayflick medium (Wise and Watson 1983) containing 20% (v/v) heat-inactivated horse serum (Life Technologies, Inc., Rockville, MD) to mid-exponential phase, as indicated by the metabolic color change of the medium. The number of viable mycoplasmas in a suspension was determined by plating serial dilutions onto modified Hayflick medium containing 1 % (wt/vol) Noble agar. After six to eight days of incubation at 37℃, the number of colony forming units (CFU) was counted using an SMZ-U stereomicroscope (Nikon Corp., Tokyo, Japan). *M. gallisepticum* clones used in this study were picked from solid media, expanded in 1 ml of liquid medium and kept at -80℃ for further analyses. Competent cells of *Escherichia coli* DH10B (Invitrogen) were used as host to clone recombinant products and grown at 37ºC in Luria-Bertani (LB) broth supplemented with 100 µg ampicillin per ml for plasmid preparation.

Colony immunoblotting and hemadsorption assay. Colony immunoblotting was performed as previously described (5) using antibodies and conditions described below for

Western blot analyses. The hemadsorption assay was conducted directly on agar plate. After partially lifting the mycoplasma colonies onto nitrocellulose membranes, the colonies were overlaid with 15 ml of fresh sheep blood washed and resuspended in phosphate-buffered saline (PBS) solution (2.7mM KCl, 1.2mM KH_2PO_4, 138mM NaCl, 8.1mM $Na_2HPO_47H_2O$, pH 7.4) to a final concentration of 0.5 % (vol/vol). After incubation at 37 °C for 30 min, the suspension was then carefully discarded and unbound erythrocytes were gently removed by washing with PBS. Mycoplasma colony immunostaining and binding of the erythrocytes were observed using a SMZ-U stereomicroscope (Nikon Corp., Tokyo, Japan).

SDS-PAGE and Western blot analysis. Protein profile analysis of *M. gallisepticum* populations and clones used in this study was performed by SDS-PAGE followed by Coomassie staining or Western blotting using the whole cell extract or fractions obtained by Triton-X114 (Sigma) partitioning as described elsewhere (Wise and Watson 1993).

Antibodies. Antibodies (Abs) used for Western blot analysis were previously reported and included (i) a rabbit anti-GapA antiserum (Goh et al. 1998), diluted 1:8,000, and (ii) the anti-Pvpa monoclonal antibody (MAb) 1E5 (Yogev et al. 1994), diluted 1:50. Detection of antibody binding was achieved using peroxidase-conjugated swine antiserum to rabbit immunoglobulin (Ig) (Dako, Copenhagen, Denmark) or to mouse IgM (Jackson Immuno Research Laboratories Inc. West Grove, PA). Abs were diluted in tris-buffered saline solution (150 mM NacCl, 10 mM Tris-base) containing 0.05 % (vol/vol) Tween 20. Immunological reagents used in the double immunofluorescence (DIF) assay were also previously described and included an anti-*M. gallisepticum* antiserum, diluted 1:150 in PBS-BSA, as well as Texas Red-labeled and fluoresceinisothiocyanate (FITC)-labeled goat antibodies to rabbit Ig (Harlan Sera-Lab, LTD, Loughborough, England), diluted 1:150 in PBS-BSA (Winner et al. 2000).

Cell cultures. All cell culture reagents were obtained from Gibco BRL, Life Technologies. The human epithelial-like cell line HeLa-229 (ATCC CCL-2.1) and the human colonic cell line CaCo-2, (ATCC CRL-1590), were both purchased from the American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were grown in a 5% CO_2 atmosphere at 37℃, HeLa cells in minimum essential medium contai ning 2 mM L-glutamine and Earl's balanced salts, supplemented with 7.5% (v/v) fetal calf serum, 5% (v/v) tryptose phosphate broth, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml of penicillin, 100 µg/ml of streptomycin and 10 mM Hepes buffer (MEMS) and CaCo-2 cells in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10%(v/v) fetal calf serum, 0.1 mM non-essential amino acids, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Propagation of the cell lines was performed in cell culture flasks (Iwaki Glass Co., LTD, Gyoda, Japan). Cell monolayers were detached from cell culture vials by trypsinization as recommended by ATCC and seeded at 10 to 20% confluency into Lab Tech II chamber slides (Nalge Nunc International, Naderville, IL) for confocal laser scanning microscopy (see

below) 24 hours prior to mycoplasma infection, and at 30 to 40% confluency into 24-well microdilution dishes (Corning Costar Europe, Badhoeverdorp, The Netherlands) for the gentamicin assay (see below) three days prior to infection. Translocation assays basically followed the protocol described by Konkel et. al. 1992. Briefly, filter units (Millicell-PCF, PITP 012 50, Millipore, Bedford, MA) containing 0.6 cm^2 membranes with 3 µm pore-size were placed into the wells of a 24-well microdilution dish. After 30 min of pre-incubation in DMEM, the filter units were transferred into fresh medium and inoculated with 2x 10^5 CaCo-2 cells. The medium in both, the upper and the lower chamber was changed every three days. The monolayer integrity was measured using the dextran blue exclusion assay described by Birkness et al. 1999. Briefly, filter units were placed into fresh medium and 0.5 ml of a 0,5% (w/v) solution of blue dextran 2000 (Amersham Pharmacia Biotech, Uppsala, Sweden) was added to the upper chambers. After 2 h of incubation in a 5% CO_2 atmosphere at 37℃, the optical density (OD) of the medium of the lower chamber was measured at 620 nm with DMEM only as standard OD. Filter units without cells or seeded with HeLa cells were used as negative controls. Tight monolayers were usually ready (OD≤0.08) for translocation experiments after 14 days. Cell cultures were regularly shown to be free of mycoplasma contamination by plating the eukaryotic cells on mycoplasma agar medium as described above.

Cell invasion assays. The capacity of different *M. gallisepticum* strain R subpopulations or clones to enter eukaryotic cells was assessed using the gentamicin invasion (Elsinghorst 1994) and/or the double immunofluorescence assays (Heesemann and Laufs 1985), as previously described for this mycoplasma species (Winner et a.. 2000). With the gentamicin assay, the frequency of invasion was estimated as the ratio between the number of organisms that have survived the antibiotic treatment and the number of mycoplasmas used as inoculum. Each experiment was done with HeLa cells and was performed in triplicate. In the double immunofluorescence assay, detection of intracellular and extracellular mycoplasmas was carried out as previously described using rabbit anti-*M. gallisepticum* antiserum in combination (i) with FITC-labeled anti-rabbit Ig as secondary antibody to stain extracellular organisms before permeabilization of the cells, and (ii) with Texas Red-labeled anti-rabbit Ig as secondary antibody to stain extracellular and intracellular mycoplasmas after permeabilization.

Translocation assays. Mycoplasma cells were suspended in cell culture medium using a syringe as described elsewhere (Winner et al. 2000) to a final concentration of approximately 10^7 CFU/ml. CaCo-2 monolayers were washed in antibiotic-free DMEM and infected on the apical side with 250µl of the mycoplasma solution, giving a multiplicity of infection of approximately 20:1 (mycoplasmas to cells). The filter units were placed into a well containing fresh antibiotic-free DMEM and incubated for 2 h in a 5% CO2 atmosphere at 37℃. Serial dilutions of medium of the lower ch amber were plated on solid medium as described above to assess the number of mycoplasmas that have crossed the monolayer.

The number of CFUs of the inoculum was calculated before incubation following the same procedure.

DNA manipulations. Standard methods were used for DNA manipulations including agarose gel electrophoresis, restriction endonuclease digestion, ligation, chemical transformation, and electroporation as described or according to manufacturer's instructions. Southern hybridizations were performed according to the Genius System User's Guide for Membrane Hybridization, Version 3.0 (Roche Molecular Biochemicals, Mannheim, Germany).

Tagging of selected clonal variants. The plasmid pISM2026 was kindly provided by Dr. C. Minion (Iowa States University, Ames, IA) and carries the *Tn4001* in which a *Bam*H1 restriction site has been previously inserted in one inverted sequence (IS) (Knudtson and Minion. 1993). This plasmid was modified for other studies not reported here by inserting, into the *Bam*H1 restriction site, random tag-oligonucleotide sequences generated as previously described by Hensel et al. (1995) using the oligonucleotide 5´CTA**GGTACC**TACAACCTCAAGCTT$(NK)_{20}$AAGCTTGGTTAGAATG**GGTACC**AG´ (*Bam*HI restriction sites are indicated in bold letters). The resulting tagged plasmids, pISM2026-tag, were used to transform *E. coli*. Sequence analyses of several recombinant plasmids showed that each carried a specific distinct Tag sequence. Five of these tagged recombinant plasmids containing the unique oligonucleotide Tag7 (5´AGGTAGTTCGCTTGTAGCT 3´), Tag 9 (5´GAG AGTTGTTTCGGTATGG 3´), Tag 10 (5´ ATCGAGGTA TCGGTTGCTA 3´), Tag17 (5´ CGAGTGTTCAGGGTTGGG 3´), or Tag 20 (5´AGAGATGCTGATTTAGCGG 3´) were individually used to transform *M. gallisepticum* clones as listed in Table 3 and as previously reported (Winner et al. 2003). Briefly, *M. gallisepticum* cells (approximately 10^9 CFU) washed three times and resuspended in 100 µl of electroporation buffer (8 mM HEPES pH: 7.4, 272 mM sucrose) were incubated on ice for 10 min with 10 µg of a pISM2026-tag. The mixture was then subjected to electroporation (2.5 kV, 100 Ω, 25 µF), resuspended in 1 ml of chilled Hayflick medium and incubated on ice for 10 min and at 37 ℃ for 90 min. Gentamicin was then added to the mycoplasma cell suspension to a final concentration of 100 µg/ml, and aliquots of 25 to 100 µl were plated onto solid Hayflick medium containing 50µg/ml of gentamicin. After incubation at 37 ℃ for 8 days, colonies were picked, individually grown and analysed. Tagged *M. gallisepticum* clones (i) that have retained the parental phenotype in terms of hemadsorption, protein profile and cell invasion, and (ii) that displayed a single *Tn4001*-tag chromosomal insertion, were selected for further studies.

***In vivo* infection experiments.** Experimental infections of chickens with *M. gallisepticum* were performed as previously described (Much et al. 2002). Briefly, a total of 121 one-day old Arbor Acress chickens, were selected as previously described from a flock certified free of *M. gallisepticum* and of *M. synoviae*. A total of 21 chickens were slaughtered and examined for pathological lesions as well as for the presence of mycoplasmas. No

pathological lesion characteristic of mycoplasma infection was found, and cultivation for mycoplasmas was negative. At the age of 21 days, the remaining chickens were weighted, marked and divided into four groups of 24 or 25 chickens, so that the average body weight of each group did not significantly differ based on the Student t-test. Each group was placed into an aerosol chamber of 0.224 m^3 and inoculated respectively with 10 ml of culture media containing (i) no mycoplasmas (group 1), (ii) 9.2 x 10^8 CFU ml^{-1} of R_{low} (group 2), (iii) 8.6 x 10^8 CFU ml^{-1} of R_{high} (group 3), and (iv) 9.8 x 10^8 CFU ml^{-1} of the same mixture of genetically tagged clones (group 4) used for *in vitro* translocation assays, as described in Table 3. Each inoculum was pulverized into fine aerosol particles of 7 to 10 μm and sprayed for 2 min into the chamber. Birds were maintained in the unventilated aerosol chamber for an additional 15 min before they were transferred to their isolation units. Feeding and cleaning of the four groups were then performed by four individual crews to avoid risks of cross-contamination. After nine days, each bird was weighted, slaughtered, and necropsy was performed for pathomorphological lesions. The lesions were documented by a scoring system characterized by the amount of fibrous exudates on the serous membrane of the thoracic air sacs. Gross lesions were scored on a scale from 0 (no lesions) to 8 (severe bilateral lesions) (Czifra et al. 2000). During necropsy, swabs were collected from (i) the trachea, (ii) the lung, (iii) the left air sac, (iv) the liver, (v) the spleen, (vi) the kidney, (vii) the brain, and (viii) the heart, and directly seeded into 5 ml of Hayflick medium. After three and six days of growth, the metabolic color change of each culture was recorded and an aliquot was plated onto solid media to monitor the presence or absence of mycoplasmas as previously described (Much et al. 2002).

Statistical analysis. Invasion and translocation frequencies are expressed as mean ± standard deviation of *n* independent values. Frequencies of invasion and translocation were analyzed using the Student´s *t* test while air sac lesions scores were analyzed using the Chie Square test. The probability for significance was $p \leq 0.05$.

RESULTS

Factors involved in *M. gallisepticum* cell invasion. In a previous study, invasion of non-phagocytic eukaryotic cells by a low (R_{low}) passage in culture media of the prototype strain R was demonstrated, while organisms of a high passage of the same strain remained outside of the cells. Ten times propagation of R_{high} in HeLa cell monolayers further resulted in a population R_{high}P10 (Fig.1) that was enriched in invasive organisms (Winner et al. 2000). In an attempt to identify the molecular factors that could account for *M. gallisepticum* cell invasion, the protein profiles of single colonies randomly picked from R_{high}P10 before and after gentamicin treatment in cell invasion assays were compared by SDS-PAGE analyses. Results indicated that the only consistent detectable difference was the presence of two proteins in several clones derived from the population which has survived the antibiotic treatment (R_{high}G11) which were absent from all clones randomly picked from R_{high}P10.

These products of about 105 and 120 kDa, respectively, were identified by Western blot analysis as the GapA and CrmA cytadhesins, two proteins encoded by the same operon and not expressed in R_{high} (Papazisi et al. 2000). Since it has been previously shown that binding of erythrocytes to *M. gallisepticum* colonies correlates with the expression of GapA and CrmA (Winner et al. 2003), clones derived from R_{high}P10 and R_{high}G11 were subjected to the hemadsorption (HA) assay. As expected, results confirmed that clones derived from R_{high}G11 that expressed the two cytadhesins were HA positive [HA+] while those derived from R_{high}P10 were HA negative [HA-].

To better understand the role of GapA and CrmA in host cell invasion, two [HA-] clones, namely RHG11K5 and RHG11K6, and one [HA+] clone, namely RHG11K8, that derived from R_{high}G11 (Fig. 1 and Table 1) were subjected to the double immunofluorescence and to the gentamicin cell invasion assays. While the two clones RHG11K6 and RHG11K8 displayed a cell-invasive ability similar to that of R_{low} (further referred to as [INV+]), the RHG11K5 clone was unable to invade non-phagocytic cells (further referred to as [INV-]). Taking into account that RHG11K6 is [HA-] and does not express the two major cytadhesins GapA and CrmA, these data were unexpected and indicated that cell invasion, at least *in vitro*, may be independent from the presence of one or both proteins. To confirm this finding, the [HA-] RCL2 clone which derived from R_{low} and previously shown to lack both GapA and CrmA (Winner et al. 2003), was subjected to the gentamicin and double immunoflurescence assays. Results revealed that RCL2 was able to enter non-phagocytic eukaryotic cells with efficiency similar to that of R_{low} supporting the hypothesis that the GapA and CrmA are not required *in vitro* for *M. gallisepticum* cell invasion.

In R_{low}, the expression of GapA and CrmA undergoes phase variation in expression, an event that is driven by a highly reversible point mutation occurring in the beginning of the *gapA* gene (Winner et al. 2003). In the [HA-] R_{high} population, a point mutation located in the beginning of *gapA* also is responsible for the absence of GapA and CrmA but is located elsewhere (Papazisi et al. 2002) and is reversible with a low frequency. Sequencing of the 5' end of the RHG11K5 and RHG11K6 *gapA* genes revealed the presence of a point mutation identical to that found in R_{high}.

Translocation of *M. gallisepticum* through cell monolayers with tight junctions. In the chicken host, *M. gallisepticum* cell invasion has been proposed to play a role in escaping the host immune defenses but also in allowing the pathogen to cross epithelial barriers in order to disseminate throughout the body and to cause systemic infection (Winner et al. 2000, Much et al. 2002). This observation prompted us to assess *in vitro* the ability of R_{low} and R_{high} to pass through an epithelial barrier with tight junctions. For this purpose, CaCo-2 cells were grown in a two-chamber system onto a permeable support membrane until formation of tight intercellular junctions, so that the resulting intact monolayer represented a limiting barrier against passive diffusion of small and large molecules. Infection of intact CaCo-2 monolayers was performed with R_{low} and R_{high}, respectively, by the apical side of the system

(upper chamber), and the presence of mycoplasmas in the lower chamber was assessed after 2 h by direct plating of serial dilutions of the medium onto agar plates. Controls included chamber systems without cells and with confluent HeLa cell monolayers, which unlike the CaCo-2 cells are unable to form tight junctions. Results presented in Table 2 indicate that R_{low} and R_{high} significantly differ (P <0.01) in their capacity to translocate through the polarized CaCo-2 cell monolayer. While about 4 % of the inoculum of R_{low} were recovered in the lower chamber after 2 h of infection, only 0.2% of the inoculum of R_{high} were recovered. In contrast, controls showed that about 19% and 29 % or 34% and 28% of the inoculum of R_{low} and R_{high}, respectively, transferred from the upper to the lower chamber when confluent HeLa cell monolayers or the permeable support membrane alone were used in the assay (see Table 2). Since R_{low} is able to established intracellular residence in a variety of non-phagocytic cells (Winner et al. 2000), including the CaCo-2 cell line (data not shown), while R_{high} remains extracellular, these results suggested that *M. gallisepticum* translocation through a monolayer with tight junctions correlates with cell-invasion. This hypothesis was further supported by performing the translocation assay (i) with the RHC3 cell-invasive negative clone that derived from R_{high} by random picking, and (ii) with the R_{high}P10 population which was previously obtained by 10 serial passages of R_{high} in HeLa cell monolayers and which was described as being enriched in invasive organisms (Winner et al. 2000). Results indicate that RCH3 behaved as the parental R_{high} (Table 2), whereas the number of mycoplasmas recovered in the lower chambers was significantly higher (P <0.01) with R_{high}P10 as inoculum (Table 2).

Factors influencing translocation of *M. gallisepticum* through an epithelial barrier. Strategies employed by *M. gallisepticum* to translocate through epithelial barriers could involve passage via the cell by transcytosis or via the cell junctions, or by killing of the cells. In order to better understand which translocating route is taken by the avian pathogen and the nature of the factors involved in this process, the translocation assay was performed with a mixture of clones all derived from R_{high} but displaying different [HA] and [INV] phenotypes. This mixture includes (i) [HA-/INV-] RHC3, (ii) the [HA-/INV-] RHC3P10 clone derived from RCH3 by random picking after 10 times passaging through HeLa cell monolayers, and (iii) the RHG11K5, RHG11K6, and RHG11K8 clones described above that display the [HA-/INV-], [HA-/INV+] and [HA+/INV+], respectively, (see Tables 1 and 3). Prior to the assay, each clone was genetically tagged by chromosomal insertion of the *Tn4001*mod carrying a unique oligonucleotide tag-sequence for further identification. Expression of GapA and CrmA, growth, and invasion capability of each tagged clone was shown not to differ from that of its respective parent.

The translocation assay was performed as described above with the tagged-clones mixed in the proportion indicated in Table 3, and mycoplasmas recovered in the lower chamber after 2 h of infection were plated onto solid media. From the grown colonies, 91 were randomly picked and subjected to dot-blot hybridization with tag-specific oligonucleotide probes to

identify those that have crossed the tight monolayer. Results revealed (Fig. 2 and Table 3) that 96.70 % of the mycoplasmas that are detected in the lower chamber possessed the Tag17 carried by clone RHG11K8. This result suggests that translocation of mycoplasmas through CaCo-2 cells forming tight intercellular junctions does not affect the overall integrity of the monolayer as the clone, RHG11K8, recovered in the lower chamber was the less represented in the inoculum (8.00%). This specific translocation almost excluded the cell-invasive RHG11K6 clone which only represented 2.19% of the mycoplasmas that have translocated. Since this clone lacks GapA and CrmA, this raised the question of the direct influence of these two molecules in the translocation process. To further assess this question, the translocation assay was performed with the [HA-/INV+] RCL2 clone (Table 1). Results indicated that RCL2, like RHG11K6, is unable to cross the epithelial barrier composed of tight CaCo-2 cells (see also Table 2).

Factors involved in *M. gallisepticum* host colonization. Data described above were obtained using *in vitro* assays and might not reflect the *in vivo* situation. We therefore assessed their relevance in experimental infection using the natural host, the chicken, as infection model. Four groups of chickens were aerosol inoculated as previously described (Much et al. 2002), with (i) media alone (group 1), (ii) R_{low} (group 2), (iii) R_{high} (group 3), and (iv) with the exact same mixture (mix) of genetically tagged clones (group 4) used in the *in vitro* translocation assays and described above (see also Table 3). After nine days of infection, the presence of air sac lesions was assessed and their severity was scored (Table 4). As well, swabs were taken from the respiratory tract and from inner organs to define the presence of *M. gallisepticum* in different body sites by cultivation in liquid media followed by plating onto agar plates. Results showed that the severity of the air sac lesions is lower in birds infected with the mixed clones than in birds infected with R_{low}, but is significantly higher than that recorded in birds infected with R_{high} (Table 4). Overall, frequencies of mycoplasma re-isolation from the respiratory tract was similar in all infected groups although it is worth mentioning that it was lower in samples collected from the lung and the air sacs of birds infected with R_{high}. In contrast, the overall frequency of re-isolation from inner organs observed with samples collected from birds infected with R_{high} (2/25) dramatically differs from that obtained with groups infected with R_{low} (24/24) or with the mixed clones (24/24).

In order to define the nature of the clones recovered in samples collected from birds infected with the mixture of clones, 94 individual colonies were randomly selected from all *M. gallisepticum* positive samples collected from four birds displaying the highest rate of re-isolation from both the respiratory tract and inner organs. A total of 2,350 colonies were then individually arrayed in 96-well plates and analyzed by dot blot hybridization using tag-specific probes. Results showed that all colonies tested displayed the specific Tag17 sequence originally inserted into clone RGH11K8, indicating that this clone is predominantly if not exclusively present in the mycoplasma population re-isolated from birds infected with the mixed population of five clones. Individual colonies deriving from these four infected

birds prior to cloning were additionally subjected to immunostaining with anti-GapA antibodies and/or to the hemadsorption assay. Results indicated that colonies randomly selected from the infected birds exhibited a GapA+ and [HA+] phenotype in accordance with that of the RHG11K8.

DISCUSSION

In this study it was shown that *M. gallisepticum* is able to cross *in vitro* a cell monolayer with tight junctions and that this event relies on particular phenotypes. The assay performed here is based on the well-described CaCo-2 human colonic cell line and has often been used to assess the translocation of enteric bacteria through the intestinal epithelium (Konkel et al. 1992, Cruz et al. 1994). The CaCo-2 cell line is one of the very few that is able to form monolayer with tight junctions in vitro and is the most thoroughly described in the literature as a rate-limiting barrier against passive flux of small, large solutes (Birkness et al. 1999) or bacteria (Konkel et al. 1992). Since *M. gallisepticum* is mainly a respiratory pathogen, this cell line might not reflect the exact situation encountered in the chicken host. Despite this concern, the fact that *M. gallisepticum* nonclonal or clonal subpopulations derived from the same strain were shown to behave differently in this assay, is an important finding. Indeed, the virulent, cell-invasive R_{low} population was able to cross the CaCo-2 epithelial barrier, while the avirulent, cell-invasion deficient R_{high} was not. Interestingly, these findings correlate with previous *in vivo* data showing the dissemination of R_{low} from the respiratory tract to the inner organs of the chicken host, while R_{high} only generates local infection (Much et al. 2002). Furthermore, testing of the R_{high}P10 population (derived from R_{high} by 10 times passaging in cell cultures) revealed an intermediate translocation efficiency, a result which also correlates with our previous findings showing that R_{high}P10 is enriched in cell-invasive organisms (Winner et al. 2000) and better disseminates throughout the host than R_{high} (Much et al. 2002). Altogether, these data tend to suggest that *M. gallisepticum* cell invasion could be involved in translocation through tight cell monolayers, a phenomenon that could participate in colonization of various host organs, if occurring *in vivo*.

At the molecular level, R_{low} expresses two products, GapA and CrmA ([HA+] phenotype) which have been described as major adhesins and are lacking in R_{high}. Our previous *in vitro* data showing R_{high} in close contact at the surface of HeLa cells or chicken embryo fibroblasts (Winner et al. 2000) have suggested that other specific or unspecific factors of *M. gallisepticum* are involved in cell adhesion. In this study, close examination of R_{high}P10 displaying the [HA-/INV+] phenotype has indicated that GapA and CrmA are not critical in cell invasion (see Table 1). This hypothesis was further supported by testing a [HA-] clone derived from R_{low}, namely RCL2, that also turned out to be cell-invasive despite the lack of expression of the two cytadhesins. Since R_{high} has been obtained by serial passaging of R_{low} in artificial culture medium and is not *per se* a clonal population it presumably contains minor subpopulations with different adhesive and/or cell-invasive properties which were enriched

following serial passaging in cell monolayers. This would explain the presence of clones in R_{high}P10 displaying the [HA+/INV+] and [HA-/INV+] phenotypes not previously detected in R_{high} but selected by the gentamacin cell invasion assay (clones RHG11K6 and RHG11K8, see Fig. 1 and Table 1). The *gapA* gene sequences of the two [HA-] clones selected in this study from R_{high}P10 (clones RHG11K5 and RHG11K6) revealed a point mutation that has already been described for R_{high} and that is responsible for the absence of GapA and CrmA products (Papazisi et al. 2002). One of these [HA-] clones, RHG11K6, is [INV+] while the other, RHG11K5 is [INV-], implicating that independent mutational events are responsible for the two phenotypes.

Although *M. gallisepticum* cell invasion appeared to be independent of the GapA/CrmA expression, results obtained with the translocation assay suggest that these two proteins might play a role in the ability of the avian pathogen to cross epithelium with tight cell junctions. Indeed, [HA-/INV+] or [HA-/INV-] clones were unable to efficiently translocate through tight CaCo-2 cell monolayers indicating that the invasive phenotype alone is not sufficient to promote translocation. Due to the lack of an appropriate screening method [HA+/INV-] clones have not been isolated in this study. This raised the questions of whether the [HA+] phenotype alone would allow such event to occur and which translocating route are taken by the mycoplasma. A partial answer was provided by performing the translocation assay with a mixture of [HA-/INV+] and [HA+/INV+] clones. In one scenario, mycoplasmas displaying the [HA+/INV+] phenotype could cross the epithelium by damaging the integrity of the monolayer either by killing the cells or by breaking the tight junctions. This would result in a permeable monolayer and all mycoplasmas, regardless of their phenotypes, would then be expected to translocate. However, results obtained within two hours suggest another picture, as almost all mycoplasmas that have crossed the monolayers displayed the [HA+/INV+] phenotype. Taking into account that [HA+/INV+] clones only represented 8% of the inoculum used to infect the apical side of the cell monolayer, this rather suggests that the route taken by the mycoplasmas involves passage via the cell by transcytosis and would then imply that both the [INV+] and [HA+] phenotypes are required. Whether GapA and/or CrmA play a direct or indirect role in this event, has still to be elucidated. One has also to bear in mind that CaCo-2 cells when completely polarized present a different surface architecture at the apical side (Guignot et al. 2001) than when grown individually for cell invasion assays. Whether non-translocating [HA-/INV+] clones do not bind to CaCo-2 tight cell monolayers or whether they are trapped into the cells after invasion has still to be elucidated.

Transepithelial cell monolayer models are reductionist systems that have both the advantage and the inconvenience to mimic only part of the complex host mucosal surfaces. Aerosol inoculation of the chicken host with the same mixture of clones used in the translocation assay has shown that only the [HA+/INV+] clone is able to generate systemic infection. At the same time, [HA-/INV+] or [HA-/INV-] mycoplasmas could not be re-isolated

from the trachea. This was unexpected because the R_{high} displaying the [HA-/INV-] phenotype is colonizing the trachea, when used alone as inoculum in a parallel experiment (see Table 4). Insertion of the transposon used for tagging into a mycoplasma gene required for *in vivo* survival cannot be ruled out, but is unlikely to explain this observation since the [HA-/INV+] or [HA-/INV-] clones used in the *in vivo* infection experiment have inserted the *Tn4001*mod in different genomic locations some of which mapped into a silent gene of the large VlhA family (Papazisi et al. 2003) or in hypothetical proteins. Rather, re-isolation from all organs of only the clone displaying the wildtype phenotype [HA+/INV+] which was even under-represented in the inoculum used for infection might reflect a more predictable situation in which mycoplasmas with the highest fitness overtake their less competitive siblings. Nevertheless, these *in vivo* results correlate with the *in vitro* data and indicate that a classical translocation assay can be relevant to assess mycoplasma factors involved in pathogen-host cell interactions.

This is the first time that a strategy combining *in vitro* translocating assays and *in vivo* experimental infection experiments with mixed, genetically tagged organisms as inoculum have been applied to mycoplasma studies. These approaches are promising as they offer the option to track down in *in vitro* or *in vivo* experiments organisms that only slightly differ in their genetic background and to assess the behavior of complex mycoplasma populations in interactions with their respective hosts.

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TABLE 1. Phenotypic features of subpopulations and clones derived from *M. gallisepticum* strain R.

Designation	Origin [a]	Phenotype		Cell invasion [d]
		HA [b]	GapA/CrmA [c]	
Uncloned subpopulations				
R_{low}	R	+ (v)	+	+
R_{high}	R	-	- (1)	-
R_{high}P10	R_{high}	-	-	+
Clonal subpopulations				
RCL2	R_{low}	-	- (2)	+
RHC3	R_{high}	-	-	-
RHC3P10	RHC3	-	-	-
RHG11K5	R_{high}G11	-	- (1)	-
RHG11K6	R_{high}G11	-	- (1)	+
RHG11K8	R_{high}G11	+	+	+

[a] R_{low} and R_{high} derived from the original strain R by passaging in artificial media; R_{high}P10 derived from R_{high} after 10 times passaging through HeLa cell monolayers, and RGH11 corresponds to R_{high}P10 after gentamicin treatment in cell invasion assays.
[b] Hemadsorption based on binding of erythrocytes on the surface of mycoplasma colonies. + and – indicate positive or negative binding of erythrocytes; (v) indicates that in R_{low}, proteins involved in HA (GapA and CrmA) are undergoing phase variation in expression, however in R_{low} the [HA+] phenotype predominates.
[c] Based on SDS-PAGE and Western blot analyses. The presence of GapA and CrmA was previously shown to correlate with the ability of the mycoplasma cells to bind erythrocytes. (1) and (2) indicate the presence of nonsense mutations in the *gapA* gene at nt 769 and nt 1393, respectively (Papazisi et al 2000, Winner et al. 2003). Nucleotide positions correspond to the sequence published by Papazisi et al. 2000.
[d] As defined by the gentamicin cell invasion assay and by double immunofluorescence (DIF) followed by confocal laser scanning microscopy using HeLa cells. + intracellular sighting using DIF and frequency of invasion similar to R_{low} as defined in Winner et al. 2000, - no intracellular sighting using DIF and frequency of invasion similar to R_{high} as defined in Winner et al. 2000 (see Materials and Methods).

TABLE 2. Translocation frequencies of subpopulations and clones derived from *M. gallisepticum* strain R.

Strain R subpopulations and clones	% Translocation [a]		
	CaCo-2	Permeable support membrane	HeLa-229
R_{low}	3.73 ± 0.77	34.27 ± 5.31	18.74 ± 3.31
R_{high}	0.19 ± 0.05*	28.59 ± 3.05	28.97 ± 4.59
R_{high}P10	2.11 ± 0.20[§]	38.53 ± 6.32	35.33 ± 1.61
RHC3	0.18 ± 0.06*	31.42 ± 6.78	n.d.[c]
RCL2	0.19 ± 0.04*	23.33 ± 3.06	18.83 ± 2.97
Mix [b]	0.87 ± 0.15*[§]	24.50 ± 4.24	14.85 ± 3.28

[a] SD calculated with n>12
[b] Proportions of individual clones contained in the mixture and their phenotypes are indicated in Tables 3 and 1, respectively
[c] not done
* Values that are significantly different from those obtained with R_{low}.
[§] Values that are significantly different from those obtained with R_{high}.

TABLE 3. Outcome of translocation assays using a mixture of genetically tagged clones derived from *M. gallisepticum* R_{high}.

Designation	Cell Invasion [a]	Tag [b]	HA [c]	Proportion (%)		
				in inoculum	after translocation	after infection
RHC3	- (0.36±0.09)	10	-	30.8	1.1	0.0
RHC3P10	- (4.72±1.16)	20	-	16.0	0.0	0.0
RHG11K5	- (0.14±0.03)	9	- (1)	25.5	0.0	0.0
RHG11K6	+ (5.70±1.00)	7	- (1)	19.7	2.2	0.0
RHG11K8	+ (5.20±1.60)	17	+	8.0	96.7	100.0

[a] See Table 1; SD calculated before tagging for HeLa cells with n =9
[b] Tags are detailed in Materials and Methods
[c] See Table 1; (1) Mutations in the *gapA* gene are identical to that of R_{high}

TABLE 4. Outcome of aerosol inoculation of chickens with *M. gallisepticum* R_{low}, R_{high} or a mixture of genetically tagged clones derived from R_{high}.

Inoculum	Control [a]	R_{low}	R_{high}	Mix [b]
Group	1	2	3	4
Total number of birds	25	24[c]	25	24[c]
Number of chickens presenting air sacculitis:				
Total lesion scores per group	0	187	2	96
Mean lesion score per bird	0	7.79	0.08	4
Number of birds with air sacculitis	0	24	2	18
Mean lesion score per bird with air sacculitis	0	7.79	0.08	4.57
Frequency of re-isolation from:				
Trachea	0/25	24/24	18/25	21/24
Lung	0/25	23/24	13/25	22/24
Air Sac	0/25	17/24	9/25	19/24
Liver	0/25	13/24	0/25	5/24
Spleen	0/25	12/24	0/25	9/24
Kidney	0/25	21/24	2/25	20/24
Heart	0/25	22/24	0/25	19/24
Total frequency of re-isolation from:				
Respiratory tract	0/25	24/24	23/25	24/24
Inner organs	0/25	24/24	2/25	24/24

[a] Sterile broth medium used for aerosol inoculation

[b] Proportions of individual clones contained in the mixture and their phenotypes are indicated in Tables 3 and 1, respectively

[c] R_{low}- and mix- infected groups were composed of 25 chickens. In each of these groups, one bird died and the corresponding values were not included

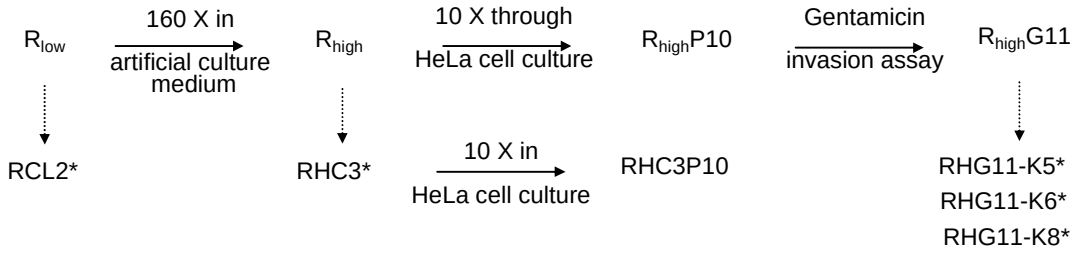
Figure Legends

FIG. 1. Panel A: Flow chart representing the relationship between subpopulations derived from *M. gallisepticum* strain R. R_{low}, R_{high} and R_{high}P10 have been previously described (Winner et al. 2000) and derived from strain R by serial laboratory passage in artificial culture media or through HeLa cell cultures. R_{high}G11 was obtained after subjecting HeLa cells infected with R_{high}P10 to gentamicin treatment. Clones that were selected from *M. gallisepticum* populations are indicated by an asterisks. **Panel B**: The expression of the GapA and PvpA proteins was assessed by Western blotting and is illustrated for RHG11K5 (lane 1), RHG11K6 (lane 2), RHG11K8 (lane 3), RHC3 (lane 4), and RHP10 (lane 5).

FIG. 2. Identification of *M. gallisepticum* clones that have translocated through CaCo-2 cell monolayers by hybridization. A translocation assay was performed using a mix of five clonal variants described in Table 3, each carrying a different oligonucleotide tag (Tag 7, 9, 10, 17 and 20). After translocation, mycoplasmas contained in the lower chamber were recovered, plated and 91 single colonies were randomly picked and cultured into a 96-well microtiter plate (Panel A). The 91 cultures were analyzed by dot blot hybridization using oligonucleotide probes P7, P9, P10, P17 and P20 corresponding to each tag, as indicated below Panels B through D. Except for the experiment illustrated in Panel D, the membrane was stripped in between hybridization with the different probes. Controls in positions D12, E12, F12, G12 and H12 (boxed) corresponded to clones containing individual Tags 7, 9, 10, 17 and 20 as indicated.

Fig. 1

A


R_{low}
160 X in
artificial culture
medium
R_{high}
10 X through
HeLa cell culture
R_{high}P10
Gentamicin
invasion assay
R_{high}G11
RCL2*
RHC3*
10 X in
HeLa cell culture
RHC3P10
RHG11-K5*
RHG11-K6*
RHG11-K8*

B

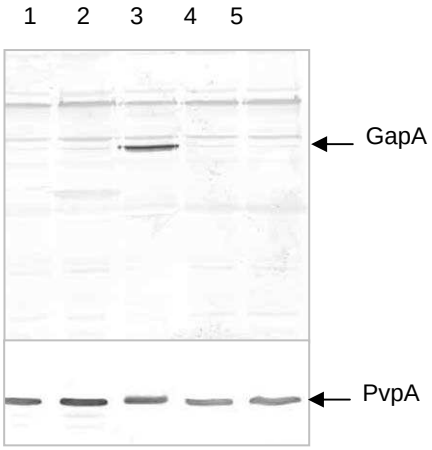
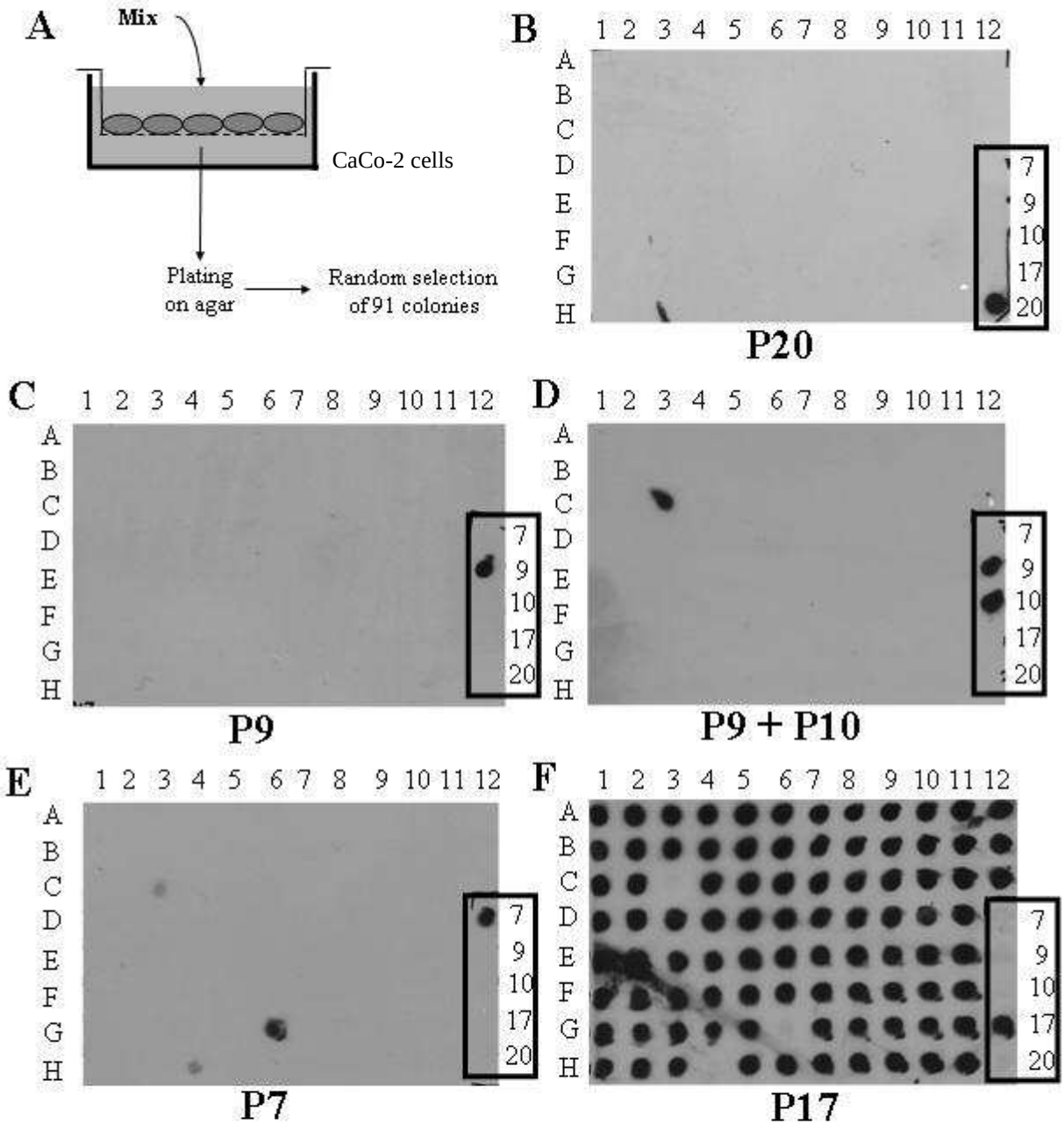

1 2 3 4 5
GapA
PvpA

Fig. 2


A
Mix
CaCo-2 cells
Plating on agar
Random selection of 91 colonies
B
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
7 9 10 17 20
P20
C
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
7 9 10 17 20
P9
D
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
7 9 10 17 20
P9 + P10
E
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
7 9 10 17 20
P7
F
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
7 9 10 17 20
P17

4.3. Role of the GapA and CrmA cytadherence proteins *in vivo*

Role of *Mycoplasma gallisepticum* GapA and CrmA cythadhesins in promoting virulence and in colonization of the natural chicken host

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Abstract

Mycoplasma gallisepticum is an important avian pathogen that commonly induces chronic respiratory disease in chickens. To better understand the mycoplasma factors involved in host-colonization, chicken were infected via aerosol with two hemadsorption negative [HA-] mutants, mHAD3 and RCL2, that derived from a low passage of the pathogenic strain R, R_{low}, and are both deficient in the two major cythadhesin, GapA and CrmA. After 9 days of infection, chickens were monitored for air sac lesions and for the presence of mycoplasmas in various organs. Data showed that the mHAD3 in which the *crmA* gene has been disrupted does not promote efficient colonization or significant air sac lesions. In contrast, the spontaneous [HA-] RCL2 mutant which contains a point mutation mutation in the *gapA* structural gene successfully colonized the respiratory tract and display an attenuated virulence when compared to R_{low}. In a previous study, the RCL2 point mutation was shown *in vitro* to spontaneously revert with a high-frequency resulting in ON and OFF switching of the HA phenotype. Detailed analyses further revealed that such event is not responsible of the observed *in vivo* outcome since 98.4% of the mycoplasma populations recovered from RCL2-infected chicken still display the mutation and the associated phenotype. Unlike R_{low}, RCL2 was however unable to colonize inner organs. These findings demonstrate the major role played by the GapA and CrmA products in *M. gallisepticum* host colonization and virulence.

Introduction

The wall-less bacterium *M. gallisepticum* is an important avian pathogen that commonly induces chronic respiratory disease in chickens (16, 18, 27, 33) and sinusitis in turkeys (9). In chickens, other clinical manifestations such as arthritis and salpingitis can also be observed (1). *M. gallisepticum* infections are responsible for considerable economic losses worldwide and consequently a large number of studies have been dedicated to the better understanding of its biology and of the factors involved in host-interactions.

M. gallisepticum mainly colonizes its host via the muscosal surfaces of the respiratory tract causing air sacculitis within a few days (20). The mycoplasma propensity to then disseminate throughout the body and to generate systemic infection is reflected by the high rate of *M. gallisepticum* re-isolation from inner organs such as the liver, the heart, the spleen or the kidney, in birds experimentally infected via aerosol (20). *M. gallisepticum* successful infection may rely on two independent phenomenons that may provide *M. gallisepticum* with means to circumvent the specific host defenses. These are the high versatility of its surface architecture that occurs via phase variable expression of *vlhA* family members (7, 22), and the mycoplasma ability to establish facultative intracellular residence in non phagocytic host-cells (29).

For colonization, dissemination or cell invasion, the attachment of *M. gallisepticum* to host-cells is a crucial event that is mediated via a unipolar terminal organelle or bleb-like structure containing at least two potential cytadhesin-related proteins. These are the phase variable PvpA molecule (3) and a 32 kDa product encoded by the *mgc*2 gene (15) that both display homology to the P30 adhesin of the human pathogen *M. pneumoniae* (26). Interestingly, *mgc*2 is located a few hundred nucleotides upstream of a cluster of four cytadherence related genes designated (from 5' to 3') *gapA* (or *mgc1*) (13, 17), *crmA* (or *mgc3*) (25, 35), *crmB* and *crmC* (24). While the role of CrmB and CrmC in adherence has only been predicted in silico (24), several independent *in vitro* studies have shown that the GapA and CrmA products are two major cythadhesins of *M. gallisepticum* strain R (25, 28). Indeed, a high, avirulent passage of strain R (R_{high}) that lacks both GapA and CrmA has a diminished capacity in adhering to MRC5 human cells (25) and is deficient in binding erythrocytes on colonies (28) when compared to a low, virulent passage of the same strain (R_{low}). In R_{high}, the lack of GapA and CrmA is due to an additional adenine residue in the beginning of the structural *gapA* gene that results in a premature stop codon and has a negative polar effect on the transcription of the *crmA* gene located downstream (25). Interestingly, complementation of R_{high} with wild-type *gapA* or *crmA* genes did not restore *in vitro* adhesion to MRC5 cells while introduction of the wild-type *gapA-crmA* operon did (23). Although R_{high} transformants that have reacquired expression of both products induce air sacculitis in chickens, lesion scores were lower than those observed with R_{low} and no tracheal lesions

were detected (23). Collectively, these data suggest that both GapA and CrmA are required for adhesion but also that other factors lacking in R_{high} may contribute to virulence.

Recently, a reversible point mutation occurring at beginning of the structural *gapA* gene and corresponding to a base substitution was shown to govern the spontaneous high-frequency phase variation in expression of GapA (28). This mutational event, which is distinct from that described above for R_{high}, also has a polar effect on the transcription of the *crmA* resulting in the concomitant phase variation of the CrmA product. Oscillation in expression of the GapA and CrmA products is responsible for the variable binding of erythrocytes (HA) on MG colonies (28). This was demonstrated using by a lineage derived from R_{low} and composed of clonal variants that alternatively displayed the [HA+] and [HA-] phenotype and expressed, or not, the two cythadesines, respectively. Phase variation in expression of surface components in pathogenic mycoplasma species has been extensively described and a collection of genetic systems generating high-frequency has been reported (5, 7, 30, 34). These mainly include gene family encoding related surface proteins and single-copy gene encoding molecules with adhesive properties. However, the significance and consequences of this phenomenon have been rarely addressed *in vivo* and only in the case of complex gene family (11, 14).

The aim of this study was to assess *in vivo* the colonizing ability and the virulence of a well characterized [HA-] variant, namely RCL2 that was part of the clonal lineage described above, lacks the GapA and CrmA products and displays a base substitution in the *gapA* gene (28). For this purpose, chickens were experimentally inoculated via aerosol to monitor the virulence of RCL2 and the nature of the organs that were colonized. Since the two GapA and CrmA cythadhesins are believed to be important in infection we postulated that a back-switching from [HA-] to [HA+] might confer a selective advantage *in vivo*, and therefore we also monitored the presence of these products and of the *gapA* gene mutation, in populations re-isolated from RCL2 infected chickens. The same experiment was conducted with the parental R_{low} strain, with R_{high} and with the previously described mHAD3 mutant that derived from R_{low} by insertion of the Tn*4001*mod in the *crmA* structural gene (28). In this mutant, disruption of the *crmA* gene lead to a [HA-] phenotype identical to that of RCL2 due to the lack of CrmA product and to the production of minute amount of GapA. Results presented in this study revealed that RCL2 and mHAD3 differ in virulence and in colonization of the birds. Interestingly, mHAD3 showed no virulence and a low frequency of re-isolation when compared to RCL2. Populations recovered and analyzed from RCL2 infected chickens all displayed the [HA-] phenotype but one. The role of GapA and CrmA in colonization of the lower and upper respiratory tract of chickens is discussed.

MATERIAL AND METHODS

Mycoplasma strains and growth conditions. *M. gallisepticum* laboratory passages R_{low} and R_{high} used in this study were kindly provided by S. Levisohn, Kimron Veterinary Institure, Bet Dagan, Israel. R_{low} and R_{high} correspond to the prototype strain R propagated 10 and 160 times in culture medium, respectively (19). The clonal variant RCL2 and the mutant mHAD3 derived from R_{low} by spontaneous mutation in the *gapA* gene (RCL2) or by insertion of the Tn4001mod in the *crmA* gene (mHAD3), respectively (28). Consequently these two mutants display the hemadsorption negative phenotype that correlates with the lack of expression the *gapA* and *crmA* genes. Specific rabbit antisera against the CrmA protein were produced as described below using antigens prepared from the clonal variant RCL1 which has been previously described and shown to express the CrmA product (28).

Mycoplasmas were grown at 37℃ in modified Hayflick medium (32) containing 20% (v/v) heat-inactivated horse serum (Invitrogen Life Technologies, Rockville, MD) to mid-exponential growth phase, as indicated by the metabolic color change of the medium. The number of viable mycoplasmas in a suspension was determined by plating serial dilutions on Hayflick medium containing 1% (w/v) agar, followed by incubation at 37℃. After 6-8 days, the number of colony forming units (CFU) was counted using an SMZ-U stereomicroscope (Nikon, Tokyo, Japan). The presence of the Tn*4001*mod in mycoplasma populations recovered from infected chickens was assessed by plating appropriate dilutions on solid media without or with 100 µg per ml of gentamicin.

Escherichia coli DH10B (Invitrogen) was used as host to clone recombinant products and grown at 37℃ in Luria-Bertani broth supplemented with 100 µg of ampicillin per ml for plasmid preparation.

Experimental infection procedure. The experimental infection procedure performed in this study is identical to that previously described by our group (20). Briefly, one hundred and fifty Arbor acress chickens of 21-day old and certified free of mycoplasmas were divided into five groups of 30 birds each. Each group was placed in an aerosol chamber and inoculated with 10 ml of culture medium pulverized into fine aerosol particles of 7 to 10 µm and sprayed for 2 min into the chamber. Inoculum consisted of (i) 9.6×10^9 CFU of R_{high}, (ii) 10.5×10^9 CFU of R_{low}, (iii) 9.7×10^9 CFU of mHAD3, (iv) 4×10^9 CFU of RCL2 or (v) medium alone with no mycoplasma. Infected birds were slaughtered nine days post-infection and necropsy was performed for pathomorphological lesions of the thoracic and abdominal air sacs that were documented by a scoring system previously described (8) with a maximum theoretical score of 12 per bird.

During necropsy, swabs were collected from several body sites of the respiratory tract (the trachea, the lung and the left thoracic air sac) and of deeper inner organs (the spleen, the liver, the kidney and the heart). Samples were directly transferred into culture medium and

incubated at 37℃. Samples that showed a metabolic color-change within 14 days were further processed while the remainings were kept at 37℃ for six additional days. All samples were stored at –80℃ for further analysis after an aliquot from each grown culture was plated in two dilutions onto Hayflick agar plates. Mycoplasmas were identified as *M. gallisepticum* on randomly selected cultures by colony immunobloting using anti-*M. gallisepticum* rabbit polyclonal serum in dilution 1:300 (29) and then during phenotyping of the mycoplasma populations.

Antibodies to CrmA. *M. gallisepticum* clonal variant RCL1 was subjected to Triton X-114 fractionation as previously described (31) and proteins that partitioned into the insoluble pellet were resolved by 9% SDS polyacrylamide gel electrophoresis (SDS-PAGE). The 116 kDa CrmA protein of interest was localized by Negative staining using the Zinc Stain Kit (BioRad, Hercules, CA) and the corresponding gel slice was excised, lyophilized and homogenized in an equal volume of 0.9% NaCl. Half of this solution was then injected to to both Popliteal Lymph Node of New Zealand White rabbits while the remaining was inoculated subcutaneously in three different locations. The rabbits were given subcutaneously two booster injections of the protein in NaCl at monthly intervals so that each rabbit had received a total amount of protein corresponding to approximately $1.5x10^{11}$ CFU. Eleven days following the last inoculation, the rabbits were anesthetized with ketamine hydrochloride (25 mg per kg of body weight) and Xalazine (3 mg per kg of body weight) and bled by cardiac puncture. The specificity and the working dilution of the rabbit antibodies directed against CrmA were assessed by Western blot using proteins extracted from CrmA-negative and CrmA-positive clones, namely RCL2 and RCL1, respectively.

Colony immunoblotting and hemadsorption assay. Colony immunoblotting was performed as described elsewhere (4) with the antibodies and under the conditions described below for Western blot analyses. The hemadsorption (HA) assay was used to confirm that mycoplasmas reisolated from infected chickens have conserved the phenotype of the inoculum. To avoid artifacts due to removal of surface material through washing, colonies were first blotted on nitrocellulose discs (Schleicher & Schuell, Protran BA 83, Dassel, Germany) and remaining of the colonies left on the agar plate were overlaid with a 0.5% (vol/vol) suspension of sheep erythrocytes in 1x phosphate-buffered saline solution (PBS: 2.7 mM KCl, 1.2 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4. $7H_2O$, pH 7.4) and incubated for 30 min at 37℃. After two gentle wash es with PBS, the colonies were examined microscopically for hemadsorption.

SDS-PAGE and Western blot analysis. Protein profile analyses of populations and clones used in this study were performed by SDS-PAGE followed by Coomassie staining and/or Western blotting using fractions obtained after Triton-X114 (Sigma-Aldrich, St. Louis, MO) partitioning as described elsewhere (31). After blocking with 2% Non Fat Dry Milk (BioRad, Hercules, CA, USA) in Tris-buffered saline solution (TBS) (150m M NaCl, 10mM Tris-base)

for 1 h at room temperature, the membranes were incubated overnight at 4℃ with gentle rocking (i) with rabbit anti-GapA antibodies (25) and/or (ii) with rabbit anti-CrmA antibodies diluted 1:8000. After three washes with 1x TBS membranes were incubated with peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako, Copenhagen, Denmark) at a final dilution of 1:2000 for 2 h at room temperature with gentle rocking, followed by three consecutive washings with 1x TBS. Reactions were visualized by addition of 4-chloro-1-naphtol and hydrogen peroxide (BioRad, Hercules, CA).

DNA manipulations. Mycoplasma genomic DNA was extracted as previously described (6). Plasmid DNA was extracted using the PeqLab E.Z.N.A. Plasmid Miniprep Kit II (PeqLab Biotechnologie GmbH, Erlangen, D). Restriction endonuclease (Promega GmbH, Madison, WI) digestion, ligation, electroporation were preformed according to manufacturer's instructions and to standard procedure.

PCR assays. The presence of the transposon in mycoplasma populations recovered from infected birds was assessed using a PCR assay previously described (28) that contained (i) 1 mM of the forward Tn1 primer (5' ACATGAATTACACGAGGGC 3') and 1 mM the reverse Tn2 primer (5' GTTCTTCTTCTGACATAGTAG 3'), (ii) 100 ng of chromosomal DNA as template, (iii) Taq DNA polymerase (Promega) in 1x buffer supplied by the manufacturer and supplemented with 1,5 mM of $MgCl_2$, and (v) 0.2 mM dNTP (Promega). Thermocycling was performed in a Perkin-Elmer DNA Thermo Cycler with the following conditions: 3 min of denaturation at 95℃ followed by 30 cycles of 1min denaturation at 94℃, 1 min annealing at 50℃ and 2 min extension at 72℃, and finally 5 min of final extension at 72℃. As an internal control, two primers TufG15 (TTCGATCGTAGTAAACCTCACG) and TufC26 (GACGATTTTGAGTTGCGTATTC) which amplify a 210 bp of the house keeping *tuf* gene (12) were added before starting the PCR cycles.

The localization of the transposon was confirmed by long-range PCR (LR-PCR) using the Expand ™ Long Template PCR system (Roche, Basel, Switzerland) to amplify the region located between the beginning of *crm*A gene and the IS sequence of the Tn*4001*mod. For this purpose, primers pIS256rev (GGTCATGTAAAAGTCCTCCTGGG) (28) and pJF (TAAGAAGACTCCACAAATGCT) (25) were used at a final concentration of 1.5 mM each in 1x buffer (System I) and 0.35 mM dNTPs using the following conditions: denaturation step at 94℃ for 2 min, followed by 10 cycles of 30 s denaturation at 94℃, 30 s annealing at 48℃, 4 min extension at 68℃; 20 cycles of 30 s denaturation at 94℃, 30 s annealing at 48℃, 4 min extention at 68℃ with cycle elongation for 20 s per cycle, and a final extension cycle at 68℃ for 7 min.

The presence of the point mutation located at nucleotide 1393 of the RCL2 *gapA* gene ((position defined based on Papazisi et al. (25)) was assessed using a PCR assay followed by digestion of the PCR product by *Mse*I (NEB Inc., USA) and the resulting fragments were resolved on a 10% polyacrylamide gel as described elsewhere (28). Briefly, the PCR assay

was performed using 100 ng of chromosomal DNA as template, 1 mM forward primer GapA4 (TTCGGAAAATCCCTTTGCAGTAG) and 1 mM reverse primer GapA5 (TAGAGGAGTAGTTGTTTGAGTTTC), and in 1x buffer containing 2mM $MgCl_2$ and 0.2 mM dNTP. Thermocycling conditions were the followings: one denaturation step at 95℃ for 3 min, followed by 30 cycles of 1 min denaturation at 94℃, 1 min annealing at 45℃, 1 min of extension at 72℃; and a final extension cycle at 7 2℃ for 5 min (28).

Statistical analysis. The free SISA software was used for statistical analysis. The numbers of infected birds, air sac lesion scores and the frequency of re-isolations were analyzed by the Fisher exact test (n = 30). Both challenged groups (birds infected with RCL2 or mHAD3) were compared to control groups (R_{low} and R_{high}) and to each other. Otherwise indicated in the text, the probability for significance was $p \leq 0.05$.

RESULTS

Virulence of the two distinct [HA-] *M. gallisepticum* mutants. To better understand the role of the two cytadhesins GapA and CrmA in *M. gallisepticum* virulence and host-colonization, two groups of chickens (Table 1) were infected by aerosol with the RCL2 and the mHAD3 mutants, respectively. Three additional control groups were included in the experiment and corresponded to chickens inoculated with (i) sterile broth media, (ii) the virulent parental strain, R_{low}, or (iii) a high passage of the strain R, R_{high}, which has lost its virulence properties and lacks both the GapA and CrmA products.

Nine days after inoculation, chickens were slaughtered and air sacs lesions (AS) were scored as described in Material and Methods. As illustrated in Table 2, control groups behaved as expected (20) since (i) no AS lesions were found in birds of the group inoculated with media, (ii) 30 birds out of 30 displayed air sacculitis in the R_{low}-infected group, with a total lesion score of 220 and (iii) only 3 out of 30 birds of the R_{high}-infected group presented mild AS lesions with a total score of 4. Results obtained with the mHAD3-infected group revealed that only 8 out of 30 chickens displayed air sac lesions with a total score of 12. A closer examination revealed that among these, mycoplasma populations recovered from one bird exhibiting severe air sac lesions (score of 5) had lost the transposon and reverted to the parental R_{low} phenotype (see below). These data indicates that the number of birds presenting AS lesions and the severity of the lesion did not significantly differ between groups infected by R_{high} and by mHAD3* (Table 2). In contrast, more than half of the RCL2-infected chickens (19 out of 30) displayed air sac lesions with a total score of 40. These values are significantly higher than those observed in groups inoculated with mHAD3 or R_{high} ($p<0.05$), however they are also significantly lower than that obtained with the parental R_{low} strain suggesting that RCL2 displayed an attenuated virulence.

Host colonization ability of the two distinct [HA-] *M. gallisepticum* mutants. In order to define the ability of the two *M. gallisepticum* [HA-]mutants to colonize the chicken host, swab

samples were collected at necropsy from the respiratory tract and from the inner organs. A total of 150 swabs from the trachea, the left thoracic air sac, the lung, the liver, the spleen, the kidney or the heart were grown in media until metabolic color-change and plated on solid media. A large number of samples showed color change within the first week of incubation and samples were considered negative after no color change was observed for 14 days of incubation. The identity of *M. gallisepticum* was assessed on randomly selected cultures by colony immunobloting using anti-*M. gallisepticum* rabbit polyclonal serum (29) and further confirmed during phenotyping of the recovered mycoplasmas (see below).

The frequency of re-isolation of *M. gallisepticum* in each group is presented in Tables 3 and 4, and shows that (i) no mycoplasma was recovered from chickens inoculated with media, (ii) R_{high} predominantly colonizes the upper respiratory tract, (iii) R_{low} colonizes the respiratory tract and various inner organs, and (iii) that the degree of colonization varies among birds of a same group (i. e. not all birds displayed mycoplasmas in all organs). Overall, the highest frequency of MG reisolation was observed in the group infected by R_{low} with 70% of positive samples. In contrast, the mHAD3-infected group displayed the lowest frequency, 13.2 % (ca 10% for mHAD3*), a value representing less than half of that obtained with the R_{high}-infected group (29.7 %). Finally, MG was reisolated in 43.0 % of the samples collected from RCL2-infected birds suggesting that mHAD3 and RCL2 significantly differ ($p<0.05$) in their ability to colonize or to survive the host although both derived from R_{low} and displayed a similar GapA and CrmA negative phenotype.

Overall, mHAD3-infected birds displayed the lowest frequency of mycoplasma re-isolation from the respiratory tract (ca. 20 %, see table 3) when compared to that calculated in groups infected by RCL2 (ca. 80 %) or even by R_{high} (ca. 60%). Most specifically, the frequency of re-isolation from the lung was particularly low in the mHAD3 group in respect to that obtained from the trachea.

Finally, re-isolation from the inner organs was lower in all groups than that of the respiratory tract, with R_{low} presenting the highest rate and mHAD3 the lowest. This suggests that the ability of MG to colonization or persist within the respiratory tract might correlate with its dissemination throughout the body.

***In vivo* reversion of the mHAD3 and RCL2 mutations.** Data described above suggested that RCL2 exhibits attenuated virulence properties when compared to the parental R_{low} while mHAD3 that derived from the same strain is avirulent. This raised the question of whether these difference may be explained by the presence in RCL2-infected chickens of mycoplasma populations that have reverted to the HA positive phenotype.

To assess this question, we first monitored the 27 mycoplasma-positive samples that were collected from mHAD3-infected group (i) for their resistance to gentamicin [$Gm^{R/S}$] by plating appropriate dilutions on solid agar plates with and without gentamicin, (ii) for the detection of GapA and CrmA in SDS-PAGE and Western blot analysis [$GapA^{+/-}$ $CrmA^{+/-}$] and (iii) for the

presence of the Tn*4001*mod and its location within the *crmA* gene by PCR using the two couples of primers described in Material and Methods [$Tn^{+/-}$]. Results revealed that 7 out of 27 positive samples contained mycoplasmas that were gentamicin sensitive [Gm^S], did not contain the transposon and produced both GapA and CrmA. Most particularly, populations recovered from bird #8076 which exhibited the highest AS lesion score, all expressed the GapA and CrmA products regardless of the origin of the sampling (trachea, air sac, lung, liver and heart). This is illustrated in Figure 1 that shows a perfect correlation between the expression of GapA and CrmA (Panel A, lanes 2 and 3) and the absence of the transposon within the *crmA* gene in population recovered from the trachea and the heart of bird #8076 (panels B and C, lanes 2 and 3). Interestingly, the same picture was obtained for samples collected from the trachea of two birds from which positive samples composed of mycoplasmas exhibiting the original mHAD3 features [Gm^R, $GapA^-CrmA^-$, Tn+] were also recovered from another body site. This is illustrated in Fig. 1 for the two samples recovered from the trachea (lane 4) and from the kidney (lane 5) of bird #8079 that respectively presented the R_{low}- and the original mHAD3-features

Similarly, we monitored 62 out of the 86 samples (72 %) which were positive for *M. gallisepticum* in birds infected by RCL2 (i) for the expression of GapA and CrmA and (ii) for the presence of the point mutation located at the beginning of the structural *gapA* gene by combining PCR with restriction analyses as previously described (28). This analysis revealed that 61 samples displayed molecular features identical to the RCL2 inoculum. This is illustrated in Fig. 2A and 2B lanes 1-4, 6 and 7. In one bird, bird #9607, from which mycoplasmas were reisolated from the trachea, the air sac and the lung, analysis of the sample of the trachea (#A673) revealed the presence of a mycoplasma population that was different from the RCL2 inoculum. Plating of this sample and analysis of 10 randomly picked colonies revealed that one colony displayed the CrmA and GapA products and no additional *Mse*I restriction site within the beginning of the structural *gapA* gene. This indicates that sample #A673 is composed of a mixed population, ca. 10% of which displayed the parental R_{low} features. Although the *in vitro* ON and OFF switching of the *gapA* gene expression has been previously estimated to $5x10^{-2}$ to $2x10^{-4}$ per cell per generation, the emergence of revertants in sample #A673 is likely to reflect an *in vivo* event as it not been observed for any other sample.

As controls, samples derived from R_{low} and R_{high} were also analysed. No difference was observed between the R_{low} inoculum and samples recovered in R_{low} infected chickens. Two samples re-isolated from the trachea and the air sac of one chicken infected by R_{high} exhibit the GapA and CrmA products. As discussed below, these results are not surprising since R_{high} is not a clonal population per se and was previously suspected to contain a minor sub-population presenting the R_{low} phenotype (20, 29).

Overall, most phenotypic changes were recorded in MG population recovered from birds infected by mHAD3 and it is noteworthy to mention that in more than 50 % (6 out of 11) of the samples presenting a phenotype different from the inoculum were collected from the trachea.

DISCUSSION

Data presented in this study demonstrate that the GapA and CrmA adhesins of *M. gallisepticum* play a key role in colonization and virulence of the natural chicken host. Infections of chicken with two mutants presenting a same phenotype but distinctive mutations led to different outcomes *in vivo*. More specifically, the [HA-] mHAD3 mutant in which the *crmA* gene has been disrupted (28) is unable to promote efficient colonization of the chicken-host following inoculation via aerosol. Results were particularly striking when considering that mHAD3 could be re-isolated from the air sacs in only one bird while the frequency of re-isolation from the trachea was approximately 10 times higher in the same group of infected birds. In one mHAD3-infected chicken (#8076), the lesion score was surprisingly higher than for others of the same group. Detailed analysis of mycoplamas reisolated from #8076 revealed that they display the wild type phenotype and that they have lost the transposon Tn*4001*mod initially inserted in *crmA*. *In vitro*, the Tn*4001*mod, which carries its own transposase, is stable, however the selection pressure imposed *in vivo* by the host may promote the emergence of survivor, wild-type populations. Whether undetected wild-type mycoplasmas pre-existed in the mHAD3 inoculum cannot be ruled out. In such scenario, one would have expected the wild-type mycoplasmas to quickly overtake the mutant and be transmitted to other birds of the same confined group resulting in predominant reisolation of wild-type *M. gallisepticum*. The picture, which we observed in our experiment, rather suggests that loss of the transposon has occurred over time. This is supported by a previous, independent work by Mudahi-Orenstein and collaborators (21), in which infection of chicken with a *M. gallisepticum* mutant also containing the Tn*4001* in the *crmA* gene was performed. Data presented in this study were limited to analyzing tracheal colonization but revertants were also noted several days after intratracheal inoculation and evaluation of mycoplasma CFU recovered over time from this body site was significantly lower than in birds infected with the wild-type strain.

Aerosol inoculation of chicken with the spontaneous [HA-] RCL2 mutant that contains a nonsense mutation in the *gapA* structural gene offers a different picture. In contrast to the situation previously described, RCL2 successfully colonized the upper and lower respiratory tract although frequency of reisolation from the air sacs was slightly lower than in birds infected by R_{low}. The overall lesion score indicates that RCL2 display an attenuated virulence compared to R_{low} but is significantly higher than in birds infected by R_{high}. Once again, appearance of the lesions seems to correlate with the ability of *M. gallisepticum* to reach or

to survive within the air sacs. As well, RCL2 better performed than R_{high} or mHAD3 in reaching inner body sites however with less efficiency than the “wild-type”, R_{low}. Overall, this suggests that RCL2 exhibits a pattern of infection that reflects an intermediate situation when compared to R_{low} and to R_{high} or mHAD3 and that RCL2 might only be impaired in virulence. Point mutations located in the *gapA* gene of RCL2 and R_{high} are distinct but both affect the stability of the polycistronic mRNA that encode GapA and CrmA and both result in absence of these two products (25, 28). Besides of their exact location, the only attribute that distinguishes these mutations is their frequency of reversion. *In vitro*, the point mutation in *gapA* of RCL2 was shown to spontaneously revert with a high-frequency resulting in ON and OFF switching of the *gapA* expression while that of R_{high} is reversible with a low frequency (28). Unexpectedly, the infection outcome observed with RCL2 when compared to R_{high} cannot be attributed to such an event as 98.4% of the populations recovered from RCL2-infected birds still possess the non-sense mutation. This indicates that switching ON of the *gapA* and *crmA* expression via a reverse mutation does not spontaneously occur with a high frequency *in vivo*, over a period of 9 days. This unexpected finding raised the question of whether the mutation is stable in the chicken host or of whether it provides the organism with an advantage in the beginning of the infection, before a specific immune response is built up.

Our data indicate that other factors than GapA and/or CrmA might be required in order for *M. gallisepticum* to promote successful infection. This supports the finding by Papazisi et al. (23) showing that complementation of R_{high} by wild type GapA and CrmA does not fully restore virulence. In this respect, RCL2 derived from R_{low} which one was shown to differ from R_{high} by its capacity to establish intracellular residence in non-phagocytic cells. Since RCL2 is also cell-invasive (data not shown), this property may provide the organism with a means to better disseminate from the respiratory tract to the inner organs. *In vivo*, colonizing microorganisms are faced to a hostile environment. Under these stress conditions, the occurrence in RCL2, within the context of the point mutation, of unconventional events that would result in a temporary production of GapA and/or CrmA products cannot be rule out. For instance, misreading of termination codons has been extensively described for viruses and usually involves unusual modification of normal cellular tRNAs and depends on sequences neighboring the leaky stop codon (2). As well, tRNA hopping by re-pairing of tRNA on cognate or near-cognate codon can generate frameshift by increment as large as 50 nt and is stimulated by the sequence context (10).

A common trend observed between RCL2, mHAD3 and R_{high} infected birds is the reduced colonization of the air sacs when compared to R_{low}. This observation suggests that GapA and CrmA might be particularly required for *M. gallisepticum* to reach, to adhere or to maintain himself in this particular body site. As well, there is a correlation between colonization of the air sacs and re-isolation from the inner organs suggesting that this site maybe be a portal for dissemination of the avian mycoplasma.

The presence of mycoplasmas in body sites other than the mucosal surfaces has long been underestimated although this occurrence could have important consequences for treatment and diagnosis. Understanding how mycoplasmas are able to spread from the mucosal surface to deeper organs may provide new valuable information for the control of mycoplasmosis.

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TABLE 1. Phenotype of *M. gallisepticum* populations and mutants used as inoculum in experimental infection.

Infection groups	Inoculum	HA	Expression of GapA and CrmA	Presence of an additional *Mse*I site	Presence of Tn*4001* by PCR
I	media	nd *	nd	nd.	nd
II	R_{low}	+	+	-	-
III	R_{high}	-	-	-	-
IV	RCL2	-	-	+	-
V	mHAD3	-	-	-	+

* not done

TABLE 2. Number of chickens presenting air sacculits and/or infected by *M. gallisepticum.*

Infection group	Number of birds with AS lesions (per group)	Scoring of AS lesions		Number of birds in which MG was recovered		
		Total	per bird	Total	RT	IO
Media	0	0	0	0	0	0
R_{low}	30	220	7.33	30	30	28
R_{high}	3	4	1.33	26	26	7
RCL2	19†	40†	2.1	30	30	14
mHAD3	8	12	1.5	18‡	17	5
mHAD3*	7¶	7¶	1.0	17	14**	4

*In one birds all *M. gallisepticum* populations reisolated from five body sites were shown to displayed the wild type parental R_{low} phenotype (See text)

** Mycoplasma samples recovered from the trachea of two birds displayed the R_{low} parental [HA+] phenotype while samples recovered in the same birds from other organs displayed the mHAD3 [HA-] phenotype

¶ Significantly different ($p \leq 0.05$) from values obtained with R_{low}- or RCL2-infected birds; Not significantly different ($p = 0.272$) from obtained with R_{high}-infected birds.

† Significantly different ($p \leq 0.01$) from values obtained with R_{low}-infected birds and significantly different ($p \leq 0.05$) from values obtained with R_{high}- or mHAD3-infected birds.

‡ Significantly different ($p \leq 0.05$) from values obtained with R_{low}-, R_{high}- or RCL2-infected birds which displayed among themselves no significant differences.

TABLE 3. Frequency of re-isolation of *M. gallisepticum* from the respiratory tract and from inner organs after 9 days of infection.

Organs		Inoculum†					
		Media	R_{low}	R_{high}	RCL2	mHAD3	mHAD3*
RT	trachea	0/30	23/23	23/26	25/25	15/29	12/29
	AS	0/29	27/30	7/30	16/30	2/30*	1/30*
	Lung	0/29	29/29	21/30	27/30	4/29*	3/29*
	Total	0/88	79/82	51/86	68/85	21/88	16/88
	Total %	0.0	96.3	59.3	80.0	23.9	18.2
IO	Spleen	0/30	7/30	0/27	1/29	0/28	0/28
	Liver	0/30	8/29	0/30	1/29	2/28	1/28
	Heart	0/30	18/30	2/30	6/29	1/30	0/30
	Kidney	0/30	28/29	7/29	10/28	3/30	3/30
	Total	0/120	61/118	9/116	18/115	6/116	4/116
	Total%	0.0	51.7	7.8	15.7	5.2	3.5
RT+IO	Total	0/208	140/200	60/202	86/200	27/204	20/204
	Total%	0.00	70.0	29.7	43.0	13.24	9.80

* The number of samples from which mycoplasmas were re-isolated is significantly lower in the group infected by mHAD3 than in that infected by RCL2

†Contaminated samples were withdrawn

TABLE 4. Phenotype of the mycoplasmas recovered after infection.

Infection group	Number of MG* positive samples / total	Number of samples analyzed	Number of samples presenting the phenotype of the inoculum (%)
Media	0/208	0	0
R_{low}	140/200	24	24 (100%)
R_{high}	60/202	29	27 (93%)
RCL2	86/200	62	61 (98.4%)
mHAD3	27/204	27	19 (70.4%)

*MG: *M. gallisepticum*

Figure Legends

FIG. 1. Analyses of mycoplasma populations recovered from mHAD3-infected chickens. **Panel A**: Total proteins of mycoplasma populations were separated by SDS-PAGE and stained with Coomassie blue. **Panel B**: Total DNA from mycoplasma populations analyzed in Panel A were subjected to a multiplex PCR assay and the resulting products specific of the Tn*4001* (tn) and of the house keeping EfTu gene (tuf) were detected by ethidium bromide staining after agarose gel electrophoresis. **Panel C**: Total DNA from mycoplasma populations (analyzed in Panel A) were subjected to a Long Range PCR assay using a forward primer located in the *crmA* gene and a reverse primer located in the Tn*4001* to amplify a 1.6 kb DNA which was detected by ethidium bromide staining after agarose gel electrophoresis. Mycoplasma populations were recovered from the trachea (T), the kidney (K) or the heart (H) of mHAD3-infected chickens as described in Materials and Methods. R_{low} and mHAD3 were used as controls. Numbers above the lanes correspond to the number that was assigned to each bird.

FIG. 2. Analyses of mycoplasma populations recovered from RCL2-infected chickens. **Panels A and C:** Total proteins of mycoplasma populations (A) or clones (C) were separated by SDS-PAGE and stained with Coomassie blue. **Panels B and D**: Total DNA from mycoplasma populations (B) or clones (D) analyzed in Panel A and C were subjected to a PCR assay to amplify the *gapA* region containing the RCL2 point mutation and the resulting products were digested by *MseI*. Restriction fragments were detected by ethidium bromide staining after agarose gel electrophoresis. Mycoplasma populations were recovered from the left air sac (AS), the trachea (T), the lung (L), or the kidney (K) of RCL2-infected chickens as described in Materials and Methods. Numbers above the lanes correspond to number that were assigned to each bird. RCL2 and RCL1 that are two isogenic variants derived from R_{low} that differ by (i) their HA phenotype, (ii) the expression of GapA and CrmA and (iii) a base substitution at the beginning of the gapA gene, were used as controls. Lanes 1 to 10 correspond to individual clones randomly selected from the mycoplasma population collected from the trachea of bird #9607. Asterisks point out restriction DNA fragments that are only detected in [HA-] variants lacking the GapA and CrmA products.

Fig. 1

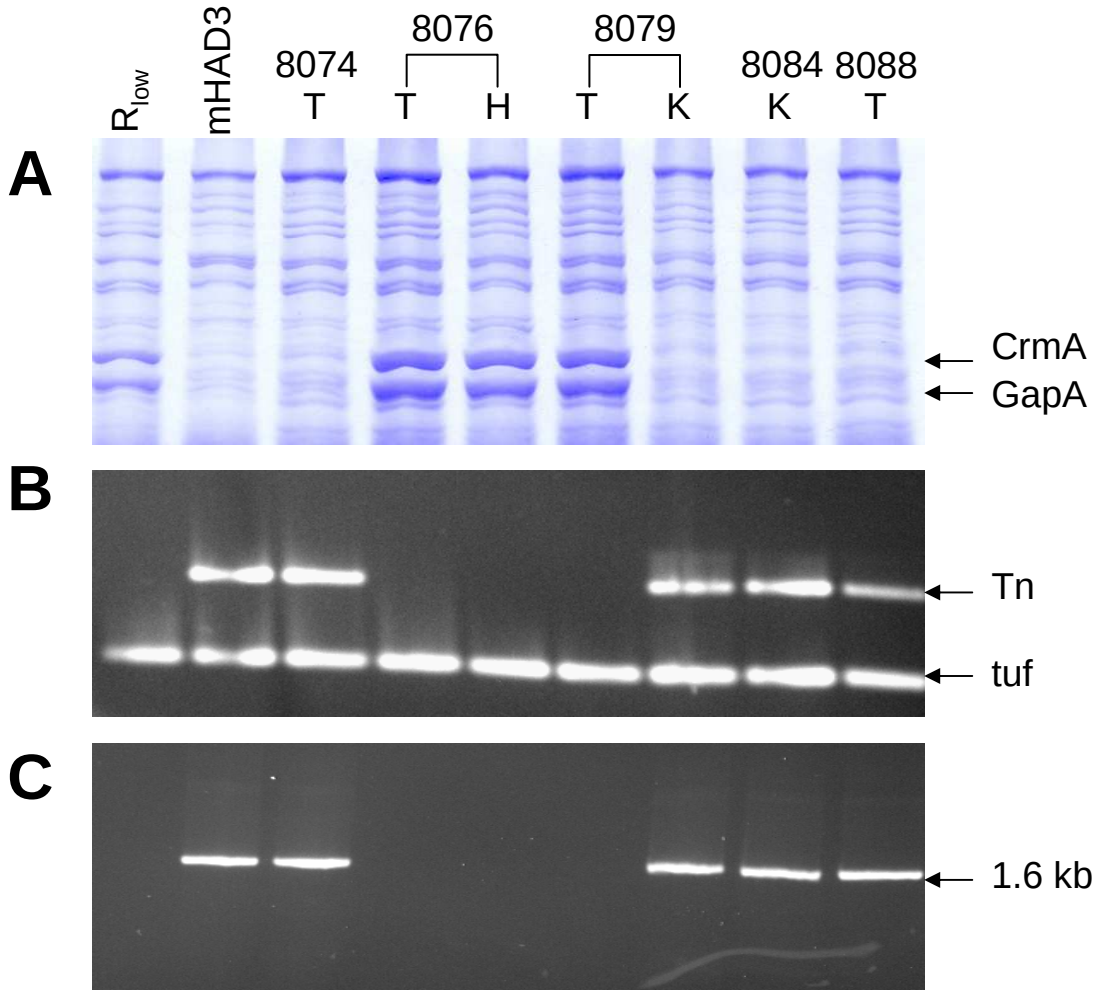
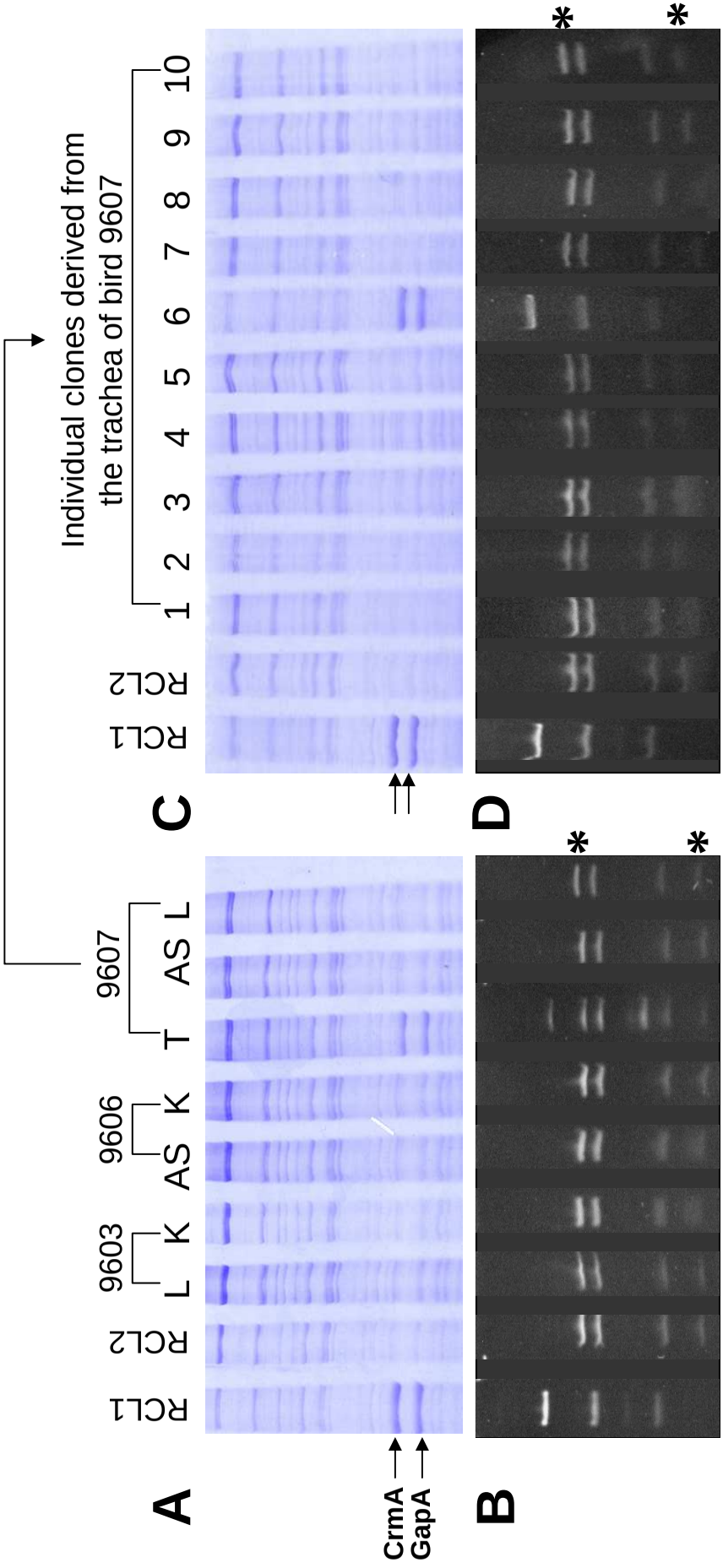

R low
mHAD3
8074
T
8076
T
H
8079
T
K
8084
K
8088
T
A
CrmA
GapA
B
Tn
tuf
C
1.6 kb

Fig. 2


A
B
C
D
CrmA
GapA
RCL1
RCL2
9603
L
K
9606
AS
K
9607
T
AS
L
Individual clones derived from
the trachea of bird 9607
1
2
3
4
5
6
7
8
9
10
RCL1
RCL2
*
*

5. MANUSCRIPTS IN PREPARATION

5.1. Targeted disruption of cytadherence-related genes of *Mycoplasma gallisepticum*

Collection of Data (see attached)

5.2. Preparation of antibodies against cytadherence-related proteins of *Mycoplasma gallisepticum*

Collection of Data (see attached)

5.1. Targeted disruption of cytadherence-related genes of *Mycoplasma gallisepticum* (Collection of Data)

Introduction

In contrast to the large wealth of data obtained from the analyses of mycoplasmal genome sequences (Dandekar et al., 2000; Fraser et al., 1995; Herrmann and Reiner, 1998; Himmelreich et al., 1996; Chambaud et al., 2001; Jaffe, Miyata, and Berg, 2004; Papazisi et al., 2003; Sasaki et al., 2002), there is still a general need of efficient genetic tools when it comes to study the functional genomics of mollicutes. The generation of mutants by gene disruption is a crucial step for the understanding of protein function and for uncovering their involvement in complex processes such as pathogenesis. However, gene disruption in mycoplasmas involving any method to transfer DNA into the bacteria, was not practical until 1987. It was Dybvig and Cassell (Dybvig and Cassell, 1987) who described for the first time the transformation of a member of the mollicutes. Using the streptococcal transposon Tn*916*, they succeeded to transform *Acholeplasma laidlawii* and *Mycoplasma pulmonis.* Since that time transposon-based strategies have been used to generate random insertion mutants also in other *Mycoplasma* species. A certain disadvantage of this method is the laborious and time-consuming screening for mutants carrying the transposon in a specific locus of the mycoplasma genome.

The targeted gene knock-out is an important strategy that can help to unravel the function of a particular protein in the organism. Recently, the targeted inactivation of a gene by site-directed homologous recombination has been described for *A. laidlawii* (Dybvig and Woodard, 1992), *M. gallisepticum* (MG) strain S6 (Markham et al., 2003), and *M. genitalium* (Dhandayuthapani et al., 2001; Dhandayuthapani, Rasmussen, and Baseman, 1999). In these studies suicide vectors carrying a resistance gene were used which are unable to replicate in the target organism, hence the drug-resistant transformants could be obtained only after successful integration of the plasmid into the chromosomal DNA. In *S. citri* (Duret et al., 1999) and *M. pulmonis* (Cordova et al., 2002), for which gene inactivation using non-replicating plasmids could never be obtained, vectors carrying sequences of the chromosomal origin of replication, *oriC*, have been successfully used to drive the homologous recombination. The aim of this study was to find out the prerequisite of homologous recombination and the construction of gene knock-outs in MG strain R in order to study the involvement of several genes in cytadherence.

Material and Methods

Bacterial strains and growth conditions

Mycoplasma gallisepticum (MG) RCL1 (Winner et al., 2003) selected from strain R_{low} (Lin and Kleven, 1984) was grown at 37℃ in a modified H ayflick medium (Wise and Watson, 1983) containing 500 U/ml of penicillin. Mycoplasma tranformants were selected on Hayflick agar plates containing 4 µg/ml of tetracycline (Hayflick/Tet). *Escherichia coli* DH10β(Invitrogen, Paisly, UK) and GM119 (NEB, Frankfurt am Main, Germany) were grown at 37℃ in LB (10 g/l tryptone, 5 g/l NaCl, 5 g/l yeast extract, pH 7) broth. Transformants were selected on LB agar plates containing 100 µg/ml of ampicillin (LB/Amp) or both, 50 µg/ml Amp and 7 µg/ml Tet (LB/Amp,Tet).

DNA Isolation, Manipulations, Southern Blotting

Recombinant plasmid DNA was isolated from *E. coli* cultures using PeqLab E.Z.N.A. Plasmid Mini Kit (PEQLAB Biotechnologie GMBH, Erlangen, Germany) according to the manufacturer's protocol. Mycoplasma genomic DNA was obtained by standard methods as described previously (Winner et al., 2003). Restriction endonucleases (Promega, Mannheim, Germany) were used in accordance to the manufacturer´s instructions. Neutral Southern blotting was performed using nitrocellulose membrane (Amersham, GE Healthcare Europe GmbH, Vienna, Austria) according to the manufacturer's instructions. Southern blot hybridizations using digoxigenin (DIG)-labelled probes corresponding to either a part of the *oriC* region of MG, or partial *gapA* and *tetM* sequences were performed according to the Genius System User's Guide for Membrane Hybridization, Version 3.0 (Roche Molecular Biochemicals, Mannheim, Germany).

PCR

Long-range PCR (LR-PCR) using the Expand™ Long Template PCR System (Roche, Vienna, Austria) was used to amplify selected genes from 300 ng of chromosomal DNA of RCL1. For this purpose, primers listed in Table 1 were used. The amplification mixture consisted of the appropriate primer pair at a final concentration of 1.5 mM each in 1x buffer (System 1) and 0.35 mM dNTPs. The following conditions for an amplification were used: a denaturation step at 94℃ for 2 min followed by 10 cycles of 30 s denaturation at 94℃, 30 s annealing at 56℃ and 4 min extension at 68℃; then followed 20 cycles of 30 s denaturation at 94℃, 30 s annealing at 56℃, 4 min extension at 68℃ with a cycle elongation for 20 s per cycle, and a final extension cycle for 7 min at 68°C. The tetracycline resistance gene *tetM* was obtained by amplification of the full-length gene using plasmid pMM20-1 (Chopra-Dewasthaly et al., 2005) as the source and the primers McTetfor and McTetrev at the same conditions as described above.

Table 1. Oligonucleotide primers used in this study. Highlighted sequences (bold) represent restriction sites.

Designation	Sequence	Restriction Site(s)
Ori1	CTTTGTTGT**CGATCG**TAATATAAAG	*Pvu*I
Ori2	TATTAATAGAAAAA**CGATCG**TCTATAAAC	*Pvu*I
Ori3	TGATGC**CGATCG**CATTAGGTTTTTTC	*Pvu*I
McTetfor	GATTT**GATATCAGATCT**GAACGGGAGTAATTGGAAG	*Eco*RV, *Bgl*II
McTetrev	CTTAT**AGATCTGATATC**CATATTTATATAACAACAT	*Eco*RV, *Bgl*II
TetF	CATGTGGAGATAGAAC	
TetR	GATATTCCTGTGGCGC	
Mgc2_1	ATGGATTCAAGTTGGTAATTGTTCAATC	
Mgc2_2	TAGGATCCTAATGCACCTGGGTTGG	
GapA10	ATATTA**CTCGAG**GAAATGAATTCACAAGGCCAATC	*Xho*I
GapA11	ATTTAA**CTCGAG**GAAGTCATTGGTTGCTCTAGAACG	*Xho*I
CrmA3	ATCGTTCTAGAGCAACCAATGAC	
CrmA4	TCATTTCTAGACCGTTTGGATTTG	
CrmB3	TAATGTTAAAAGCTCACATCAAAAG	
CrmB4	CTAATTAGTATTTATTTTCACTAATC	
CrmC1	CAATGATTAAAAAACAACTAAGAC	
CrmC2	CTATTGATTGGGTTGTTTAGTTC	
H2f	ATTCACAAGGCCAATCTAATC	
H2r	TTGATCTTGAGTAGCTTCTAC	

The presence of the *tetM* gene in the transformants was confirmed by PCR using primers TetF and TetR as described elsewhere (Chopra-Dewasthaly et al., 2005). Briefly, a denaturation step was performed at 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 40 s, annealing at 54℃ for 40 s, extensio n at 72℃ for 40 s, and a final extension step at 72℃ for 5 min.

A fragment of the *oriC* origin of MG was amplified using genomic DNA of strain RCL1 and primers Ori1 and Ori2. The conditions for the PCR amplification were as followed: a denaturation step at 95℃ for 2 min, followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and extension at 72℃ fo r 1 min, and a final extension step at 72℃ for 5 min.

Cloning procedure – basic vectors, disruptor vectors

To confirm that only the selected gene fragments were amplified by the PCR process, samples of the PCR products were digested using appropriate restriction endonucleases, namely *Pvu*II and *EcoR*I for "*mgc2*", *Pvu*II for "*gapA*", *Eco*RI and *Hind*III for "*crmA*", *Hind*III for "*crmB*", *Eco*RV and *Hind*III for "*crmC*", and *Hind*III and *Sac*I for "*tetM*". PCR products were

purified by agarose gel electrophoresis using the QiaQuick Gel Extraction Kit (QIAGEN, Vienna, Austria) according to the manufacturer's instructions and eluted by ddH_2O. Purified fragments were subcloned into the pGEM-T Easy Vector (Promega, Mannheim, Germany) following the manufacturer´s instruction manual for T/A cloning or into the vector pGEM-5Zf(+) (Promega, Mannheim, Germany) via the newly introduced restriction sites (details see below). The resulting plasmids were transformed into the *E. coli* strain DH10β (Invitrogen, Paisly, UK) and selected on LB/Amp or LB/Amp,Tet agar plates.

In case of using the Klenow fragment of DNA polymerase I (Promega, Mannheim, Germany) to fill in 5´ protruding ends, the desalted digestion mixture (pCC1 digested by *Eco*RI or pISMTet digested by *Bam*HI/*Sma*I) was incubated with 7.5 U of the enzyme in a Klenow buffer containing 40 μM of dNTPs and 20 μg/ml BSA. After formation of the blunt ends, the insert and vector, both purified by agarose gel electrophoresis, were ligated (vector:insert ratio 1:3) using 1 U of T4 ligase (Roche Vienna, Austria) for 16 h at 4℃. After electroporation into *E. coli* DH10β, the resulting transformants were checked for the orientation of the inserted genes by performing a double-digest of the plasmids with *Hin*DIII/*Pst*I.

Those plasmids bearing the target genes in both, positive and negative, orientations in respect to the *lacPO* promoter were chosen for further use. After confirming the orientation by sequencing the region around the insertion site, the target genes were disrupted by inserting a tetR cassette consisting of the gene *tetM* under its native promoter *tetPO* in the middle of the target gene. The tetracycline resistance gene *tetM* was obtained by amplification of the full-length gene using plasmid pMM20-1 (Chopra-Dewasthaly et al., 2005) Finally, disruptor plasmids bearing the tetR cassette in both orientations were produced. For purification of recombinant plasmid DNA the Plasmid E.Z.N.A. Minikit I (PEQLAB Biotechnologie GmbH, Erlangen, Germany) was applied. DNA sequencing was performed at VBC Genomics BIOSCIENCE Research GmbH, Vienna, Austria.

Transformation of mycoplasmas

Mycoplasmas were transformed as described previously (Winner et al., 2003). Briefly, a frozen stock of MG RCL1 was cultivated overnight in 1 ml of liquid medium at 37℃ and then transferred into 4 ml of fresh prewarmed medium; after 24 and 36 h 1 ml aliquots were used to inoculate 9 ml of fresh medium and all samples were cultivated along overnight. Afterwards, the two overnight cultures were combined, centrifuged (9,400 g, 10 min, 4℃) and washed three times in 20 ml, 10 ml and 1 ml of ice-cold electroporation buffer (8 mM HEPES pH 7.4; 272 mM sucrose). The final 1 ml suspension of electrocompetent MG cells was divided into 100 μl aliquots (approx 10^8-10^9 cells per 100 μl). Aliquots were mixed with 2-10 μg of plasmid DNA, incubated on ice for 1-10 min and subjected to electroporation (2.5 kV, 100 Ω, 25μF) in a Bio-Rad Gene-Pulser^{R}II Apparatus using 1 mm gap electroporation

cuvettes. After electroporation, cells were immediately resuspended in 1 ml of chilled Hayflick medium and incubated on ice for 10 min, before placing them at 37℃ for 30-90 min. After regeneration, 100 µl aliquots were plated on Hayflick agar plates at a 10^{-6} dilution to determine the numbers of total CFU. The remaining transformed cells were plated on Hayflick/Tet agar, and incubated at 37℃ in the dar k. The plates were examined 10 to 14 days later using a stereomicroscope. Single colonies were picked and seeded in 1 ml of Hayflick/Tet broth for further analysis.

Results

This study attempted to develop a system with which to inactivate a specific gene of MG by homologous recombination. This process involves the alignment of identical sequences, a crossover between the aligned DNA strands, and breaking and repair of the DNA resulting in an exchange of material between the strands. Such a targeted gene disruption has been described for many bacterial species, although for MG done only by Markham and co-workers (Markham et al., 2003) in which the authors succeeded to inactivate specific gene of MG S6 by homologous recombination.

In order to target the sequences of genes in the gene cluster around the cytadherence gene *gapA*, namely *mgc2*, *gapA*, *crmA*, *crmB* and *crmC*, it was decided to create plasmids in which the target gene would be disrupted by insertion of the tetracycline resistance cassette, tetR, consisting of the *tetM* gene under its native promoter *tetPO*. In general, the target gene fragment was amplified by PCR, purified by agarose gel electrophoresis and inserted via T/A cloning into the pGEM-T Easy vector. This procedure takes advantage from the fact that the Taq DNA polymerase used to create the PCR product adds a single adenine nucleotide to the 3´ end of the otherwise blunt end PCR fragment which perfectly fits to the pGEM-T Easy vector that has been linearized with *Eco*RV and equipped with thymidine overhangs at the 3´ termini. This cloning routine results in plasmids carrying the insert in both orientations with respect to the *lacPO* promoter of the pGEM-T Easy vector. In the next step, the tetR cassette was inserted within the target gene by using suitable restriction sites in both orientations with respect to the *lacPO*. The names of resulting plasmids were designated in such a way that the gene or insert (tetR cassette) running in the same (positive) or opposite (negative) orientation as *lacPO* was given even or odd number, recpectively (for the scheme see Appendix 1).

Construction of the disruptor plasmids

pTet

The gene supposed to disrupt the genomic target sequence and to be used as a marker in the subsequent selection process is one of the two resistance genes most commonly used in mycoplasma recombinant DNA work, the *tetM* gene of *Steptococcus faecalis* (Franke and

Clewell, 1981). In order to obtain the gene with its upstream promoter *tetPO* a LR-PCR was performed using primers McTetfor and McTetRev (Table 1) and plasmid pMM20-1 (Chopra-Dewasthaly et al., 2005) as a template. A PCR product of the expected size of 2.38 kb was obtained and subjected to a control restriction digest using enzymes *Hin*dIII and *Sac*I. As expected, digestion with *Hin*dIII resulted in fragments of 1.9 kb and 0.48 kb. Similarly, fragments of correct sizes (1.8 kb and 0.58 kb) were obtained with *Sac*I, thus confirming the specificity of the PCR reaction. At the same time the PCR product was subcloned into the pGEM-T Easy vector by T/A cloning. Two of the resulting plasmids carrying the *tetM* gene in different orientations with respect to the *lacPO* promoter of pGEM-T Easy were designated pTet1 and pTet2 (for the plasmid map see Appendix 2). To obtain an insert usable for the creation of the disruptor plasmids, the plasmid pTet2 was digested by *Eco*RV or *Bgl*II creating fragments with either blunt or sticky ends. These fragments of 2.37 kb containing the *tetM* gene under control of its native promoter were purified by agarose gel electrophoresis and stored for further use.

pISM-TetII

In order to prove that the tetR cassette used to disrupt the target genes is indeed conferring resistance to tetracycline in MG, plasmid pISM-TetII was constructed. The pISMTet plasmid (kindly provided by W. Jechlinger, unpublished data) was used as a donor for the tetR cassette. This plasmid contains the Tn*4001*mod transposon with its gentamicin-resistance conferring *aadC-aphD* gene (Knudtson and Minion, 1993) and was modified by inserting a *tetM* gene with its *tetPO* promoter in the same orientation to extend the applicability of this transposon. Plasmid pISMTet was digested by *Bam*HI and *Sma*I in order to delete the 2.8 kb fragment containing the original tetR cassette. Incubation with the Klenow fragment of DNA polymerase I made the DNA ends blunt and the vector was ligated with the *Eco*RV-digested 2.37 kb fragment of pTet2 containing the shorter version of the tetR cassette. The resulting transformants were screened for the plasmids carrying the insert in the same direction of the gentamicin resistance gene to resemble the situation of the original pISMTet.

Mgc2

To create a plasmid for disrupting the *mgc2* gene of MG, plasmid pMC2 harbouring the full length *mgc2* gene was used. Genomic DNA of MG strain RCL1 was used as a template for LR-PCR together with primers mgc2_1 and mgc2_2 (Table 1) to amplify a sequence of 1.8 kb, covering the entire coding sequence for the *mgc2* structural gene, and upstream and downstream flanking sequences. The upstream sequence of about 0.5 kb contained the 3´ end of the structural gene *licA* and a short 58 bp intragenic region. The downstream sequence of about 0.4 kb consisted of the 156 bp *mgc2-gapA* intragenic region and 5´ fragment of a *gapA* structural gene.

The PCR product was subcloned into the pGEM-T Easy vector, and the resulting recombinant plasmids carrying the insert in different orientations with respect to the *lacPO* promoter were named pMC1 and pMC2. The further cloning required the plasmids to be transformed into the methylase-negative *E. coli* strain GM119 (*dam*$^-$, *dcm*$^-$). Subsequently, plasmid DNA was cut by *Bsa*BI (NEB) and ligated with the 2.37 kb pTet2/*Eco*RV fragment resulting in the fission of the *mgc2* sequence into a 0.27 kb 5´ box and a 0.44 kb 3´ box. Recombinant disruptor plasmids were named pDMC1-1, pDMC1-2, pDMC2-1 and pDMC2-2 (see plasmid maps in Appendix 2).

GapA

A disruption plasmid targeting the major cytadherence gene *gapA* of MG was constructed as followed: Primers GapA10 and GapA11 (Table 1) were used to amplify a fragment of 2.75 kb covering an internal part of the *gapA* gene. The PCR product was subjected to a control restriction digest using enzyme *Pvu*II resulting in fragments of 1.64 kb and 1.11 kb, thus confirming the specificity of the PCR reaction. The DNA was then digested by *Xho*I and subsequently ligated into the vector pGEM-5Zf(+) previously linearized by *Sal*I. The digestion of the resulting plasmids pGA1 and pGA2 with *Hinc*II resulted in 5.35 kb fragment dividing the *gapA* sequence into 0.75 kb box and 2.62 kb box. In between the 2.37 kb pTet2/*Eco*RV fragment (see above) was cloned. Resulting disruptor plasmids were designated pDGA1-1, pDGA1-2 and pDGA2-1, pDGA2-2, respectively (see plasmid maps in Appendix 2).

CrmA

The construction of the *crmA* disruptor plasmid started with the amplification of a great part of the *crmA* gene of MG RCL1. Primer CrmA3, located 550 bp upstream of the *crmA* start codon to cover up a hypothetical promoter, and primer CrmA4 (Table 1), located within the 3´ region of the *crmA* coding sequence, were used to amplify a 2.56 kb fragment coding for most of the 3.2 kb *crmA* gene. The PCR product was subjected to a control restriction digest using enzymes *Eco*RI and *Hin*dIII. As expected, digestion with *Eco*RI resulted in fragments of 1.55 kb and 1 kb. Similarly, fragments of correct sizes (1.15 kb, 0.75 kb and 0.67 kb) were obtained with *Hin*dIII, thus confirming the specificity of the PCR reaction. The PCR product was subcloned via T/A cloning into the pGEM-T Easy vector yielding plasmids pCA1 and pCA2 differing in the orientation of the insert with respect of the *lacPO* promoter. The plasmids were digested with *Bgl*II cutting inside the *crmA* gene (see plasmid map, Appendix 2) releasing a 0.37 kb fragment and thereby dividing the *crmA* gene into a 0.3 kb 5´ box and a 1.36 kb 3´ box. The 5.22 kb DNA-fragment of the *Bgl*II-digested pCA1 or pCA2 plasmid, respectively, was ligated with the 2.37 kb fragment of the *Bgl*II-digested pTet2 plasmid carrying the tetR cassette. The resulting disruptor plasmids were named pDCA1-1, pDCA1-2, pDCA2-1 and pDCA2-2.

CrmB

To amplify the *crmB* gene, primer pair CrmB3 and CrmB4 (Table 1) was used in a PCR reaction generating a fragment of 2.76 kb, which covers the complete *crmB* gene sequence. A control restriction digest of the PCR product using *Hin*dIII resulted in the expected fragments of 1.55 kb and 1.23 kb. The desalted amplicon was ligated with the linear pGEM-T Easy vector resulting in plasmids pCB1 and pCB2 differing in the orientation of the insert with respect to the *lacPO* promoter. Digestion of the plasmids pCB1 and pCB2, respectively, by *Bgl*II led to a loss of 0.44 kb fragment and thereby dividing the *crmB* gene into a 1.8 kb 5´ box and a 0.5 kb 3´ box. In order to create knock-out vectors for the *crmB* coding sequence, the 2.37 kb *Bgl*II-fragment of the pTet2 ligated with the 5.32 kb *Bgl*II fragments of plasmids pCB1 and pCB2. The resulting disruptor plasmids were designated pDCB1-1, pDCB1-2, pDCB2-1, and pDCB2-2 (for plasmid maps, see Appendix 2).

CrmC

The entire *crmC* gene of MG strain RCL1 was amplified by using primers CrmC1 and CrmC2 (Table 1) yielding an amplicon of 2.6 kb. The PCR product was subjected to a control restriction digest using enzymes *Eco*RV and *Hind*III. Digestion with *Eco*RV resulted in fragments of 1.4 kb and 1.2 kb. Similarly, fragments of correct sizes (1.63 kb and 0.95 kb) were obtained with *Hind*III, thus confirming the specificity of the PCR reaction. The purified amplicon was subcloned into the pGEM-T Easy resulting, surprisingly, in recombinant plasmids carrying the insert exclusively oriented against the transcription direction of the *lacPO* promoter in all transformants screened. Hence, this plasmid was named pCC1. To obtain the *crmC* in the same orientation like the *lacPO*, another cloning strategy was followed: *Eco*RI was used to excise the *crmC* sequence from plasmid pCC1. Purified fragments of the *crmC´* insert and of the vector backbone were subjected to a fill-in reaction using the Klenow fragment of DNA polymerase I and then religated to force a frameshift of the *lacZ´* part. A selected recombinant plasmid with the *crmC* in the same orientation like the *lacPO* was designated pCC2.

To finally disrupt the *crmC* gene, the *Eco*RV-linearized plasmids pCC1 and pCC2 were ligated with the 2.37 kb *Eco*RV fragment of the pTet2 harbouring the tetR cassette. The resulting disruptor plasmids were denominated pDCC1-1, pDCC1-2, pDCC2-1, and pDCC2-2 (for plasmid maps see Appendix 2).

Transformation of Mycoplasma gallisepticum

Transformation with suicide plasmids

Before transformation with *tetM*-containing suicide disruptor plasmids, the sensitivity of MG strain RCL1 to the antibiotic Tet was assessed. A Teț concentration of 4 μg/ml was found to completely suppress the growth of MG RCL1 both on plates or in liquid broth. This result is in

accordance with other studies, where tetracycline was used as a selection marker at the same concentration (Dybvig, French, and Voelker, 2000; Markham et al., 2003)

Several preliminary transformation experiments were conducted using the tetracycline-resistance conferring plasmid pISMTet, which was also used as a transformation control throughout all following experiments. This plasmid is based on pISM2062 (Knudtson and Minion, 1993), with a *tetM* resistance gene inserted into the *Bam*HI/*Sma*I-site of transposon Tn*4001*mod in the same orientation as the gentamicin resistance gene. The best transformation frequencies were obtained with 6 µg of pISMTet, yielding in average $5x10^{-6}$ tet-resistant colonies per µg DNA. Similarly, the construct pUCTnTet was tested (kindly provided by W. Jechlinger, unpublished data). This construct is based on the pUC18 backbone and contains Tn*4001*, whereas the original gentamicin resistance gene was replaced by the tetracycline resistance gene *tetM*. The transformation with the pUCTnTet gave results comparable to the pISMTet. The morphology of transformed colonies grown on Hayflicks/Tet plates resembled that of nontransformed mycoplasmas (see below). Also, the plasmid pISM-TetII was tested to confirm that the tetR cassette used for creation of the disruption plasmids is conferring resistance to tetracycline in MG. The transformation efficiencies were in average $5.5x10^{-5}$ per µg DNA. The transformants grown on Hayflick/Tet plates formed colonies with the typical "fried-egg" character of MG. The presence of the *tetM* gene in the colonies was confirmed by PCR as described previously (Chopra-Dewasthaly et al., 2005).

Several of the suicide constructs (pDGA1-1, pDGA2-2, pDCB2-2, pDCC1-1 and pDCC1-2) described above containing genes of the "mgc region" disrupted by the tetracycline resistance cassette tetR were used in a number of independent transformations. A large number of MG colonies were observed on selective plates after transformation even when no DNA was used (ddH_2O instead) for transformation. Likewise, such colonies were observable on the plates when plasmid DNA was present in the transformation mixture as well. The morphology of these colonies was different from non-transformed mycoplasma colonies, which are smooth, circular and may have a dense central area ("fried-egg appearance"). Whereas the transparent colour and size (0.2-0.3 mm in diameter) were as expected, the characteristic shape was not seen and no centre of the colony could be detected. Moreover, these colonies failed to grow further in liquid Hayflick/Tet broth. To avoid growth of such colonies, transformed cultures were incubated for 2.5 hour in a non-selective broth, then were brought up to 5 ml of medium with Tet at 4 µg/ml and grown overnight in the presence of the antibiotic. Plating was performed either directly or the cultures were harvested and cells were resuspended in a suitable amount of fresh selective broth. Unfortunately, no transformant showing the characteristic mycoplasma morphology with the ability to grow further onto the agar plate was found during all transformations.

Site-directed mutagenesis via homologous recombination in MG was described only in a single report in which strain S6 was mutagenized via PEG-mediated transformation with a suicide vector carrying a *tetM*-disrupted *p47* gene (Markham et al., 2003). From the two transformants that came out in total, one was tested positive for site-specific integration in the *p47* gene. Although the author did not disclose the number of independent transformations, it seems that the frequency of integration might be rather low. Our attempts to reproduce the results of the above mentioned study have not been successful. When plasmid *tetM*/*p47*/pGEM-T was used In our laboratory, transformation of MG strain R failed, too. Although it might be conceivable that the different outcomes of Markham´s and our transformations are due to the different MG strain used in the study, other reasons cannot be ruled out.

Transformation with OriC- modified disruptor plasmids

As the effort of homologous recombination in MG using suicide vectors was not successful, it was decided to upgrade the disruptor plasmids by adding a fragment of MG´s origin of replication. The idea behind was not to create a replicative plasmid as this might not lead to integrate any sequence into the genome, but rather to prolong the residence of the disruptor plasmid so that the neccessary homologous recombination events acquire more time. The fragment was selected based on the publication of Papazisi and co-workers (Papazisi et al., 2003), where the authors speculated about the MG origin of replication on the basis of AT-rich sequences, containing AT-repeats and DnaA boxes, which are – at least in *E. coli* – necessary to bind the DnaA protein known to be the initiator of replication. It was assumed that a plasmid containing a part of the putative MG *oriC* could longer exist in the cell than a suicide vector. Thereofore two PCR approaches were designed. However, despite multiple attempts, no PCR reaction using primers Ori1 and Ori3 (Table 1) with RCL1 genomic DNA of as template did result in the expected 1.15 kb product. However, a smaller PCR product of 0.42 kb could be amplified using the primer pair Ori1 and Ori2 (Table 1). The PCR product was subcloned via T/A cloning into the pGEM-T Easy vector resulting in plasmid pGEM-oriC. This plasmid was then digested by *Sac*II and *Fsp*I and the 2 kb fragment carrying the MG-*oriC* as well as the plasmid´s *ColE1* origin was ligated with the 4.9 kb fragment of pDGA1-1 (described above) obtained by digestion with *Sac*II and *Bsr*BI containing the *gapA* gene disrupted by *tetM* (Fig. 1). The resulting final construct, pDGA-oriC (6.9 kb), was used for transformation of MG RCL1.

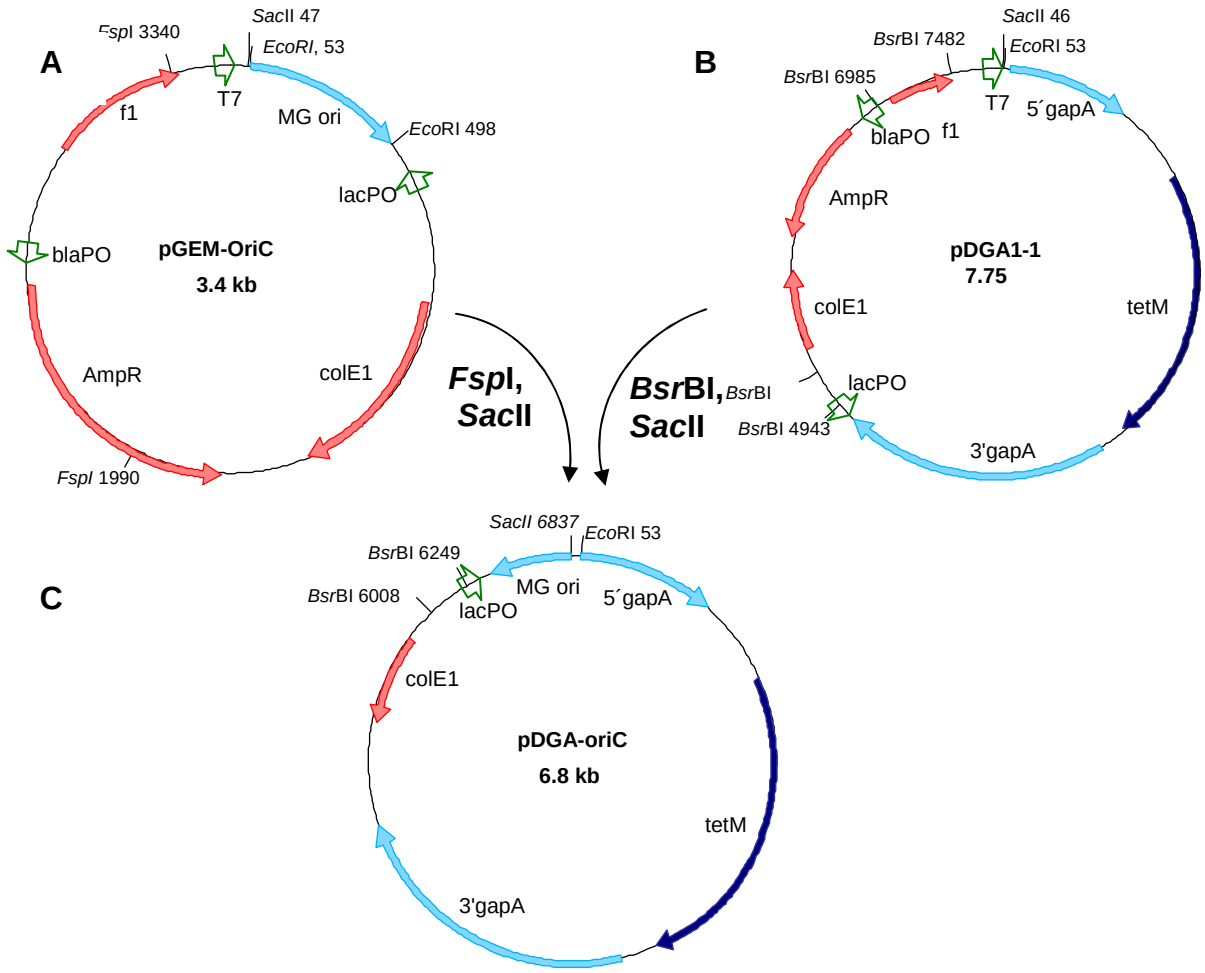


Fig. 1. Scheme of cloning of the pDGA-oriC´ vector. (A) Plasmid pGEM-oriC carrying a small fragment of MG *oriC* was digested by *Fsp*I and *Sac*II. (B) Plasmid pDGA1-1 carrying a part of the MG *gapA* gene disrupted by *tetM* was digested by *Bsr*BI and *Sac*II. (C) The 2 kb fragment of pGEM-oriC was ligated with the 4.8 kb fragment of pDGA1-1 generating plasmid pDGA-oriC.

When MG strain R_{low} was electroporated with plasmid pDGA-oriC, transformation frequencies were rather low resulting in 1-10 transformants per 10^8 competent cells. Twenty tetracycline-resistant colonies arising from the transformation with pDGA-oriC were isolated and propagated in Hayflick/Tet medium. Genomic DNA from strain R_{low} and the pDGA-oriC transformants was prepared and digested by *Eco*RV. The digests were analysed by Southern blot technology and probed with a specifically Dig-labelled internal fragment of the *tetM* gene obtained by PCR using primers TetF and TetR (Table 1). As expected, no signal was observed for the *Eco*RV-digested DNA of R_{low}. Surprisingly, a band of approx. 9.1 kb was detected in all pDGA-oriC transformants (data not shown). The size of the fragment did not correspond to the 10.8 kb of the expected *Eco*RV-fragment harbouring the *gapA* gene. This suggested that the recombination event took place in another genomic region than around *gapA*. Since the plasmid used for transformation carried also another homologous MG sequence, a fragment of the *oriC´*region, we thought to investigate whether the recombination took place in this locus.

Therefore the genomic DNA of R_{low} or R_{high} and pDGA-oriC transformants was digested by *Cla*I and probed with a specifically Dig-labelled *oriC* probe, obtained by PCR using primers Ori1 and Ori2 (Table 1). As a result, a band of 5.8 kb, corresponding to the native *Cla*I-fragment containing the *oriC* region of MG, was detected in strain R_{low} only. In contrast, a band of approx. 12 kb was detected in all the transformants, strongly indicating that a recombination event occurred within the *oriC* region, therefore increasing the size of the oriC-fragment for the size of the plasmid (Fig. 2).

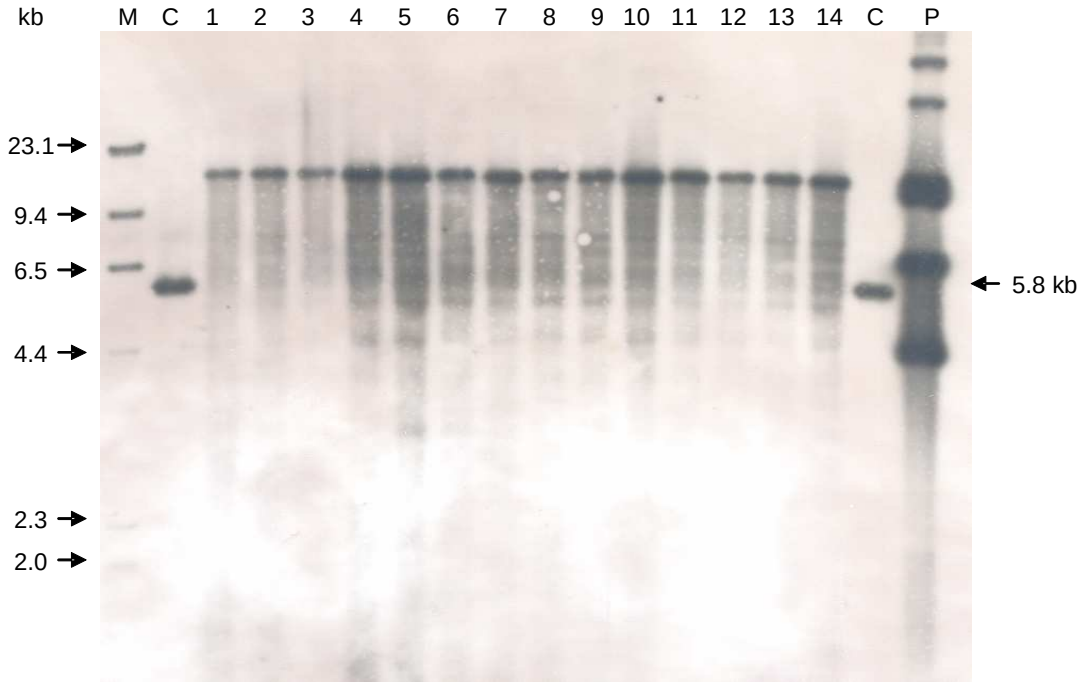


Fig. 2. Southern blot using Dig-oriC probe. The genomic DNA of MG R_{low} or R_{high} and pDGA-oriC transformants was digested by *Cla*I and probed with a Dig-labelled *oriC* probe. A band of 5.8 kb corresponding to the fragment containing the native *oriC* of MG was detected in strain R_{high}. In all the transformants, a band of 12 kb was detected. (M) Molecular size marker, (C) DNA of MG R_{high}, (lanes 1-14) DNA of pDGA-oriC´ transformants, (P) plasmid pDGA-OriC´

This finding was further confirmed by an additional Southern blot using a Dig-labelled *gapA* probe, obtained by PCR using primers H2f and H2r (Table 1). As expected, two bands of about 14 kb and 12 kb, representing the genomic *gapA* as well as the *gapA* being part of the pDGA-oriC vector were detected in all the tested transformants (data not shown). To our surprise, no recombination event took place in the *gapA* region and no transformants were found having an extrachromosomally replicating plasmid. Moreover, in all transformants analyzed no deletions occurred in the plasmid DNA, which is integrated in the very same location for every colony tested in its entire length, presumably by a single cross-over recombination event.

In this study we have verified that the disruption of a given target gene by means of homologous recombination is an extremely rare event in MG, although it might be still feasible as the addition of a small fragment of 0.4 kb directed the integration of a 7 kb plasmid into the genomic DNA in every single transformant analyzed. To circumvent the limitations of the existing suicide disruption vectors it might be sufficient to modulate the length of the homologous target sequences. The full length gene was subcloned order to facilitate the expected homologous recombination events. Contrary to our first assumptions, we finally observed that for homologous recombination to take place also a shorter sequence is sufficient. Further attention will be focused on the targeted disruption of genes in the "mgc cluster" to obtain mutants for further elucidation of the complex cytadherence process.

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5.2. Preparation of antibodies against cytadherence-related proteins of *Mycoplasma gallisepticum*
(Collection of Data)

Introduction

The investigation of MG cytadherence requires the identification of molecular players directly or indirectly involved in this process and their localization within the cell. Determination of mycoplasma genome sequences (Dandekar et al., 2000; Fraser et al., 1995; Herrmann and Reiner, 1998; Himmelreich et al., 1996; Chambaud et al., 2001; Jaffe, Miyata, and Berg, 2004; Sasaki et al., 2002) together with the deciphering of the total genome of one MG strain (Papazisi et al., 2003) have provided the basis to predict gene products potentially involved in cytadherence-related processes, e. g. by comparing the genome of MG with other mycoplasms allowing the identification of functional homologues involved in various processes including cytadherence. The identified orthologues are good candidates for the development of antisera. The antibodies can then be used for determination of gene expression in various types of assays e. g. colony immunoblotting, Western blots, nearest-neighbour analysis, immunostaining, immuno-fluorescence microscopy and confocal laser-scanning microscopy.

For the generation of antibodies specific for MG proteins mainly two methods of choice can be followed: either the preparation and purification of MG proteins from a polyacrylamide (PAA) gel that has been used to separate proteins contained in the cell lysate of a freshly grown MG culture or the recombinant expression of specific MG genes in e. g. *E. coli* with the subsequent purification of the recombinant protein. The use of epitope tags in recombinant DNA techniques simplifies the purification of recombinant proteins by affinity chromatography. An epitope tag is a small peptide sequence, typically 3-14 amino acids in length, and encoded by the expression vector either upstream or downstream of the cloning site for the gene of interest. Protein synthesis finally produces the target protein with the epitope tag as a hybrid protein, whereas the epitope tags do not generally affect normal protein folding or function. Another important advantage is the availability of antibodies specific for the tag that allow the detection of the fusion protein.

The most widely used tags for purifying proteins expressed in bacteria, yeast, insect and mammalian systems are the polyHis-tags allowing the purification under both, native or denaturing, conditions and the Maltose Binding Protein (MBP), allowing the easy purification of stable fusion products from bacterial extracts under mild conditions.

Though, expression of mycoplasma sequences in *E. coli* is often hindered by an unusual mycoplasmal codon usage pattern: the UGA codon is utilized for tryptophan by all mycoplasmas resulting in the truncation of recombinant MG-specific proteins if expressed in

E. coli that uses this codon as a stop codon. This premature termination often makes it impossible to obtain MG gene products in the proper conformation or with the proper functionality in *E. coli* expression systems.

The aim of this study was to generate polyclonal antisera against proteins encoded within and surrounding the cytadherence-associated "mgc locus", namely *mgc2*, *crmB* and *crmC* using recombinant DNA technology. To circumvent the translation barrier, each of the tryptophan-encoding TGA codons that were contained at least once in each of the genes was mutagenized to the tryptophan encoding TGG triplet that is legible for *E. coli*. The modified sequences were then subcloned downstream of the N-terminal poly-His tag of plasmid pRSET or downstream of the MBP of vector pMal-c2 to enable the purification of the expressed proteins, which were subsequently used for the immunization of rabbits.

Material and Methods

Bacterial strains, growth conditions and media

Mycoplasma gallisepticum (MG) strains R_{low}, R_{high} (Lin and Kleven, 1984), RCL1, RCL2, RCL3, RCL4, mHAD3 (Winner et al., 2003), R_{high}A3 (picked as a single clone from strain R_{high}) and R_{high}GT5 (Papazisi et al., 2000) were cultivated at 37℃ in modified Hayflick medium (Wise and Watson, 1983) containing 500 U/ml of penicillin. *E. coli* strain DH10β (Invitrogen, Paisly, UK), SCS110 (NEB, Frankfurt am Main, Germany) and TB1 (NEB, Frankfurt am Main, Germany) were cultivated in LB broth (10 g/l tryptone, 5 g/l NaCl, 5 g/l yeast extract, pH 7) at 37℃ with vigorous shaking. Special strains of *E. coli* such as XL10-Gold (Stratagene, La Jolla, USA) were cultivated according to the manufacturer's instructions (LB, 10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract, pH 7). After transformation, the *E. coli* cells were stabilized in SOC medium (20 g/l tryptone, 5 g/l yeast extract, 10 ml 1M NaCl, 2.5 ml 1M KCl, 10 ml sterile 2M Mg++ (stock solution 1M $MgCl_2.6H_2O$ + 1M $MgS0_4.7H_2O$); 10 ml sterile 2M glucose, pH 7). For the production of the fusion protein His-Mgc2 superbroth (32 g/l tryptone, 20 g/l yeast extract, 5g/l NaCl) (Minion, VanDyk, and Smiley, 1995) was used. *E. coli* BL21(DE3)pLysStar(pRCC) were grown on SOB agar plates (20 g tryptone, 5 g yeast extract, 0.5 g NaCl; pH 7; 15 g agar, 10 ml sterile 2M Mg++ /stock solution 1M $MgCl_2.6H_2O$ + 1M $MgS0_4.7H_2O$/). In case of selective media, antibiotics were used at final concentrations of (i) ampicillin (Amp) 100 µg/ml, and (ii) chloramphenicol (Cam) 35 µg/ml.

DNA manipulation

Standard methods were used for DNA manipulations. For screening numbers of *E. coli* transformants crude plasmid extractions were performed as follows: After harvesting 1 ml of the bacterial culture, cells were collected by centrifugation (6,000 g) and the pellet was resuspended in 250 µl of the resuspension buffer (50 mM Tris-Cl, pH 8; 10 mM EDTA, pH 8; 100 µg/ml RNaseA). 300 µl of the lysis buffer (200 mM NaOH; 1% SDS (w/v)) was added

and mixed by inversion followed by addition of 300 µl of the neutralization buffer (3 M potassium acetate, pH 5.5). The solution was centrifuged (10 min, 10,000 g, 4℃) in order to remove bacterial proteins and chromosomal DNA. The supernatant was then mixed with 600 µl isopropanol and centrifuged (30 min, 16,000 g, 4℃). The pellet containing plasmid DNA was washed with 70% EtOH, air-dried, and resuspended in 15 µl ddH_2O. Other plasmid DNA extractions were performed by using the following commercial kits according to the manufacturers´ instructions: Plasmid Miniprep PeqLab Kit I (PEQLAB Biotechnologie GmbH, Erlangen, Germany), QiaQuick Gel Extraction Kit (QIAGEN, Vienna, Austria), Wizard Clean Up System and restriction endonucleases from Promega (Mannheim, Germany) or NEB (Frankfurt am Main, Germany). Electrotransformation of *E. coli* was performed according to standard procedure. Sequence analysis of DNA was performed at the VBC-Genomics BIOSCIENCE Research GmbH, Vienna, Austria.

PCR

In order to introduce restriction sites suitable for cloning into vector pRSET-B, the *mgc2* sequence of the plasmid pWMC was amplified using primers mgc2_3 and mgc2_4 (Table 1) and the following conditions: denaturation at 94℃ for 2 min, 30 cycles of denaturation at 94℃ for 30 s, annealing at 50℃ for 30 s, extensio n at 68℃ for 1 min, and a final extension step at 68℃ for 2 min.

Table 1. Oligonucleotide primers used in this study to enable cloning of DNA fragments into expression vectors.

Primer	Sequence	Restriction site
mgc2_3	ACGCAG**GAATTC**ATAACAATTATG	*Eco*RI
mgc2_4	TTTACA**AAGCTT**GTCTTATCTAGG	*Hin*dIII
B1F	AG**GAATTC**AATGCTAATTTTTCATC	*Eco*RI
B1R	AT**GTCGAC**TAATCATGTAATGAG	*Sal*I
B2F	AG**GAATTC**TATATTACTAATTTAGC	*Eco*RI
B2R	AT**GTCGAC**AAAGTTGTTGTTCAAG	*Sal*I
B3F	AG**GAATTC**CAACCTAATATTGATGC	*Eco*RI
B3R	AT**GTCGAC**TCAATTGTTCTGTAACTC	*Sal*I
B4F	AG**GAATTC**CAGAACAATGAAACGATC	*Eco*RI
B4R	AT**GTCGAC**TATTCACTAATCATTTTGG	*Sal*I

The highlighted sequences (bold letters) represent the newly introduced restriction sites

Fragments of the *crmB* gene were amplified using primer pairs B1F/B1R, B2F/B2R, B3F/B3R, B4F/B4R, B2F/B3R and B3F/B4R (Table 1) applying the following conditions: denaturation step at 93℃ for 2 min, 25 cycles of d enaturation at 93℃ for 1 min, annealing at 50℃ for 1 min, extension at 72℃ for 2 min, and a final extension step at 72℃ for 5 min.

Site-directed mutagenesis

A single-site mutagenesis was performed to correct the stretch of adenines in plasmid pRCB. This PCR-based mutagenesis employed 3 U *Pfu* polymerase (Promega, Mannheim, Germany) and primers CB5Afw and CB5Arev (Table 2) at a final concetration of 1.5 mM, each introducing restriction sites facilitating direct screening for the desired mutation, 300 ng of plasmids pRCB1 and pRCB2, and 0.2 mM dNTPs. The thermal cycling conditions implied a denaturing step at 94℃ for 1 min and 30 cycles o f denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and elongation at 68℃ for 12 min. A 19 µl of the PCR product was subjected to a ligation reaction using 1 U of T4 DNA ligase (Roche, Vienna, Austria). Butanol-precipitated ligated products were transformed by electroporation into *E. coli* DH10β (Invitrogen, Paisly, UK), which were plated on LB/Amp agar plates.

Table 2. Synthetic oligonucleotides primers used for site-directed mutagenesis.

Designation	Sequence	Position	Gene / Restriction site
mgc_tga	GAAAGATTACCTCCGAACC**A**TG**G**TTTTATCCAGTAGTGGG	172-210	*mgc2* / *Nco*I
cb_1tga	CTTTAGATTCGATTAGATG**G**AATGCTAATGCTAATTTTTC	185-224	*crmB* / *Bsm*I
cb-2tga	GATTCTAATTTTATCTCATTACCATG**G**TTACAATATATTAC	766-806	*CrmB* / *Nco*I
cb_3tga	CGTTTCGCTTAATTGCACC**A**TG**G**ACAACAACTTTAGCC	1400-1437	*crmB* / *Nco*I
cc_1tga	GGGACAACCAACCTTAACTG**G**GCGTTAAATAATATTAG	808-845	*crmC* / *Bsr*I
cc_2tga	CGCACTAAAAGATGGGAAATG**G**TACCTAAGTTTCTTTG	1443-1480	*CrmC* / *Kpn*I
cc_3tga	CTCCTGGTGAGATCGA**C**TG**G**AAACCTAGAGTAGAAG	1892-1927	*crmC* / *Bsr*I
CB5Afw	TTTATTAATAATGTTTCG**A**AAAAAGTGGTAGCTGAAG	415-451	*crmB* / *Csp*45I
CB5Arev	CTTCAGCTACCACTTTTT**T**CGAAACATTATTAATAAA	415-451	*crmB* / *Csp*45I

Mutated nucleotides are shown in bold, introduced restriction sites are underlined. The positions of the primers are according to GenBank entry AE015450, with the coodinate 1 corresponding to the beginning of each gene.

The QuikChange Multi system (Stratagene, La Jolla, USA) allows the mutagenesis of a DNA sequence at multiple sites at the same time, using a single oligonucleotide per site. The single steps of this method are outlined in Fig. 1.

Mutagenic oligonucleotide primers mgc_tga for *mgc2*, cb_1tga, cb_2tga and cb_3tga for *crmB* and cc_1tga, cc_2tga and cc_3tga for *crmC* genes were designed according to the manufacturers´ instructions (Table 2). Briefly, all the primers anneal to the same strand of the template plasmid, the GC content is 40-50%, and the desired mutations are in the middle of the primer with 15 nucleotides being complementary to the template at both sides. The

length of the designed primers is between 36 and 41 nucleotides. Additionally, each primer introduces a restriction site facilitating direct screening of transformants for the mutation.

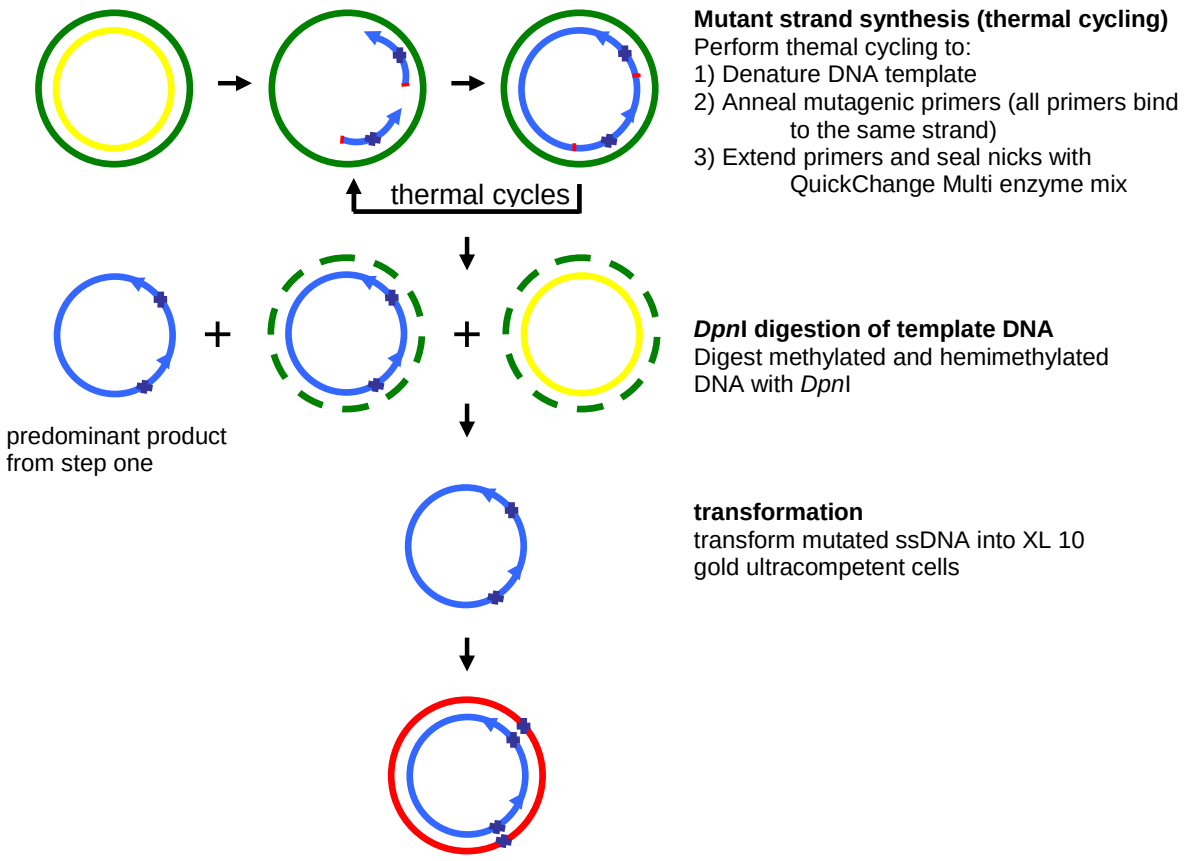


Fig. 1. Scheme of the QuikChange Multi site-directed mutagenesis method (Stratagene, La Jolla, USA). Step 1 consists of a thermal cycling procedure that results in multiple rounds of mutant strand DNA synthesis. Components of the PCR reaction include the supercoiled and methylated dsDNA template, one or more synthetic oligonucleotide primers containing the desired mutations, and an enzyme blend containing *Pfu* Turbo DNA polymerase and a thermostable T4 DNA ligase provided with the kit. First, the mutagenic primers, which are designed to bind the same strand of the template, are annealed to the corresponding strand of the denatured template DNA. In step 2 of the procedure, primers become elongated to longer fragments, which are ligated to establish the full-length circles containing the desired mutations. During step 3, the endonuclease *Dpn*I, which is specific for methylated and hemimethylated DNA digests the parental template DNA leaving behind full-length single strand DNA circles, which harbour the desired mutations. In step 4, the reaction mixture is transformed into *E. coli* XL10 Gold.

The kit also provided an internal control reaction, which was processed simultaneously to verify that all components were working properly. The control reaction is based on the 4 kb pBluescript® II SK (-) phagemid harbouring the *lacZ´* gene that encodes the first 146 amino acids of the β-galactosidase. This *LacZ´* alpha peptide is responsible for the production of blue colonies when expressed in the appropriate *E. coli* background (lacZ Δ M15) and grown on media containing X-gal and IPTG. The QuikChange Multi control template was modified to contain stop codons at three positions in the *lacZ* coding sequence. Each of the mutations

should prevent the production of active β-galactosidase. The QuikChange Multi control primer mix consists of three primers, each of which reverts one of these stop codons to the codon found in the original *lacZ* gene. Restoration of active β-galactosidase requires that all three stop codons get mutagenized in the same molecule.

For the multi-site mutagenesis of the selected MG genes, all the plasmids used as templates were of similar size (pMC2 4.8 kb, pCC2 5.6 kb, pCB2 5.8 kb), so the same conditions were used. A mutant strand synthesis reaction and a control reaction were performed according to the manufacturer's recommendations (Table 3).

Table 3. Components used in the control and the experimental reactions

Mutagenesis Reactions Component	**Control Reaction**	**pMC2**	**pCB2/pCC2**
10x QuikChange Multi reaction buffer	2.5 µl	2.5 µl	2.5 µl
ddH_2O to a final volume 25 µl	18.5 µl	14.25 µl	12.25 µl
QuikSolution	0 µl	0.75 µl	0.75 µl
ds-DNA template (100 ng)	1 µl	4 µl	4 µl
Mutagenic primers (100 ng/µl each)	0 µl	1 µl	3 µl
Mutagenic control primers mix	1 µl	0 µl	0 µl
dNTP mix (10mM each)	1 µl	1 µl	1 µl
QuikChange Multi enzyme blend	1 µl	1 µl	1 µl

The cycling parameters were the same for all four reactions: a denaturing step at 95℃ for 1 min followed by 30 cycles of denaturation at 95℃ for 1 min, annealing at 55℃ for 1 min and elongation at 65℃ for 8 min.

After the PCR, 1 µl of restriction enzyme *Dpn*I (10 U/µl) (cutting specifically methylated and hemimethylated DNA and therefore suitable for degrading the parental plasmid DNA used as the template for the PCR) was added directly to each amplification reaction, which was gently mixed and incubated at 37℃ for 1 h. A 45 µl aliquot of electrocompetent frozen *E. coli* XL10-Gold cells was thawed on ice and mixed with 2 µl of β-mercaptoethanol (provided with the kit) in 15 ml polypropylen tubes (Sterilin, Staffordshire, UK) for 2 min. Then, 1.5 µl of the digestion mixture was transferred into the *E. coli* XL10 Gold cells. After 30 min of incubation on ice the cell mixture was transferred into the 42℃ water bath. The tubes were heat-shocked for 30 s, cooled immediately on ice for 2 min and gently resuspended in 500 µl SOC medium preheated to 42℃. The transformed culture was incubated at 37℃ for 1 h under

gentle agitation at 240 rpm. Finally, cells were plated at different amounts onto LB/Amp (Stratagene) agar plates containing 80 µg/ml X-gal and 20 mM IPTG. From the mutagenesis control sample 10 and 20 µl were plated whereas from the experimental mutagenesis 150 µl were used per plate.

Induction of MBP-fusion protein expression (NEB)

Vectors carrying *malE-crmB* sequences were maintained in *E. coli* TB1. Expression of the MBP fusion proteins was induced according to the manufacturer's instructions, briefly: clones grown on LB/Amp-plates were picked and cultured overnight in broth for 16 hours. One ml of each overnight culture was used to inoculate 10 ml of fresh LB/Amp broth. When the cultures reached OD_{600} 0.3, they were induced by IPTG at a final concentration of 0.3 mM and aliquots were collected in 1 h intervals. Induction was tested by SDS-PAGE of cell lysates followed by Western blotting using an anti-MBP antiserum (NEB) in dilution 1:10,000

Protein purification with magnetic beads

This rapid method for purification of polyhistidine (poly-His) tagged proteins is based on paramagnetic precharged nickel particles used to isolate the protein directly from a crude cell lysate of bacterial cells (Fig. 2). Fusion proteins can be purified under native and/or denaturing conditions.

For the purification, cells from an induced bacterial culture (500 ml) were harvested by centrifugation (6,000 g, 5 min, RT). The pellet was resupended in 50 ml FastBreak™ cell lysis reagent and DNaseI (5 µg/ml) was added to the lysate to reduce the viscosity. Aliquots of 5 ml were then gently mixed by inversion with 20 µl magnetic particles (5 min, RT). The mixture was placed into a magnetic stand to recover the particles coated with His-tag fusion protein. The supernatant was aspirated, and another aliquot of the lysate was mixed with the beads. These steps were repeated until the whole cell lysate was mixed with the beads. Finally, the magnetic particles were washed three times with 2 ml MagneHis™ binding/washing buffer (100 mM HEPES, 10 mM imidazol, pH 7.5) and the His-tagged fusion protein was eluted using 150 µl MagneHis™ elution buffer (100 mM HEPES, 500 mM imidazol, pH 7.5).

To improve the efficiency of this method, the bacterial lysate was prepared as follows: pelleted cells of a 500 ml culture were resuspended in 50 ml binding/washing buffer, sonicated on ice using ten bursts of 10 s (BANDELIN SONOPULS HD2070, titan microtip MS73, 70% power) with a cooling period of 10 s between each burst. The 5 ml aliquots were then treated with MagneHis™ Ni-particles as described above.

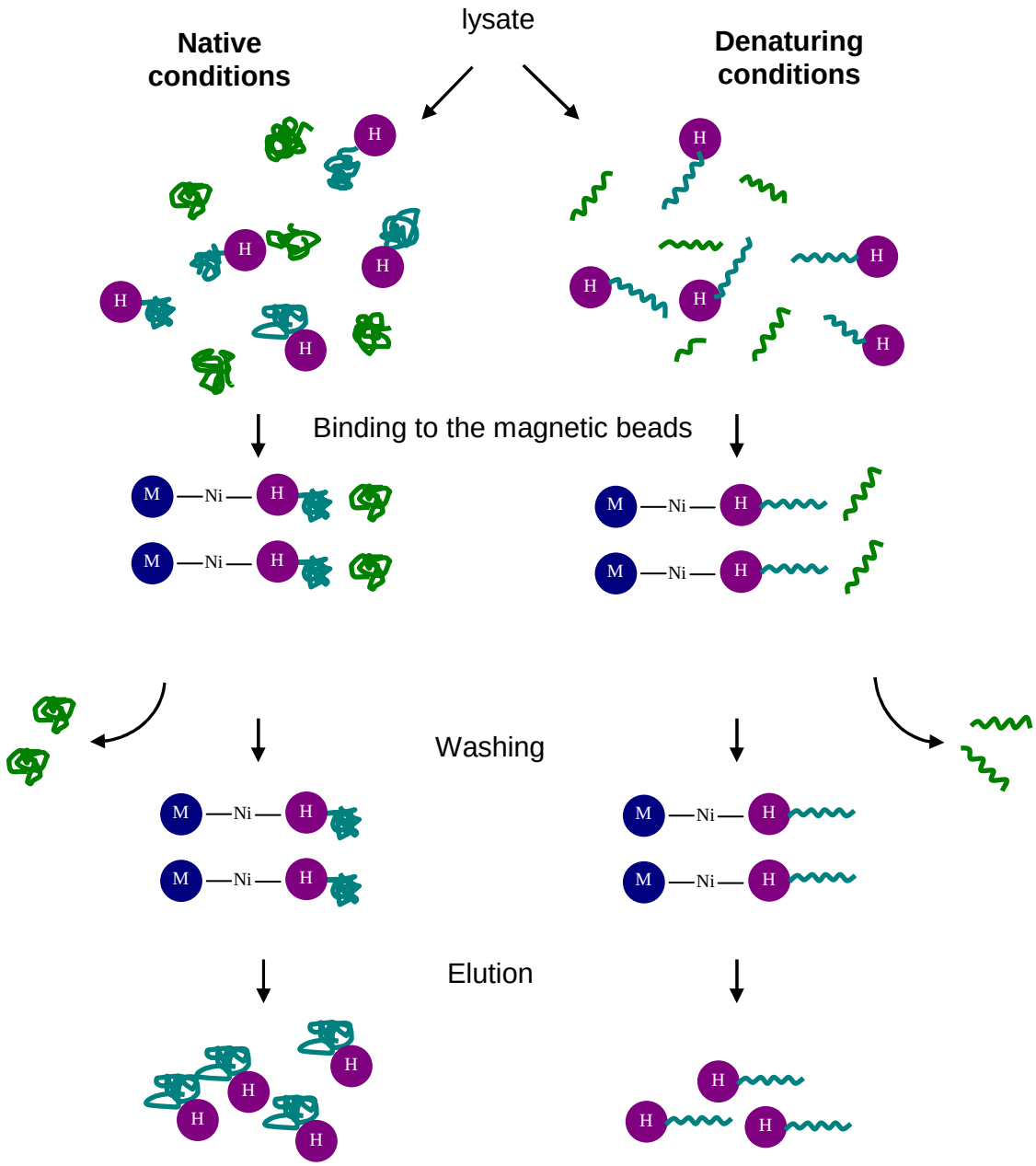


Fig. 2. Scheme of the MagneHis™ Protein Purification System (Promega, Mannheim, Germany). Bacterial cells containing a poly-His tagged protein are lysed directly in the culture medium or after centrifugation using the provided FastBreak™ cell lysis reagent (A). MagneHis™ Ni-particles are then added to the lysate, which are said to bind poly-His tagged proteins in a matter of minutes (B). Unbound proteins are washed away (C), and the target protein is recovered by elution with imidazole (D).

Resin-based purification

The ProBond™ purification system (Invitrogen, Paisly, UK) is designed for purification of poly-His tagged recombinant proteins produced by bacteria utilizing the high affinity and selectivity of ProBond™ nickel-chelating resin for recombinant fusion proteins containing six tandem histidine residues (poly-His).

The method described below is the general method; modifications used for purifying selected fusion proteins in this study are described in the Results section.

Preparation of resin

2 ml of the ProBond nickel-chelating resin (Invitrogen, Paisly, UK) was gently resuspended before use in the bottle, transferred onto a 10 ml column provided with the kit and allowed to settle completely by gravity. The supernatant was gently aspirated. Six ml of sterile water was added and mixed with the resin by gently inverting or tapping the column. Again, the resin was allowed to settle by gravity. Additional two washes were performed using either the native or the denaturing binding buffer and the supernatant was aspired.

Preparation under native conditions

Cells from a 50 ml culture were harvested by centrifugation (6,000 g, 5 min, 4℃), and resuspended in 8 ml of native binding buffer (50 mM NaH_2PO_4, pH 8, 0.5 M NaCl, 10 mM imidazol, pH 6). The solution was sonicated on ice using six bursts of 10 s (BANDELIN SONOPULS HD2070, titan microtip MS73, 70% power) with a cooling period of 10 s between each burst. The lysate was centrifuged at 3,000 g for 15 min to pellet the cellular debris. At this point the lysate was either stored at –20℃ or further processed immediately. The lysate was then incubated with the resin in the previously prepared column by gentle agitation for 30-60 min. The resin with the bound fusion protein was then allowed to settle by gravity for 15 min and the supernatant was aspirated and stored for further SDS-PAGE and Western blot analysis. The resin coated with the fusion protein was washed three times with 8 ml native wash buffer (50 mM NaH_2PO_4, pH 8, 0.5 M NaCl, 20 mM imidazol, pH 6) as described above. Finally, the column was clamped in a vertical position, the cap was removed and the fusion protein was eluted using app. 10 ml native elution buffer (50 mM NaH_2PO_4, pH 8, 0.5 M NaCl, 250 mM imidazol, pH 6). Fractions of 1 ml were collected and analyzed with SDS-PAGE. Fractions containing eluted protein were concentrated using an evaporator (Univapo 100 H, UniEquip), if necessary.

Preparation under hybrid conditions

For hybrid conditions, the lysate was first prepared under denaturing conditions (see below) followed by the binding of the fusion protein to the resin and a first wash under denaturing conditions. The last washes and the elution were performed with native buffers which should allow a refolding of the protein. The preparation of the lysate followed the same steps as described before (native conditions), with the following exceptions: Bacterial cells were resuspended in guanidinium lysis buffer (6 M guanidine hydrochloride, 20 mM NaH_2PO_4, pH 7.8, 500 mM NaCl) and rocked for 10 min at RT to ensure thorough cell lyses. The resin was washed in denaturing binding buffer (8 M urea, 20 mM NaH_2PO_4, pH 7.8, 500 mM NaCl). After the incubation of the resin with the cell lysate, the column was washed twice with 6 ml denaturing binding buffer, twice with denaturing wash buffer (8 M urea, 20 mM NaH_2PO_4, pH

6, 500 mM NaCl) and twice with native wash buffer. The protein was eluted with app. 10 ml native elution buffer (250 mM imidazol).

Recharging the resin

To recharge 2 ml of the resin in a purification column, the column was washed twice with 8 ml 50 mM EDTA (pH 8) to strip away the chelated nickel ions, twice with 8 ml sterile water and twice with 8 ml $NiCl_2.6H_2O$ (5 mg/ml in sterile water) to recharge the resin. Finally, the recharged resin was rinsed two times with 8 ml sterile water. The column containing the recharged resin was preserved with 20% ethanol and stored at 4℃.

Protein dialysis

A dialysis membrane (Invitrogen, Paisly, UK) was swiftly washed in deionized water, boiled for 10 min in distilled water to remove traces of ethanol, cooled and briefly washed in distilled water. The protein solution was filled with a glass pipette into the dialysis membrane with a fixed clip on one end and the membrane was closed. The membrane loaded with the protein solution was placed into 2 l of sterile phosphate buffered saline (PBS) at 4℃ for 12 h. Consequently, two additional washes in 1 l PBS were performed for three hours each. Finally, the protein solution was concentrated by placing the membrane in PEG powder (Roth, Lauterbourg, France) for 45 min. The final volume was 3 ml and the concentration was assessed either by SDS-PAGE analysis or by using the BCA™ Protein Assay (Pierce, Illinois, USA).

Electro-elution of proteins

Electro-elution of proteins was performed in an Electro-Eluter (BioRad, Model 422). The assembly of the cell followed the manufacturer's instructions. A gel slice of a zinc-stained (BioRad) SDS-PAA gel containing the protein of interest was minced and all pieces were loaded into the Electro-Eluter as described by the manufacturer using freshly prepared volatile buffer (50 mM NH_4HCO_3, 0.1% SDS,). The protein was eluted at a constant current of 10 mA/glass tube for 6 h. After the elution, the volatile buffer was lyophilized in a spin-vacuum (Univapo 100 H, UniEquip), leading to a concentrated protein solution.

Immunization of the rabbits, bleeding

One ml of the solution containing the fusion protein content of 150 µg was injected to the popliteal lymph nodes of New Zealand White rabbits (approx. 0.3 ml per one lymph node) while the remaining (approx. 0.4 ml) was inoculated subcutaneously around the spine of the animal. Depending on the intensity of the immune response which was checked by Western blotting, the rabbits were given several booster injections (100 µg of the protein each) subcutaneously at monthly intervals. Ten days following the last booster immunization, the rabbits were anaesthetised with ketamine hydrochloride (25 mg per kg of body weight) and xalazine (3 mg per kg of body weight) and bled by cardiac puncture. The blood was centrifuged (300 g, 5 min, RT) and the serum was collected. The specificity and the titre of

the working dilution of the rabbit antibodies directed against MG proteins were assessed by Western blot analyses using whole cell lysates of various MG variants.

Results

Anti-Mgc2

The gene *mgc2* of MG strain R contains one TGA codon (amino acid no. 65 out of 297 in total) that is translated as the amino acid tryptophan in the mycoplasma context. In order to express an untruncated Mgc2 protein in *E. coli*, the mycoplasma TGA codon had to be replaced with another tryptophan-encoding triplet, most favourable with the codon TGG. In order to do so, plasmid pMC2 (described under 4.1.) harbouring the full length *mgc2* sequence was subjected to a site-directed mutagenesis using the primer mgc_tga and thermal cycling conditions as described under Materials and Methods. Primer mgc_tga introduces two nucleotides substitutions (ccc tga to cc**a** tg**g**) thereby replacing the original TGA codon with TGG and, at the same time, creating a *Nco*I restriction site, thus allowing to distinguish a mutation carrying construct from the parental plasmid. Transformation of *E. coli* XL10 Gold gave rise to hundreds of transformants. To identify the clones carrying the mutated plasmid, randomly selected colonies were cultivated in LB/Amp medium, and the purified plasmids were digested by *Nco*I. While digestion of the parental plasmid gave rise to a single DNA fragment of 4.8 kb, digestion of the plasmids carrying the desired mutations resulted in two DNA fragments of about 1.1 kb and 3.7 kb. Screening of 8 randomly selected transformants resulted in five clones with the desired mutation where the TGA codon was replaced by the codon TGG_{Trp}. One of them was selected for further use and named pWMC. In order to subclone the mutated *mgc2* coding sequence into the expression vector pRSET-B, LR-PCR was employed using primers mgc2_3 and mgc2_4 (Table 1) providing recognition sequences for restriction enzymes *Eco*RI and *Hin*dIII, respectively. The purified PCR product was then subcloned into the *Eco*RI-*Hin*dIII sites of expression vector pRSET-B resulting in plasmid pRMC.

Several preliminary experiments were conducted to identify the proper conditions under which the expression of the fusion protein His-Mgc2 could be detected. An induction of the protein expression was initially tested in the *E. coli* strains DH10β and BL21(DE3)pLys, respectively. Since in these initial attempts no expression of the recombinant protein could be observed, it was decided to test the expression in the genetic background of the *E. coli* strain BL21(DE3)pLys Star. This bacterial strain is said to be especially suitable for the production of proteins exerting a toxic effect on *E. coli* cells, as it contains a mutation in the RNase E gene (*rne*) enhancing the stability of mRNA transcripts and thus increasing protein yield. Additionally, the pLysS plasmid carried by this strain constitutively produces a T7 lysozyme, thereby reducing basal levels of T7 RNAP, which is also encoded on the host

genome. As a consequence, no basal expression of recombinant genes that are under the control of the T7 promoter should take place.

Clones of freshly transformed *E. coli* BL21(DE3)pLys Star(pRMC) were cultivated for 16 h in LB/Amp,Cam containing 1% glucose. The overnight cultures were used for inoculation of a superbroth/Amp, Cam (Minion, VanDyk, and Smiley, 1995) containing 0.25% glucose to a final OD_{600} of approximately 0.1. At the OD_{600} 0.3-0.6, the recombinant protein production was induced by addition of IPTG (0.5 mM) for 15 min. Cells were then harvested by centrifugation (6,000 g, 5 min) and the cell pellet was resuspended in PBS (25 ml per 1 l culture). Aliquots of the non-induced and induced culture were analysed by SDS-PAGE followed by Western blot using Anti-Xpress™ antibodies, which revealed a protein of approximately 32 kDa in the induced culture only. Prolonged cultivation of the His-Mgc2-producing cells after induction led to the lysis of the bacterial cells indicated by a decrease of the OD_{600} values. Similarly, the amount of the fusion protein produced was decreasing with higher OD_{600} values (0.5 - 0.7) of the non-induced cultrure. Once the culture reached an OD_{600} higher than 0.8, the induction by IPTG did not result in any detectable His-Mgc2 production.

In order to retain a wide applicability of the antibodies, it was tried to obtain the fusion protein preferentially in its native form. The metal binding domain of the fusion protein should allow a simple purification of the recombinant proteins. Two methods for the purification of native protein were tested in parallel: (1) MagneHis™ Protein Purification System (Promega, Mannheim, Germany) and (2) Immobilized Metal Affinity Chromatography using ProBond™ Nickel-Chelating Resin (Invitrogen, Paisly, UK). While with the MagneHis™ system a crude cell lysate can be used, the resin works with a soluble fraction only. Therefore, the solubility of the fusion protein His-Mgc2 was tested. A 10 ml culture was centrifuged (6,000 g), the pelleted cells were resuspended in 1 ml PBS, sonicated, and after an additional centrifugation samples of the supernatant and the cell debris were collected. The SDS-PAGE analysis followed by Western blotting using the Anti-Xpress™ Antibodies (dilution 1:5,000) revealed that approximately 60% of the fusion protein was present in the insoluble fraction.

An overview and a schematic representation of the MagneHis™ protein purification system are given in the Materials and Methods section (Fig. 2). The bacterial lysate was prepared according to the manufacturer's instructions supplied with the system, with the following exception: the volume of the induced culture was dramatically increased from 50 ml recommended by the manufacturer to 1,000 ml on account of the rapid cell lysis and low OD_{600} of the culture susceptible for induction (see above). To utilize the full binding capacity of the magnetic beads, the particles were allowed to incubate with several aliquots of the cell lysate prior to the first washing. As the elution of the His-tagged proteins is based on highly concentrated imidazol, the eluted protein had to be dialyzed against PBS before the

immunization of rabbits. The purification method based on magnetic Ni-particles appeared to be effective in matters of the purity of the His-tagged protein, but the amount of the eluted protein was poor in relation to the volume of the culture. Additionally, the magnetic particles gradually lost their binding capacity and the amount of the pellet could not be increased, therefore the method was not considered suitable for the purification of a fusion protein which is present only at a low concentration in the soluble fraction of the cell lysate (Fig. 3, Panel A).

A second method used for the purification of the His-Mgc2 fusion protein was the ProBond^{TM} nickel-chelating resin. The soluble fraction of the cell lysate used for protein isolation was prepared as follows: the transformed cells grown in superbroth to a density of OD_{600} 0.3-0.6 were induced by IPTG and after 15 minutes the cells were harvested by centrifugation. The pellet from a 2 litres of culture was resuspended in 50 ml of native binding buffer and subsequently sonicated. The cell debris was removed by centrifugation and the supernatant was used for protein isolation. The ProBond^{TM} nickel-chelating resin (300 µl) was applied into the test tube (50 ml, Sterilin), washed twice with 5 ml ddH_2O and twice with 5 ml native binding buffer. After washing, 50 ml of the soluble fraction of the cell lysate was mixed with the washed resin and incubated for 2 h at RT. The solution was mixed in the tube by soft inversion approximately every 10 min. Afterwards, the mixture was applied onto a column (8 ml, Invitrogen, Paisly, UK) and the resin with the bound His-Mgc2 fusion protein was let to settle by gravity. The supernatant was carefully aspirated and collected in a fresh tube. The resin was washed four times with 8 ml native wash buffer and the supernatants were stored for SDS-PAGE and Western blot analysis. At the end, the His-tagged protein was eluted with 4 ml of an elution buffer containing 250 mM imidazol. The fractions containing lower amounts of the fusion protein were concentrated in an evaporator (Univapo 100 H, UniEquip). This method was found to be suitable for the purification of a His-tagged protein, which is present in the soluble fraction of the lysate, although at a low concentration. Although the elutions contained satisfying amounts of the His-Mgc2 protein (Fig. 3., Panel C), some contaminating proteins were present as well (Fig. 3, Panel B). Those contaminants were most likely derived from *E. coli* and therefore might not give rise to cross-reacting antibodies.

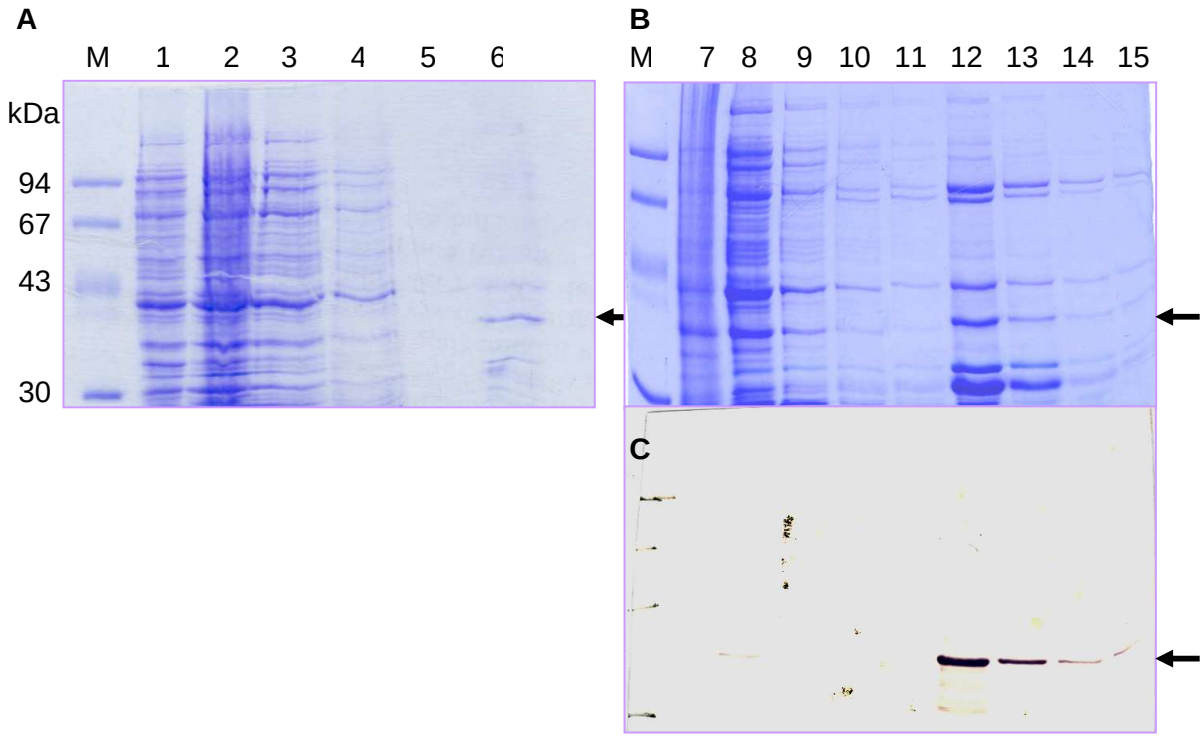


Fig. 3. The purification of His-Mgc2 fusion protein using the MagneHis™ protein purification system (Panel A) and the ProBond™ Nickel-Chelating Resin (Panel B, C). The non-induced *E. coli* cells (lanes 1 and 7), bacterial lysates after incubation with magnetic beads (lane 2) or resin (lane 8), washing steps (lanes 3-5 and 10-12) and elution (lanes 6, 12-15) were separated onto a 10% SDS-PAA gel and stained with Coomassie Blue (Panel A, B). Western blot corresponding to the SDS-PAA gel (Panel B) was stained using the Anti-Xpress™ Antibodies (dilution 1:5,000) (Panel C). The His-Mgc2 protein is indicated by arrows. (M) Molecular weight marker.

Purified His-Mgc2 protein obtained by either method was then used for immunization of two rabbits. The animals received 3 and 4 injections of the native fusion protein (100 µg per injection), respectively. The protein used for the second booster injection was a mixture of the native His-Mgc2 purified on the resin-based column and a denatured His-Mgc2. The latter was isolated from a SDS-PAA gel (as described below for His-CrmC) loaded with the insoluble fraction of the induced producer cells containing a high yield of the His-Mgc2 by electro-elution.

The reactivity of the obtained antisera to the Mgc2 protein of MG was tested by immunoblotting. A total cell lysate of the His-Mgc2 producing *E. coli* BL21(DE3)pLys Star(pRMC) as well as lysates of MG R_{low} and R_{high} were separated on a 10% denaturating PAA gel and transferred onto a nitrocellulose membrane. The blots were probed with the sera obtained from the immunized rabbits in dilutions ranging from 1:100 to 1:2,000. A protein of the expected size of 34 kDa was detected in lysates of IPTG-induced, pRMC-transformed *E. coli* cells but not in lysates of non-transformed counterparts. The best staining results showing the lowest background at the same time were obtained with a serum dilution of 1:1,000.

A slightly smaller protein of about 30 kDa was also detected in lysates of both strains of MG, R_{low} and R_{high}. The size of 30 kDa corresponds to the Mgc2 protein lacking the N-terminal poly-His tag. To test the expression of the *mgc2* gene in clonal variants and mixed populations of MG, equivalent amounts of cell lysates of the strains R_{low}, R_{high}A3, RCL1 RCL2, RCL3, RCL4, mHAD3 and R_{high}GT5 were analyzed on Western blots with the anti-Mgc2 serum. A protein of about 30 kDa was detected in all the strains tested (Fig. 4). Localization of the Mgc2 protein was assessed by colony immunobloting and by Triton X-114 phase partitioning followed by Western immunoblotting. The assays suggested that the Mgc2 protein (i) is a membrane protein as it was detected in the hydrophobic and insoluble phases of the MG cell lysate, while no protein was observed in the aqueous phase, and (ii) that the protein is localized on the cell surface as the colonies were stained with the anti-Mgc2 antibodies by colony immunoblotting. These results are in good agreement with a previously reported study (Hnatow et al., 1998).

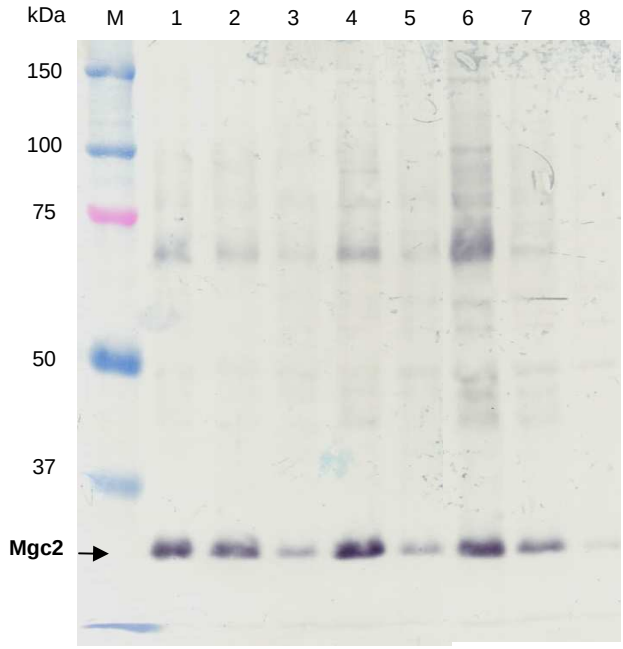


Fig. 4. Western blot analysis using anti-Mgc2 antibodies. Equivalent amounts of cell lysates of the strains R_{low} (lane 1), RCL1 (lane 2), RCL2 (lane 3), RCL3 (lane 4), RCL4 (lane 5), R_{high} A3 (lane 6), mHAD3 (lane 7) and R_{high}GT5 (lane 8) were separated by SDS-PAGE and Western blots were probed with the anti-Mgc2 serum in dilution 1:1,000. A protein of about 30 kDa was detected in all the strains tested. M: molecular weight marker.

Anti-CrmC

Gene *crmC* of MG strain R carries three TGA_{Trp} codons at positions 276, 488 and 637 of the 855 aa protein. The plasmid pCC2 (see Collecton of data under 4.1.) carrying the entire crmC gene was subjected to a multi-site directed mutagenesis using primers CC_1tga, CC_2tga and CC_3tga (Table 2). Approximately 70 randomly selected transformants were screened for the presence of restriction site *Kpn*I. Since neither *Kpn*I nor *Bsr*I restriction sites were present in the parental, non-mutated plasmid pCC2, their presence directly indicated a successful mutagenesis procedure. Plasmids which were linearized by *Kpn*I were consequently digested with *Bsr*I. The finally selected plasmid pWCC1 displayed a restriction pattern corresponding to the desired mutations of all three TGA_{Trp} codons. After digestion with *Eco*RI a 2.6 kb-fragment of plasmid pWCC1 containing the corrected *crmC* was subcloned into the expression vector pRSET-B linearized by *Eco*RI. In the resulting plasmid pRCC, the *crmC* gene was supposed to be located downstream of the poly-His tag. The proper orientation of the *crmC* and the correct fusion to the poly-His tag coding sequence of pRSET-B was confirmed by control restriction digests with *Bgl*II, *Bsr*I, *Eco*RI, *Kpn*I and *Pvu*II (Fig. 5). In parallel, the plasmid pRCC was transformed into *E. coli* BL21(DE3)pLys Star and recombinant expression was induced by adding 0.5 mM IPTG to the bacterial culture. However, the restriction digest by *Kpn*I did not result in the expected fragment pattern (Fig. 5., lane 5) suggesting that the second TGA_{Trp} codon was not mutated. Similarly, Western blot analysis with the antibody specific for the poly-His tag revealed a protein of only approximately 54 kDa, instead of the expected length for the entire fusion product of about 70 kDa. Taking these two results together, it was concluded that the second TGA codon remained unchanged, thus terminating the translation of the *His-crmC* gene in *E. coli*. This unexpected binding most probably resulted from a mistaken description of the primary digest by *Kpn*I. There the linearized form of the plasmid pWCC1 wawhen the partial digest was most probably caused by contamination present in the digestion mixture.

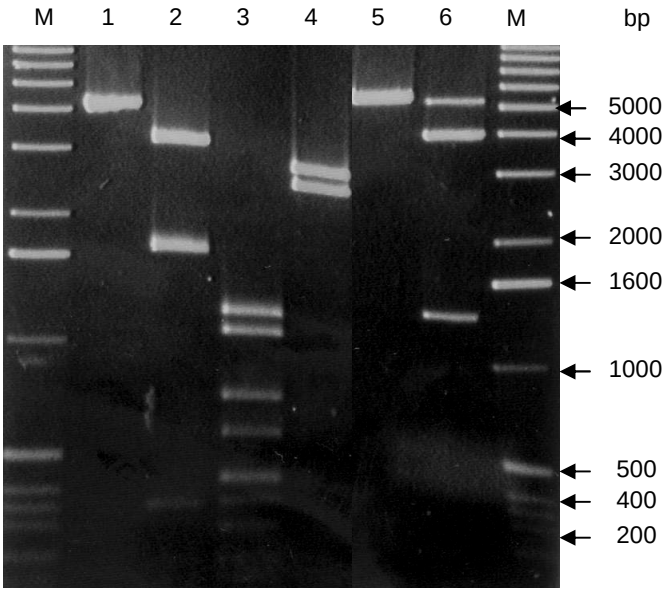


Fig. 5. Restriction digest of pRCC. Plasmid pRCC (line 1, undigested) was submitted to the control restriction digest using enzymes *Bgl*II (lane 2, fragments 3.3 kb, 1.7 kb, 0.3 kb), *Bsr*I (lane 3, fragments 1.2 kb, 1.08 kb, 0.7 kb, 0.57 kb, 0.4 kb, etc.), *Eco*RI (lane 4, fragments 2.9 and 2.6 kb), *Kpn*I (lane 5, fragments 4 kb and 1.5 kb) and *Pvu*II (lane 6, fragments 4.1 kb and 1.4 kb). M – 1 kb molecular marker, Gibco

In order to finally create a *crmC* gene with all 3 TGA codons corrected, two randomly selected plasmids from the first multi-site mutagenesis approach, namely pWCC61 and pWCC63, were used. Both plasmids carry the *crmC* gene in which only the second TGA_{Trp} is mutated to TGG_{Trp} codon as confirmed by digestion with *Kpn*I. The constructs pWCC61, pWCC63 and pRCC were transformed into *E. coli* strain SCS 110 (*dam*$^-$) and the non-methylated plasmid DNA was subsequently digested by the methylatioín-sensitive restriction enzyme *Cla*I along with *Eco*RV. Then, a 0.2 kb fragment of pWCC61 and pWCC63, respectively, containing an internal part of the *crmC* gene was ligated with the 5.3 kb fragment of the *EcoR*V/*Cla*I-digested plasmid pRCC. This ligation should assemble a full crmC gene with all three TGA codons corrected. However, despite the fact that sequence analysis confirmed the desired mutation in the second TGA_{Trp} of the *crmC* in the final constructs pR2CC1 and pR2CC2, no production of the His-CrmC fusion protein was detected after IPTG induction of transformed *E. coli* BL21(DE3)pLys Star even though several experiments under various conditions were performed. Considering all difficulties which were experienced during the construction of the plasmid pCC2 (see Collection of Data under 4.1.), when the *in frame* ligation of the TGG_{Trp}-corrected *crmC* gene with the *lacZ´* gene starting codon was not successful, it was assumed that a full-length CrmC protein is most likely harmful to the *E. coli* host. Since the expression of the full-length His-CrmC protein could not be achieved it was decided to produce truncated His-CrmC´ fusion proteins using the pRCC construct. The best induction of the His-CrmC´ protein was obtained when

LB medium was inoculated directly with colonies grown on SOB/Amp,Cam plates to reach a starting OD_{600} of 0.1-0.2 and when this culture was induced by IPTG (0.5 mM) at an OD_{600} of 0.4-0.6. The induced culture was harvested by centrifugation 60 min after induction as a prolonged cultivation resulted in a decrease of the OD_{600} of the bacterial culture. To test the solubility of the fusion protein His-CrmC´, the bacterial pellet was sonicated as described for the generation of Mgc2 antibodies. A consequent SDS-PAGE with Western blot analysis using the polyHis-specific antibodies revealed that the majority of the fusion protein remains in the insoluble fraction. Despite of that, the purification based on nickel-chelating resin was employed in order to purify the remaining of the soluble His-CrmC´ protein under native conditions. Unfortunately, all attempts to purify the truncated fusion protein failed. Additionally, also the purification under hybrid conditions (see Materials and Methods) was not successful. Thus, the His-CrmC´ fusion protein was extracted directly from the PAA-gel. The gel pieces were lyophilized, powdered, resuspended in saline and mixed with Freund´s adjuvant, and thereafter used for the immunization of two rabbits. For the booster injections, the His-CrmC´ was purified from the PAA-gel by electro-elution. One animal was sacrificed after the second booster injection as it developed abscesses; the second animal did not develop adverse reactions and was sacrificed after six immunizations.

The reactivity of the generated anti-CrmC serum was assessed similarly as described for the Mgc2-specific rabbit serum. A total cell lysate of the His-CrmC´-producing *E. coli* BL21(DE3)pLys Star(pRCC) as well as lysates of MG R_{low} and R_{high} were separated on a SDS-PAA gel and transferred onto a nitrocellulose membrane. The blots were probed with sera obtained from the immunized rabbits in dilutions ranging from 1:100 to 1:2,000. The best results with the lowest background were obtained with the serum dilution 1:400.

The CrmC protein should display a molecular weight of 97kDa as it was deduced from the sequence of the *crmC* gene disclosed by Papazisi and coworkers (Papazisi et al., 2003). To test the expression of the *crmC* gene in different clonal variants and mixed populations of MG, equivalent amounts of the total cell lysates of strains R_{low}, RCL1, RCL2, RCL3, RCL4, R_{high}A3, mHAD3 and R_{high}GT5 were analyzed on Western blots. Several bands were detected (Fig. 6) representing proteins with estimated molecular weights of 50, 97, 116 and 120 kDa. The protein p97 follows the same characteristic of ON/OFF switching as seen with GapA of the MG variants tested. The only exception is R_{high}GT5, which is GapA-positive (Papazisi et al., 2000) but appears to be negative for the p97 product. Whether the p97 protein really represents the *crmC* gene product, remains to be elucidated by other techniques, such as 2D-gel electrophoresis or MALDI-TOF analysis.

The protein p116 which migrates through the gel at the same speed as CrmA shows also a similar ON/OFF switching pattern. Hence, one might speculate that this protein is indeed the CrmA. Expression of *crmA* was found to be linked with the expression of *gapA* (Papazisi et

al., 2002; Winner et al., 2003) meaning that its transcription is disturbed in GapA-negative strains. The proteins p120 is present in all the clones tested as well as the protein p50.

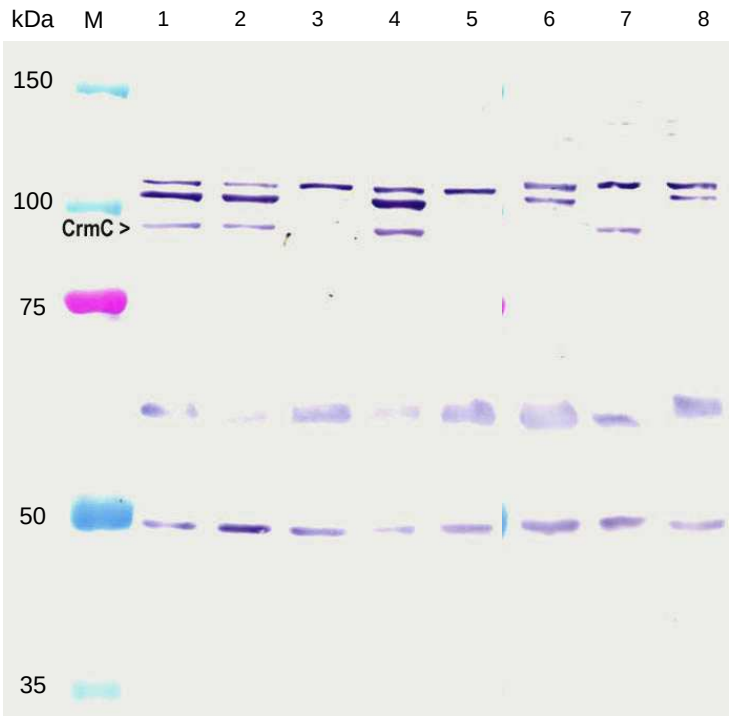


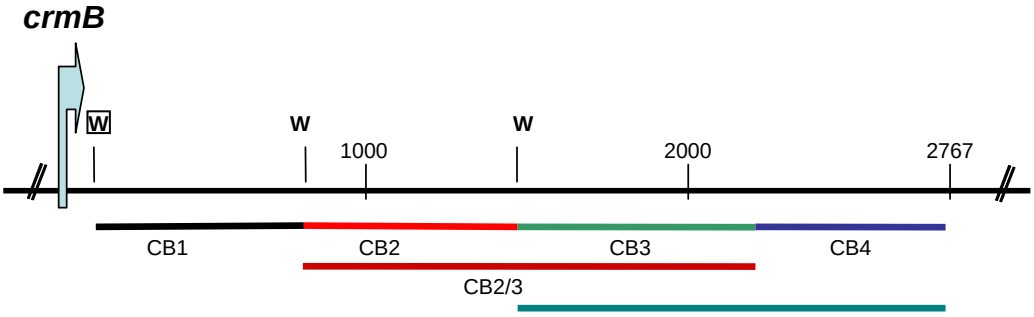
Fig. 6. Western blot analysis using anti-CrmC antibodies. Equivalent amounts of cell lysates of the strains R_{low} (lane 1), RCL1 (lane 2), RCL2 (lane 3), RCL3 (lane 4), RCL4 (lane 5), R_{high} A3 (lane 6), mHAD3 (lane 7) and R_{high}GT5 (lane 8) were separated on SDS-PAGE and Western blots were probed with the anti-CrmC serum at a dilution of 1:400. Several proteins have been detected using this serum: p120 and p50 that are present in all the clones tested, p116 and p97 that could represent CrmC. M: molecular weight marker.

Anti-CrmB

In order to express the MG *crmB* gene in *E. coli*, plasmid pCB2 (see Collection of Data under 4.1.) harbouring the complete *crmB* gene sequence inserted downstream of and *in frame* with the lacZ´ gene of pGem-T Easy was used. However, the 921 aa encoding *crmB* gene contains three TGA_{Trp} codons amino acids at positions 73, 271 and 474. Therefore, plasmid pCB2 was subjected to a multi-site directed mutagenesis using three TGA-changing primers as listed in Table 2. Plasmids of about 50 randomly selected clones were screened by digestion with *Nco*I, which should directly indicate successful mutagenesis of the second TGA_{Trp} codon. Those plasmids carrying *Nco*I sites were further digested by *Bsm*I to identify the mutations in the first and third TGA codons. Three clones with a proper restriction profile were selected and named pWCB1 to pWCB3. A direct attempt to induce the expression in the *E. coli* DH10β cells by addition of 1 mM IPTG, however, failed. Thus, in order to subclone the mutated *crmB* into an expression vector, the 2.8 kb *Eco*RI fragment of plasmid pWCB2 was ligated into the *Eco*RI-linearized pRSET-B. The resulting constructs pRCB1 and pRCB2

were transformed into *E. coli* BL21(DE3)pLys Star. Several clones grown on SOB/Amp,Cam-plates were tested for the ability to produce the recombinant protein His-CrmB. Though several concentrations of glucose and/or different growth temperatures were tested, no His-CrmB was detected after induction of expression by addition of IPTG.

Sequencing of the original plasmid pCB2 revealed a point mutation in the position 433 of the *crmB* gene. Deletion of an A nucleotide in a stretch of 6 adenines resulted in a frameshift event combined with the creation of a stop codon leading to a premature termination of translation. To restore the original coding sequence, single-site mutagenesis was applied onto plasmid pRCB using primers CB5Afw and CB5Arev (Table 2). The correct sequence in the resulting plasmid pRCB-X was confirmed by sequence analysis. Nevertheless, also with the corrected gene still no induction was detectable in transformed *E. coli* BL21(DE3)pLys Star(pRCB-X) during several attempts.



PCR	Length	Protein size	CB3/4 Fusion protein size
CB1	580 bp	21,3 kDa	64 kDa
CB2/3	1300 bp	47,7 kDa	90,4 kDa
CB3	700 bp	25,7 kDa	68,4 kDa
CB3/4	1400 bp	51,3 kDa	94 kDa
CB4	660 bp	24,2 kDa	66,9 kDa

Fig. 7. Scheme of the *crmB* fragments. The *crmB* gene was divided into four fragments, which were amplified using primer pairs B1F/B1R, B2F/B2R, B3F/B3R, B4F/B4R, B2F/B3R and B3F/B4R, which were subsequently clonec into pMalE vector using introduced *Eco*RI/*Sal*I restriction sites. Matrice is shown with corrected TGA codons (W represents Tryptophan).

A potential explanation for the unsuccessful creation of CrmB-expression vectors might be toxic effects onto the *E. coli* host cells, which was also seen before with CrmC-expression vectors. Thus, the gene *crmB* was divided into four fragments (Fig. 7), which were amplified using primer pairs B1F/B1R, B2F/B2R, B3F/B3R, B4F/B4R, B2F/B3R and B3F/B4R (see Table 1). The fragments were subsequently cloned into the *Eco*RI/*Sal*I-sites of the pMalE expression vector (di Guan et al., 1988) using newly introduced restriction sites, and the resulting plasmids were sequenced to verify the correct ligation with the *malE* sequence.

Plasmids pMalCB1, pMalCB2/3, pMalCB3, pMalCB3/4 and pMalCB4 were introduced into the *E. coli* strain TB1 and expression was induced by IPTG according to the manufacturer's instructions. The expression was detected by Western blotting using an anti-pMal antiserum (Fig. 8). A protein band with the expected size of 64 kDa for the CB1 fusion protein was observed in a culture carrying plasmid pMalCB1, while the culture containing the plasmid pMalCB4 displayed only a protein band corresponding to the MBP alone (42.7 kDa). Protein bands for the full length MBP-CB3 (68.4 kDa) and MBP-CB2/3 (90.4 kDa) as well as for smaller proteins most likely corresponding to processed forms of the fusion proteins, were observed in the protein profiles of *E. coli* TB1(pMalCB3) and *E. coli* TB1(pMalCB2/3), respectively. Testing the solubility of the fusion proteins of pMalCB1, pMalCB 2/3 and pMalCB3 revealed that most of the fusion proteins were part of the insoluble fraction of the bacterial lysate.

Finally, clones carrying plasmids pMalCB1 and pMalCB2/3 were selected to produce material for the immunization. The full-length fusion proteins MBP-CB1 and MBP-CB2/3 were excised from the SDS-PAA gel and electro-eluted. One rabbit was immunized four times with 150 µg of the MBP-CB1 and MBP-CB2/3 mixture (ratio 1:1).

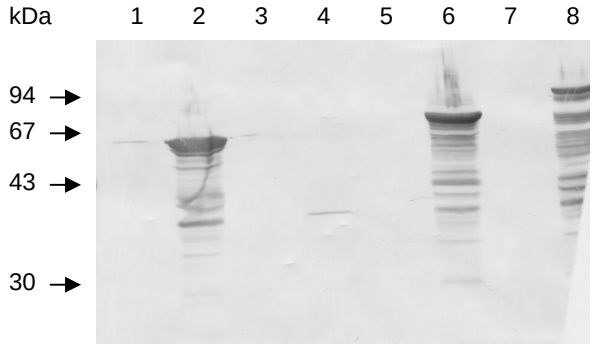


Fig. 8. Western blot analysis of the expression of MBP-CrmB proteins. The expression of MBP-CB1 (lane 1 and 2), MBP-CB4 (lane 3 and 4), MBP-CB3 (lane 5 and 6) and MBP-CB2/3 (lane 7 and 8) proteins was followed by Western bloting using bacterial extracts prepared either from IPTG-induced (lanes 2, 4, 6 and 8) or non-induced (lanes 1, 3, 5 and 7) cells and anti-MBP antibodies (1:10,000).

The reactivity of the obtained CrmB-antiserum to the proteins of MG was tested by Western blot analysis. Cell lysates of the *E. coli* TB1 producing MBP-CrmB1 and MBP-CrmB2/3, respectively, were probed with the CrmB-antiserum in dilutions ranging from 1:100 to 1:2,000. Proteins of 64 and 90 kDa, respectively, were detected in lysates of MBP-CrmB-producing *E. coli* but not in lysates of non-transformed counterparts. The best result with the lowest background was obtained when using the serum in a dilution 1:1,000. With this dilution, the expression of the *crmB* gene in cell lysates of the strains R_{low}, RCL1, RCL2, RCL3, RCL4, R_{high}A3, mHAD3 and R_{high}GT5 was analyzed by Western blotting (Fig. 9).

Several proteins were detected. A protein of 50 kDa was detected in all the clones tested as well as proteins of molecular sizes of approx. 70 kDa, 80 kDa, 85 kDa and 95 kDa (based on the sequencing data, the size of the CrmB protein was predicted to be 97 kDa). The most prominent band, however, appeared at 116 kDa which is also the size of the CrmA protein. Interestingly, also aprotein of 140 kDa was observed in all clones tested except the mutants carrying transposon, mHAD3, which carries a transposon inserted into the *crmA* gene, and R_{high} GT5, which is a clonal variant of R_{high} complemented by a *gapA* –carrying transposon. Further analysis MS/MS-analysis of the stained protein bands should help to explain this result.

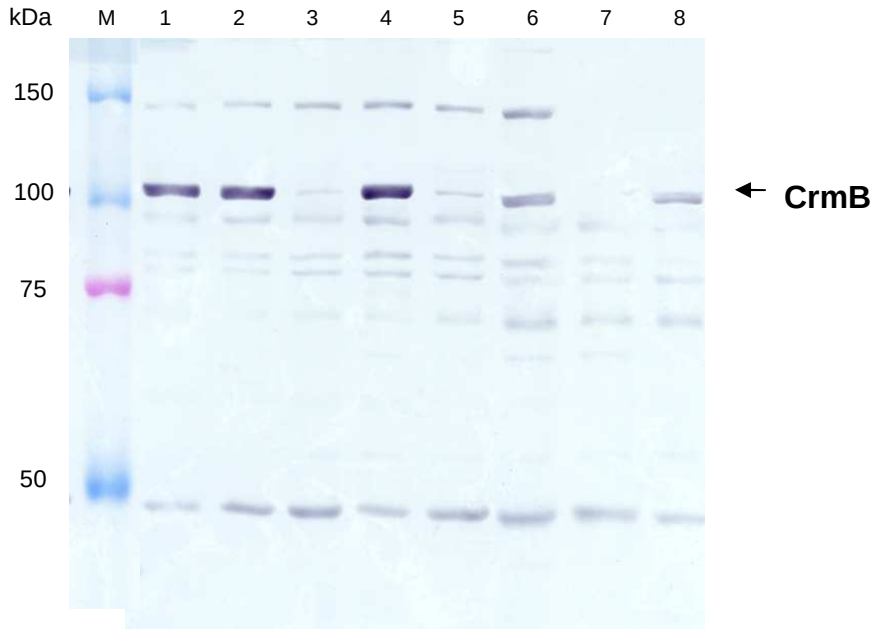


Fig. 9. Western blot analysis using anti-CrmB antibodies. Equivalent amounts of cell lysates of the strains R_{low} (lane 1), RCL1 (lane 2), RCL2 (lane 3), RCL3 (lane 4), RCL4 (lane 5), R_{high}A3 (lane 6), mHAD3 (lane 7) and R_{high}GT5 (lane 8) were separated on SDS-PAGE and Western blots were probed with the anti-CrmB serum in dilution 1:1,000. The protein of 116 kDa was detected in R_{low}, RCL1, RCL3, R_{high}A3 and R_{high}GT5 strains of MG. The proteins p50, p70, p80, p85 and p95 were detected in all the clones tested. The protein p140 is detected in all clonal variants of MG except the mutants generated by insetion of a transposon (lane 7 and 8). M: molecular weight marker.

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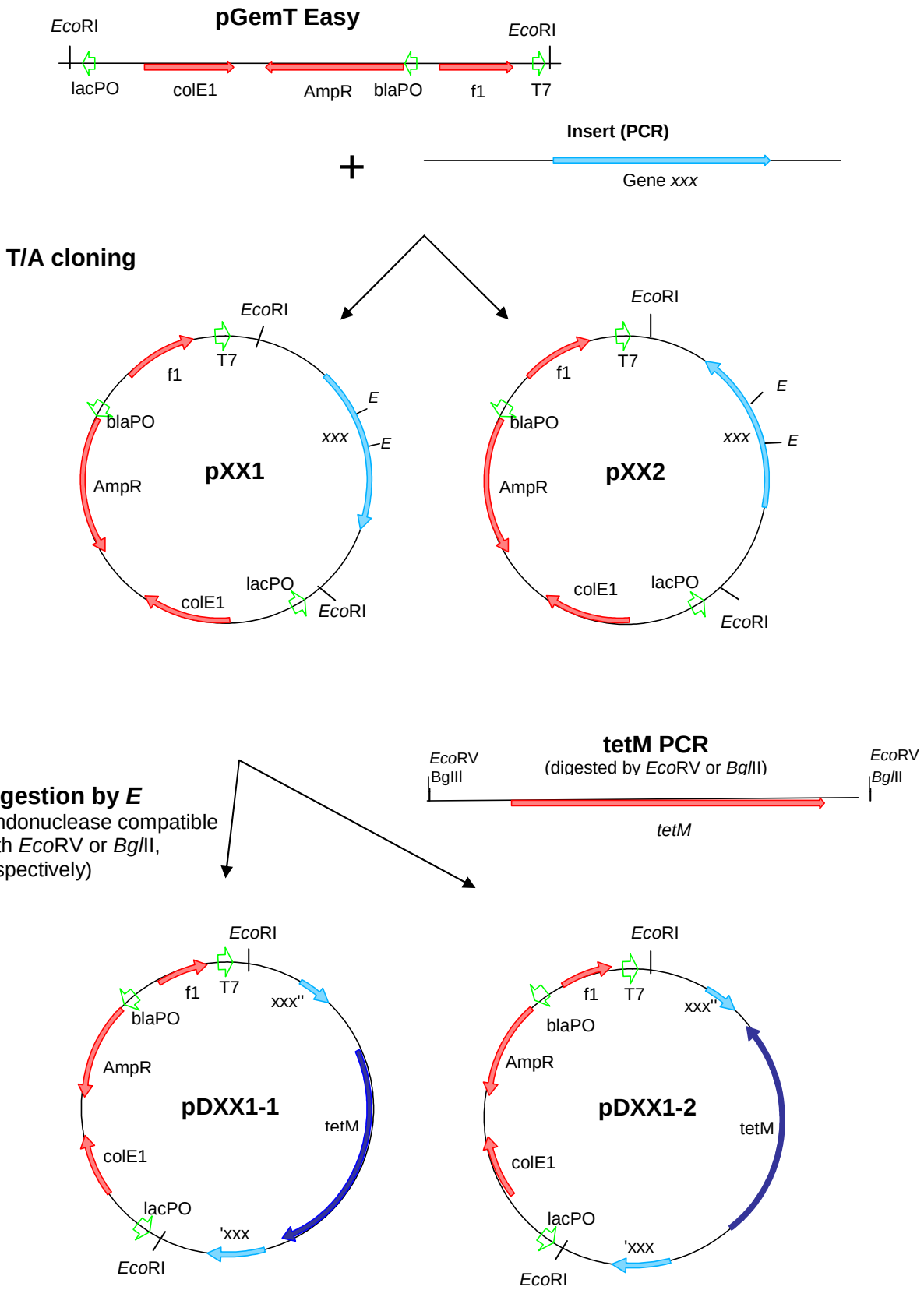
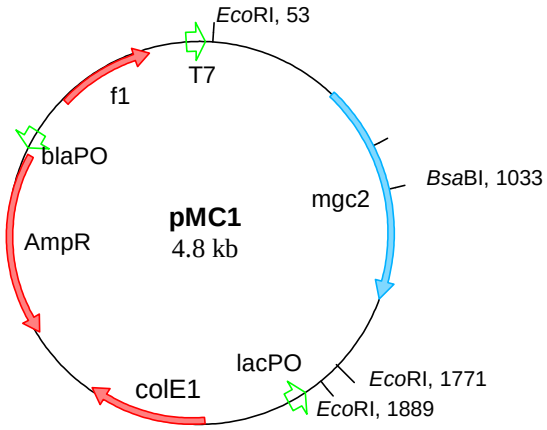
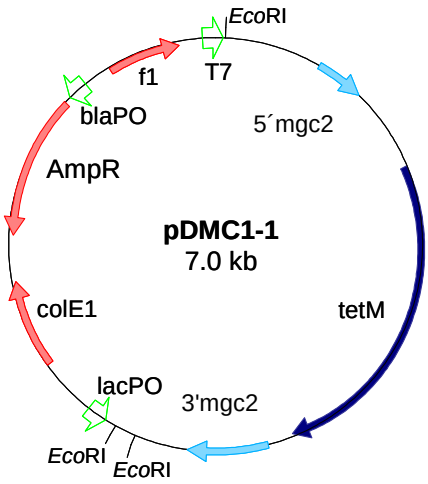
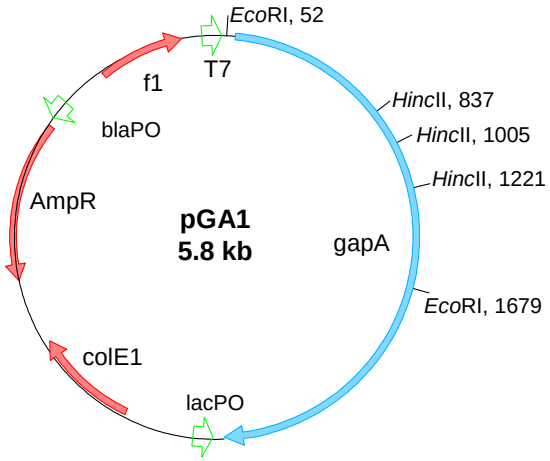
Appendix 1: Scheme of cloning of disruptor plasmids

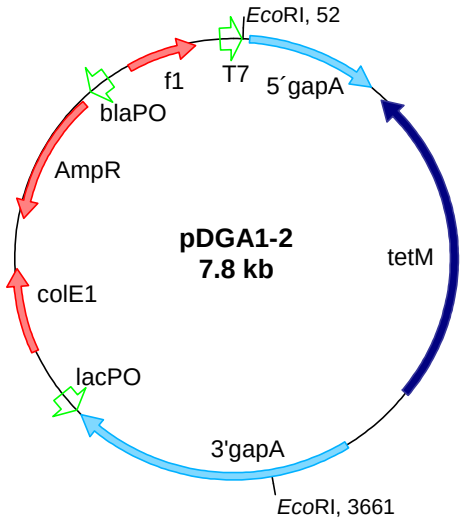


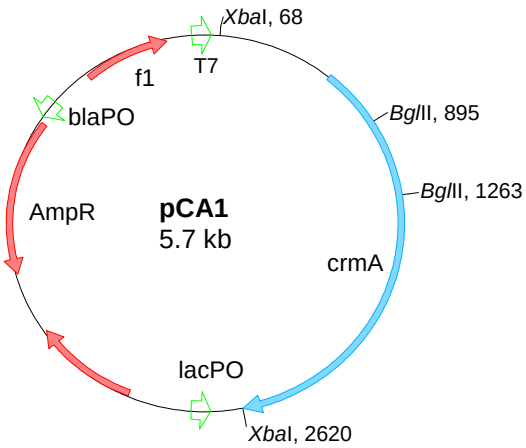
Appendix 2: Maps of selected disruptor plasmids

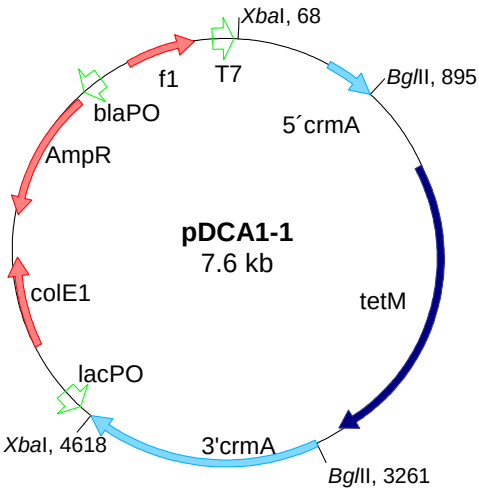


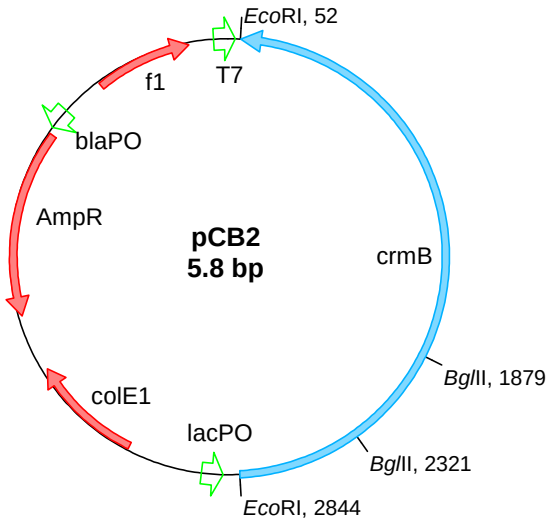








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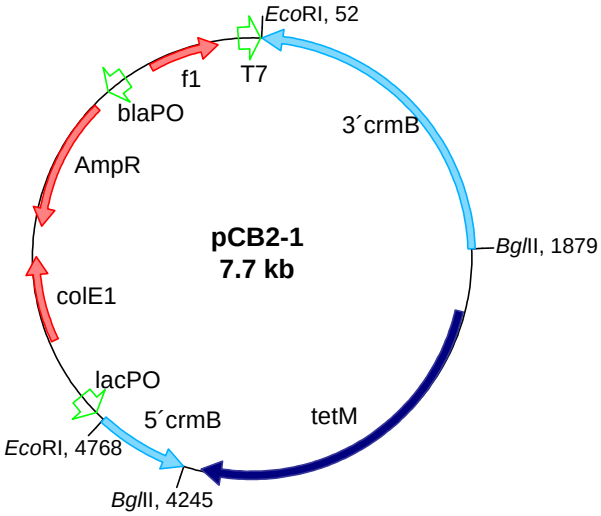
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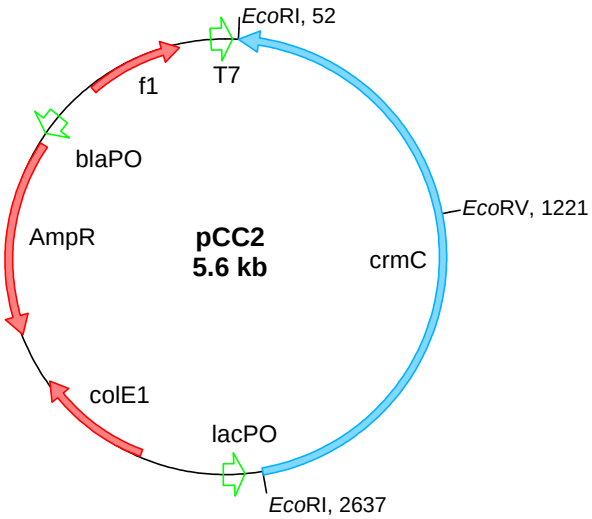
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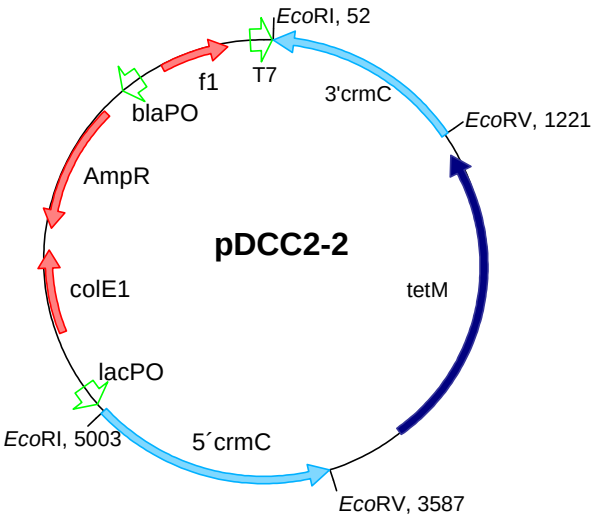
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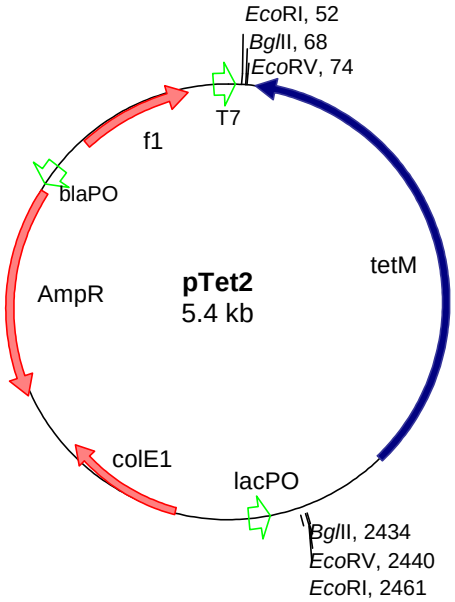
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Publications: Winner, F., Markova, I., Much, P., Lugmair, A., Siebert-Gulle, K., Vogl, G., Rosengarten, R., and Citti, C. (2003). Phenotypic switching in *Mycoplasma gallisepticum* hemadsorption is governed by a high-frequency, reversible point mutation. *Infect Immun* 71, 1265-1273

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