Cardiolipin synthetase is involved in the antagonistic interaction (reversed CAMP phenomenon) of Mycoplasma species with Staphylococcus aureus β-hemolysis.

Jonathan D. Koprnsan,1 Shlomo Rottem1 and Ran Nir-Paz2*

1Department of Microbiology and Molecular Genetics, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel.

2Department of Clinical Microbiology and Infectious Diseases, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel.

Running title: Mycoplasma reverse CAMP is cls associated

*For correspondence. E-mail: ran.nirpaz@gmail.com; Tel. (+972) 2 677 6543; Fax: (+972) 2 641 9545.
Abstract

*Mycoplasma hyorhinis*, has been implicated in a variety of swine diseases. However little is known on the hemolytic capabilities of *Mycoplasma* species in general and *M. hyorhinis* in particular. In this study we show that *M. hyorhinis* possess a β-hemolytic activity which may be involved in the invasion process. *M. hyorhinis* also possess the antagonistic cooperativity (reverse CAMP phenomenon) with *Staphylococcus aureus* β-hemolysis resulting in the protection of erythrocytes from the β-hemolytic activity of *S. aureus* (reverse CAMP). The reversed CAMP phenomenon has been attributed before to phospholipase D (PLD) activity. *In silico* analysis of *M. hyorhinis* genome, revealed the absence of the *pld* gene but the presence of the *cls* gene encoding cardiolipin synthetase which contains the two PLD active domains. Transformation of *M. gallisepticum* that has neither the *cls* gene nor the reverse CAMP phenomenon with the *cls* gene from *M. hyorhinis* resulted in the reverse CAMP phenomenon, suggesting for the first time that reverse CAMP can be induced by cardiolipin synthetase.
Introduction

Mycoplasmas (class *Mollicutes*) are the smallest self-replicating bacteria. These bacteria form a large group of prokaryotic microorganisms with over 200 species, distinguished from ordinary bacteria by their small size, minute genome and total lack of cell wall (1). Most mycoplasmas are parasites, and depend on host adhesion for infection (2, 3). Numerous pathogenic *Mycoplasma* species possess hemadsorption and cytadherence activities such as hydrogen peroxide-mediated and membrane-associated hemolytic activities which are associated with virulence potential (4, 5). Bacterial hemolysins are able to lyse red blood cells (RBCs) and a variety of other cell types, such as mast cells, neutrophils and polymorphonuclear cells (6). Hemolysins enable hemolytic microorganisms to directly damage host tissues as well as induce inflammatory responses (5, 7, 8). Many hemolysins, such as the oxygen-labile hemolysins (e.g., streptolysin O, pneumolysin O, perfringolysin O, listeriolysin O) are cholesterol dependent and require the presence of a reducing agent, such as cysteine, in order to obtain hemolytic activity (9).

Another factor, known as the CAMP factor, first described by Christie, Atkins, and Munch-Petersen (10), has been used for microbiological identification of *Streptococcus agalactiae* (group B streptococci, GBS) since it characteristically synergizes with the secreted β-hemolysin of *S. aureus* to lyse erythrocytes on blood agar plates (11). In *Clostridium perfringens* (12) and *Corynebacterium pseudotuberculosis* (13, 14) however, the rare antagonistic interaction (reverse CAMP phenomenon) was described where the β-hemolysis of staphylococci was inhibited apparently through the activity of a phospholipase D (PLD) (14).
Mycoplasma hyorhinis was first isolated from the respiratory tract of young pigs and has been implicated in a variety of diseases in swine (15, 16). *M. hyorhinis* is also one of the most common *Mycoplasma* species that contaminate various cell lines (17). Recently, we identified that a *M. hyorhinis* (strain MCLD) is able to invade nonphagocytic eukaryotic cells (18). This organism possesses a phospholipase A involved in the plasma membrane disruption, but not phospholipase C or PLD activities (19).

In the present study we show that *M. hyorhinis* possess a unique cholesterol independent heat- and protease-stable β-hemolysin activity. Interestingly, we found that *M. hyorhinis* display the rare reverse CAMP phenomenon resulting in the protection of RBCs from the β-hemolytic activity of *S. aureus*. Our results show for the first time that cardiolipin synthetase (CLS) of *M. hyorhinis*, which contains the two PLD conserved motifs, induces the reverse CAMP reaction.
Materials and Methods

Organisms and growth conditions

*M. hyorhinis* (MCLD), *M. gallisepticum* (Rlow), *M. fermentans* (JER), *M. mycoides* (PG1), *M. pneumoniae* (M129), *M. capricolum* (California kid), *M. penetrans* (GTU) and *M. hominis* (PG21) were from our strain collection. The organisms were grown for 48-72 h at 37°C in a modified Hayflick’s medium (20) containing either 10% fetal calf serum or 5% horse serum. Mycoplasmal growth was monitored by measuring the absorbance at 595 nm and by recording pH changes in the growth medium. The organisms were collected by centrifugation at 12 000 x g for 20 min, washed twice, and resuspended in a cold solution of Tris-HCl 10 mM, NaCl 250 mM (TN buffer, pH 7.5).

Total protein was determined and adjusted to a concentration of 1 mg·ml⁻¹.

*Staphylococcus aureus* and *Listeria monocytogenes* were obtained from the strain collection of the Department of Clinical Microbiology and Infectious Diseases, Hadassah Medical Center, Jerusalem, Israel, and grown on 5% sheep blood trypticase soy agar (TSA) plates (Novamed, Jerusalem, Israel).

Preparation of mycoplasmal fractions

Mycoplasma membrane and cytosolic preparations were obtained by ultrasonic treatment of washed intact cells as described (19). Membranes were separated from the supernatant fraction by centrifugation in the cold at 37 000 x g for 30 min. To obtain the cytosolic fraction, the supernatant was further centrifuged at 100 000 x g for 2 h to remove membrane fragments and ribosomes. The cytosolic fraction was kept at -70°C until used.
Hemolytic activity of *M. hyorhinis*

Qualitative hemolysis was determined by plating intact *M. hyorhinis* cells harvested at the stationary phase of growth (5 µl of 1 mg·ml\(^{-1}\) cell protein) on 5% sheep blood TSA plates (Novamed, Jerusalem, Israel) and incubated at 37°C. After 2 to 3 days, the plates were examined for hemolysis. Quantitative hemolysis was determined spectrophotometrically using sheep blood samples (Novamed, Jerusalem, Israel), pig blood samples (Lahav, C.R.O Israel) or chicken blood samples (from White Leghorn SPF chickens). The blood samples were washed twice in PBS and diluted to a final concentration of 2% packed cells. Hemolytic activity was determined as described before (4). In brief, 50 µg protein of intact *M. hyorhinis* cells, purified membranes or cytosolic fraction were incubated with 2% packed RBCs in a total volume of 1 ml (to be referred as the test mixture), in the presence or absence of 2-4 mM cysteine (Merck) for 18 h at 37°C in a rotator shaker (30 rpm). To detect RBC lysis, the test mixture was centrifuged at 1500 x g for 10 min and measurement of the released hemoglobin was spectrophotometric determined at 540 nm. Cooperative hemolysis (CAMP or reversed CAMP phenomena) was carried out as described before (10) with the following modifications. 20 µl of intact mycoplasma cells (5 mg·ml\(^{-1}\) cell protein) were inoculated onto sheep blood agar plates by making a streak down the center of each plate. The plates were incubated at 37°C for 48-72 h until β-hemolysis was clearly seen. *S. aureus* was then streaked with a loop perpendicular to the mycoplasmas being tested. CAMP activity was assessed as the enlargement of the hemolytic zones (positive CAMP) after re-incubation of the plates at 37°C for 48 h, as an inhibition of the hemolytic zone (reverse CAMP) after re-incubation of the plates at 37°C for 48-72 h or as the failure of bacteria
to exhibit an enhanced hemolysis when grown near colonies of the β-hemolytic S. aureus (negative CAMP). M. gallisepticum transformants were grown at 37°C and then heat-shocked at 42°C for 3 hours before carrying out the CAMP test.

Cloning of cls from M. hyorhinis

The cls gene (SRH_00920) was cloned from M. hyorhinis genomic DNA with a commercial PCR kit (HotMasterMix kit, 5 PRIME, Inc. Gaithersburg, MD 20878, USA) with the forward primer Mh_cls_F

(\text{ATATGCGGCCGC}AAGCAAATGAAAAATAAAAGAAGAAA) that includes a \text{NotI} restriction site and the reverse primer Mh_cls_R

(\text{TATAGGCCAGCAAGCCGTTTTCTTTCCAAGCGTAAGCAA}) that includes a \text{SfiI} restriction site. The single 1.56 kbp PCR product containing the cls gene was purified with a commercial PCR kit product (Wizard SV gel and PCR cleanup system, Promega, Madison WI, USA) and verified by sequencing.

Construction of the pMT85:cls plasmid

The purified cls PCR product was inserted into the plasmid pMT85 (5.60 kbp) containing the mini \text{Tn400l} transposon, a DNA fragment coding for the TAP-tag, the expression unit of the heat-shock inducible gene mpn531 (\text{clpB}) from M. pneumoniae and an aminoglycoside antibiotics resistance determinant as a selectable marker which confers resistance to kanamycin on E. coli and to gentamycin on mycoplasmas. Both, the plasmid and the purified cls PCR product were double-digested by \text{NotI} and \text{SfiI} (New England
Biolabs, Ipswich MA, USA) and ligated with T4 DNA ligase (New England Biolabs, Ipswich MA, USA) yielding the plasmid pMT85:cls (7.18 kbp).

Transformation of competent E. coli DH5α and plasmid purification

Plasmids were purified from E. coli DH5α with the Plasmid DNA extraction kit (iNtRON Biotechnology, Gyeonggi-do, Korea) and double-digested by NotI and SfiI. The excised inserts were amplified by PCR and verified on a 1% agarose gel. The identity of the insert was confirmed by sequencing the plasmid with primers from both ends of the cls gene: pMT-cls_Nter_F (ATGTCCCTTGTGTGAAGGT) and pMT-cls_Nter_R (CGCGTCTGGCCTTCCTGTAGC) for the N-terminal end and pMT-cls_Cter_F (ACTTTCGGCGCCTGAGCATC) and pMT-cls_Cter_R (GCCAAGAGCTTCAAAAACGAAGGAGC) for the C-terminal end.

Transformation of M. gallisepticum

Transformation of M. gallisepticum with the pMT85:cls was done by electroporation (21). After electroporation, the bacteria were allowed to recover in an antibiotics-free Hayflick’s medium and then diluted in tissue culture tubes with a medium containing gentamicin (80 μg·ml⁻¹) and incubated at 37°C until the medium changed the color from red to orange. The grown bacteria were centrifuged at 12,000 x g for 10 min, washed twice in TN buffer, diluted and plated on agar plates containing gentamicin (80 μg·ml⁻¹). Colonies were excised from the agar plate, transferred to tubes with Hayflick’s medium containing gentamicin (80 μg·ml⁻¹) and incubated at 37°C until the medium changed color from red to orange. The successful transformation of M. gallisepticum was evaluated and confirmed by PCR with the pMT85:cls sequencing primers.
Gene Expression Analysis

RT-qPCR was used to analyze the transcription levels of the cls gene in *M. hyorhinis* and *M. gallisepticum* transformants. RNA was purified from bacteria in mid-log growth using the PureLink RNA Mini Kit (Ambion). 1 μg of RNA was reverse transcribed to cDNA with the use of a High-Capacity Reverse Transcription Kit (Applied Biosystems). RT-qPCR was performed on 10 ng of cDNA using SYBER Green with the StepOnePlus RT-PCR system (Applied Biosystems). The relative expression of bacterial genes was determined by comparing their transcript levels with those of the bacterial 16S rRNA as a reference gene.

Analytical methods

Protein concentration was analyzed by the Bradford method (Bradford, 1976). Genomic DNA from *M. hyorhinis* was extracted and purified with the MasterPure Gram Positive DNA Purification Kit (Epicentre Biotechnologies Chicago, IL, USA). Thermal-treatment of intact *M. hyorhinis* was achieved by boiling for 10 min. Proteolysis of intact *M. hyorhinis* was achieved by incubation with proteinase K 5 μg·ml⁻¹ or trypsin 20 μg·ml⁻¹ for 30 min at 37°C.
Results

β-hemolytic activity of M. hyorhinis

Incubation of M. hyorhinis (equivalent to 5µg) on 5% sheep blood TSA plates for 48-72 h at 37°C revealed a clear zone of hemolysis surrounding the mycoplasma colonies indicating β-hemolysis activity (Fig. 1A). Fractionation of M. hyorhinis revealed that the membrane fraction possesses hemolytic activity while the cytosolic fraction does not (Fig. 1A). When the hemolytic activity of M. hyorhinis was monitored spectrophotometrically, both intact M. hyorhinis cells (Fig. 1B) and purified membranes (Fig. 1C) incubated with 2% packed RBCs (18 h at 37°C) were able to hemolyze RBCs from sheep, chicken and pig in a cysteine-dependent manner (2-4 mM). The hemolytic activity was not affected by glutathione (20 mM) but was completely inhibited by N-Ethylmaleimide (4 mM, data not shown) suggesting that a sulfhydryl group is essential for the activity of the M. hyorhinis hemolysin.

Many bacterial hemolysins are proteinaceous and are inactivated by heat and proteolysis (9). Interestingly, thermal treatment (boiling for 10 min) or proteolysis (treatment by 5 µg·ml⁻¹ of proteinase K or 20 µg·ml⁻¹ trypsin for 30 min at 37°C) of intact M. hyorhinis cells had no effect on the β-hemolysis activity (data not shown). These results suggest that M. hyorhinis produce a heat- and proteolysis-stable hemolysin. Some hemolysins (e.g., listeriolysin O, streptolysin O, perfringolysin O) are part of the pore-forming toxins. These hemolysins exhibit an absolute dependence on host cell membrane cholesterol resulting in the formation of extraordinarily large pores and cell lysis (9). Interestingly, pre-treatment of sheep RBCs with methyl-β-cyclodextrin (2 mM for 30 min) which depleted the membrane cholesterol level by 50% (22) had no effect on
The β-hemolysis activity of *M. hyorhinis* but inhibited the β-hemolysis activity of *Listeria monocytogenes* by 50-60% (data not shown). These results suggest that the β-hemolysin of *M. hyorhinis* acts in a cholesterol independent manner.

### M. hyorhinis induces the reverse CAMP phenomenon

β-hemolysis activity of some pathogenic bacteria (e.g., *S. agalactiae, L. monocytogenes*) is associated with a putative CAMP like factor (23, 24). Therefore, β-hemolysis of *M. hyorhinis* was further characterized using the CAMP test as described in Materials and Methods. Analysis of the CAMP phenomenon in various *Mycoplasma* species revealed a positive CAMP phenomenon with *M. fermentans, M. hominis* and *M. gallisepticum*, a negative CAMP phenomenon with *M. pneumoniae* and the rare reverse CAMP phenomenon with *M. hyorhinis, M. capricolum* and *M. mycoides* (As illustrated in Fig. 2). Interestingly, analysis of the CAMP phenomenon by *M. penetrans* revealed a unique CAMP phenotype, a positive CAMP phenomenon combined with the reverse CAMP phenomenon (Fig. 2).

The reverse CAMP phenomenon was suggested to be attributed to the activity of PLD (14). *M. hyorhinis* does not possess PLD activity (19), however, we detected in *M. hyorhinis* the two conserved PLD domains (HKD motifs; HxK(x)4D(x)6GSxN) in the *cls* gene encoding cardiolipin synthetase (CLS, accession number: AEC45753.1) residing between residues 253-270 and residues 440-457 (Fig. 3A, 3B). Both PLD domains share close homology with the PLD active sites of the cloned *Streptomyces* sp. YU100 PLD (25) (Fig. 3B). Interestingly, we have found a correlation between the presence of the *cls* gene and the induction of the reverse CAMP phenomenon (Table 1) in representative...
Mycoplasma species. In all Mycoplasma species analyzed, the cls genes analyzed contained the two HKD motifs. This correlation raised the possibility that the reverse CAMP phenomenon, attributed to PLD activity (14), might also be induced by CLS.

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**cls gene can induce reverse CAMP in M. galispticum**

230 The association of the reverse CAMP phenomenon with CLS activity was further investigated by transforming the pMT85:cls construct (7.18 kbp) (Fig. 4A) harboring the cls gene (1.56 kbp) from *M. hyorhinis* or the pMT85 plasmid (as a negative control) into *M. gallisepticum*, that has neither the cls gene nor the reverse CAMP phenomenon. Successful transformation was confirmed by PCR with pMT85:cls specific primers. The transformed mycoplasmas harboring pMT85:cls were able to induce transcription of the cls gene compared to the particularly unnoticeable transcriptional level in the transformants harboring pMT85 (Fig. 4B). The expression of cls was regulated by the heat-shock inducible promotor, clpB, therefore, the induction of cls by *M. gallisepticum* (pMT85:cls) occurred only after a heat-shock induction of 42°C for 3 h (Fig. 4B).

241 Furthermore, transformation of *M. gallisepticum* by the pMT85:cls plasmid, resulted in the rare reverse CAMP phenomenon with *M. gallisepticum* transformants characterized by an inhibition of the β-hemolytic activity of *S. aureus* at the junction between these two microorganisms (Fig. 4C). Since *M. gallisepticum* had also positive CAMP phenotype the overall picture was similar to the *M. penetrans* CAMP phenotype (Fig. 2). As expected, transformation of *M. gallisepticum* by the empty pMT85 plasmid, resulted in the positive CAMP phenotype as shown in Figure 2.
Discussion

Bacterial hemolysins are important virulence factors found in many pathogenic microorganisms. Hemolysis implies disruption of the cell membrane, and the action of hemolytic factors and hemolysins are not confined to the membrane of blood cells (7, 26). Hemolytic activity has several possible roles during invasion of host cells by *M. hyorhinis* (18). Disruption of the host cell membrane would facilitate the invasion of *M. hyorhinis* and also provide access to nutrients released from within the host cells. We show here that *M. hyorhinis* possess β-hemolysis activity resulting in total hemolysis of sheep RBCs. As shown with *Leptospira interrogans* (27), *S. aureus* (28), and certain *Vibrio* species (29-32), RBCs from different animal species have been shown to vary in their sensitivity to *M. hyorhinis* hemolysin. *M. hyorhinis* hemolysis was most pronounced with sheep and chicken RBCs. Fractionation of *M. hyorhinis* revealed that the hemolytic activity is located solely in the membrane fraction. Membrane associated hemolytic activities were described in a variety of *Mycoplasma* species including: *M. pulmonis*, *M. hyopneumoniae*, *M. bovis*, *M. capricolum*, *M. gallisepticum* and *M. pneumoniae* (5, 8). It was suggested that the wide distribution of the membrane-associated hemolytic activity in mycoplasmas contributes to the survival of these microorganisms. Because of the unique structural and biochemical characteristics of mycoplasmas, these microorganisms acquire macromolecular precursors from their environment (5). During active infections these are incorporated from host cell membranes and intracellular pools for fatty acids, phospholipids, cholesterol and nucleic acid precursors.

A reducing agent such as cysteine was required for the hemolytic activity of *M. hyorhinis*. This requirement suggests that *M. hyorhinis* possess an oxygen-labile
hemolysin. Oxygen-labile hemolysins were detected in a variety of microorganisms (33-35). The observed cysteine-dependent hemolytic activity of *M. hyorhinis* suggests the presence of a sulfhydryl group that has to be in a reduced state for lytic activity. In other oxygen-labile hemolysins, the essential sulfhydryl group is contained in a single cysteine residue near the carboxy-terminal region of the hemolysin (33).

Another property of some oxygen-labile β-hemolysins is their dependency on host cholesterol. Such hemolysins are produced by *Clostridium perfringens*, *S. pyogenes* and *L. monocytogenes* (9). These hemolysins are secreted soluble proteins, which upon encountering a eukaryotic cell, undergo a transformation from a soluble monomeric protein to a membrane-embedded supramolecular pore complex (33). The β-hemolysin of *M. hyorhinis* was neither cholesterol-dependent nor inhibited by proteolysis or by heat-treatment, suggesting the non-proteinaceous nature of this hemolysin. Heat- and/or proteolysis-stable hemolysins were described before (36-38) such as the heat-stable β-hemolysin of *Pseudomonas aeruginosa*, that was identified as a glycolipid (39).

A variety of bacterial species display a hemolytic cooperativity or a “CAMP like” activity with *S. aureus* (11, 40-42), mainly used in classical diagnostics. In the vicinity of the culture of these species and a culture of *S. aureus*, a zone of enhanced hemolytic activity occurs (synergistic hemolysis). For example, pathogenic *Listeria* species produce a synergistic hemolysis with *S. aureus* (41) attributed to a sulfhydryl-activated cytolysin and a sphingomyelinase C activity. To date, this report is the first to describe the CAMP test in mycoplasmas. Analysis of the CAMP test in various *Mycoplasma* species revealed the positive CAMP reaction with *M. fermentans*, *M. hominis* and *M. gallisepticum*; a negative CAMP reaction by *M. pneumoniae*; and with *M. hyorhinis*, *M. capricolum* and
M. mycoides is antagonistic to hemolysis with S. aureus at the vicinity of the culture of these mycoplasmas (reversed CAMP activity). Interestingly, M. penetrans showed a unique phenotype composed by two CAMP phenomena, the positive CAMP and the reverse CAMP. We were able to create this phenotype by transforming the CAMP positive M. gallisepticum with the cls gene of M. hyorhinis and therefore, we assume that these two phenomena are independent in mycoplasmas.

Indeed, previous studies have shown an association between the putative CAMP factor and virulence (43, 44). Rühlmann et al (45) have showed that the CAMP factor of GBS, a 25 kDa named protein B, binds to immunoglobulins in a way similar to that of protein A of S. aureus. The revelation of the mycoplasmal putative CAMP factor merits further investigations.

The reverse CAMP phenomenon, described before in Corynebacterium pseudotuberculosis (13, 14), was suggested to be associated with PLD activity. We have shown previously that M. hyorhinis does not possess PLD activity (19, 46) and we were unable to detect the pld gene homolog in M. hyorhinis genome. However, we identified the two conserved domains of PLD (HKD motif; HxK(x)4D(x)6GSxN) in M. hyorhinis cls gene encoding CLS, a characteristic of all prokaryotic CLSs (47). Furthermore, the conserved HKD motif-containing PLD superfamily was shown to be a part of the active site of the enzyme (48) playing a role in the catalysis of phosphatidylcholine, the major membrane component of RBCs (49). The correlation between the presence of the cls gene and the induction of the reverse CAMP phenomenon by a variety of Mycoplasma species, led to the possibility that the rare reverse CAMP phenomenon, previously shown to be associated with PLD activity (14), is induced in mycoplasmas by CLS. Indeed,
transformation of *M. gallisepticum* that has neither the *cls* gene nor the reverse CAMP phenomenon by the pMT85 plasmid harboring the *cls* gene from *M. hyorhinis* resulted in the rare reverse CAMP phenomenon with the *M. gallisepticum* transformants, suggesting for the first time that the reverse CAMP can be induced by CLS.
Acknowledgements

The authors are grateful to Dr. Anat Hershkovits and Dr. Nadejda Sigal, University of Tel Aviv, Israel, for excellent technical assistance with this work, and thank Richard Herrmann, University of Gottingen, Germany, for the gift of the plasmid pMT85.
References


Table 1. Correlation between the presence of the cls gene and the induction of the reverse CAMP phenomenon.

<table>
<thead>
<tr>
<th>Mycoplasma species (Accession number; strain)</th>
<th>Positive CAMP phenomenon</th>
<th>Negative CAMP phenomenon</th>
<th>Reverse CAMP phenomenon</th>
<th>cls gene</th>
<th>pld gene</th>
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<tr>
<td><em>M. capricolum</em> (NC_007633; California kid)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>M. hyorhinis</em> (NC_017519; MCLD)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>M. mycoides</em> (NC_005364; PG1)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>M. penetrans</em> (NC_004432; HF2)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>M. fermentans</em> (NC_014552; JER)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. gallisepticum</em> (AE015450; Rlow)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. hominis</em> (NC_013511; PG21)</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td><em>M. pneumoniae</em> (NC_000912; M129)</td>
<td>-</td>
<td>+</td>
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The CAMP test was performed using 5% sheep blood TSA plates as described in Materials and Methods. *In silico* analysis of the mycoplasma genomes, based on sequence homology, was obtained by the NCBI genome database (http://www.ncbi.nlm.nih.gov).
**Figure legends**

**Fig. 1.** The hemolytic activity of *M. hyorhinis.*

A. *M. hyorhinis* intact cells or purified membranes induced β-hemolysis of sheep blood. Either cells, membranes or cytosol (5 µg) were plated on 5% sheep blood TSA plates in the presence or absence of cysteine (0.1 mM) and hemolysis was documented after 48 h of incubation at 37°C.

B. *M. hyorhinis* β-hemolysis is cysteine-dependent. *M. hyorhinis* intact cells (50 µg) were incubated with sheep, chicken or pig red blood cells (RBCs) at a final concentration of 2% packed cells, for 18 h at 37°C. While no major hemolysis was observed in solution, the addition of cysteine (2-4 mM) increased hemolysis significantly. The hemolytic activity was monitored spectrophotometrically at 540 nm as described in Materials and Methods. The results are means ± standard deviations of three separate sets of experiments.

C. Cysteine-dependent β-hemolysis by *M. hyorhinis* purified membranes. *M. hyorhinis* purified membranes (50 µg) were incubated with sheep, chicken or pig red blood cells (RBCs) for 18 h at 37°C in the presence or absence of cysteine (2-4 mM). The hemolytic activity was monitored spectrophotometrically at 540 nm as described in Materials and Methods. The results are means ± standard deviations of three separate sets of experiments.

**Fig. 2.** CAMP effects of various *Mycoplasma* species. The CAMP test was performed using 5% sheep blood TSA plates as described in Materials and Methods. The CAMP
reactions were documented after 48hr of incubation of the *Mycoplasma* species with *S. aureus*.

**Fig. 3.** Similar to the bacterial PLD amino acid sequence, the cardiolipin synthetase of *M. hyorhinis* contains two phospholipase D domains.

A. Amino acid sequence of the cardiolipin synthetase of *M. hyorhinis* (518 AA; Accession number: AEC45753.1). Amino acid residue sequence in bold and underlined corresponds to the two phospholipase D (PLD) domains.

B. The PLD motifs in the CLS of *M. hyorhinis* share close homology with the PLD active sites of *Streptomyces* sp. YU100. The PLD motifs from the *M. hyorhinis* CLS and the *Streptomyces* sp. YU100 PLD (Accession number: AEC45753.1 and ABY71835.1 respectively) were aligned and the sequence logo was generated using the Geneious software (http://www.geneious.com). The logo shows the information content at each position (CLS1; Domain 1 of the PLD motif in CLS, CLS2; Domain 2 of the PLD motif in CLS, PLD1; Domain 1 of the PLD motif in PLD, PLD2; Domain 2 of the PLD motif in PLD).

**Fig. 4.** Cardiolipin synthetase (*cls*) induces the reverse CAMP phenomenon.

A. Description of the plasmid pMT85: *cls*. The plasmid was constructed by inserting the *cls* gene into the *Not*I and *Sfi*I sites of the plasmid pMT85 resulting in the plasmid pMT85: *cls* as described in Materials and Methods (The plasmid image was adapted from Catrein and Herrmann, 2011).
B. RT-qPCR analysis of cls by M. gallisepticum transformants. Transcription levels are presented as relative quantity (RQ), cls expression by M. hyorhinis versus M. gallisepticum transformants. mRNA levels were normalized to 16S rRNA. The data represents 2 biological repeats (N=2). Error bars indicate a 95% confidence interval.

Black bars, cls transcripts of mycoplasmas grown at 37°C; White bars, cls transcripts of mycoplasmas grown at 37°C and then heat-shocked at 42°C for 3 hours. As expected cls transcript is induced only after heat-shock due to the heat sensitive promoter of the pMT85.

C. The reverse CAMP effect is induced by M. gallisepticum (pMT85:cls) transformants. The transformants were grown at 37°C and then heat-shocked at 42°C for 3 hours before carrying out the CAMP test. The CAMP test was performed on 5% sheep blood TSA plates as described in Materials and Methods. CAMP reactions were photographed after 72 h of incubation of the transformants with S. aureus. A reverse CAMP was obtained by M. gallisepticum (pMT85:cls) and a positive CAMP reaction by M. gallisepticum (pMT85).
**A**

![M. hyorhinis intact cells, membranes, cytosol with and without cysteine](image)

**B**

<table>
<thead>
<tr>
<th>RBS species</th>
<th>No cysteine added</th>
<th>2 mM cysteine</th>
<th>4 mM cysteine</th>
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<tr>
<td>Sheep</td>
<td>0</td>
<td>0.55 ± 0.02</td>
<td>0.52 ± 0.06</td>
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<tr>
<td>Chicken</td>
<td>0</td>
<td>0.69 ± 0.04</td>
<td>0.65 ± 0.08</td>
</tr>
<tr>
<td>Pig</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th>RBS species</th>
<th>No cysteine added</th>
<th>2 mM cysteine</th>
<th>4 mM cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>0</td>
<td>0.77 ± 0.06</td>
<td>1.34 ± 0.23</td>
</tr>
<tr>
<td>Chicken</td>
<td>0</td>
<td>0.79 ± 0.08</td>
<td>0.68 ± 0.07</td>
</tr>
<tr>
<td>Pig</td>
<td>0</td>
<td>0</td>
<td>0.47 ± 0.02</td>
</tr>
</tbody>
</table>

*M. hyorhinis* purifies membranes incubated with red blood cells (RBCs)
<table>
<thead>
<tr>
<th>Mycoplasma species</th>
<th>M. penetrans</th>
<th>M. hyorhinis</th>
<th>M. pneumoniae</th>
<th>M. gallisepticum</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAMP phenomenon</td>
<td>Positive CAMP</td>
<td>Reverse CAMP</td>
<td>Negative CAMP</td>
<td>Positive CAMP</td>
</tr>
</tbody>
</table>
M. gallisepticum transformant
CAMP phenomenon
M. gallisepticum (pMT85)
C
(pMT85:cls)
M. gallisepticum (pMT85)