INFECTION AND IMMUNITY, Mar. 2003, p. 1265–1273 Vol. 71, No. 3
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.3.1265–1273.2003

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

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Received 4 September 2002/Returned for modification 23 October 2002/Accepted 21 December 2002

***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

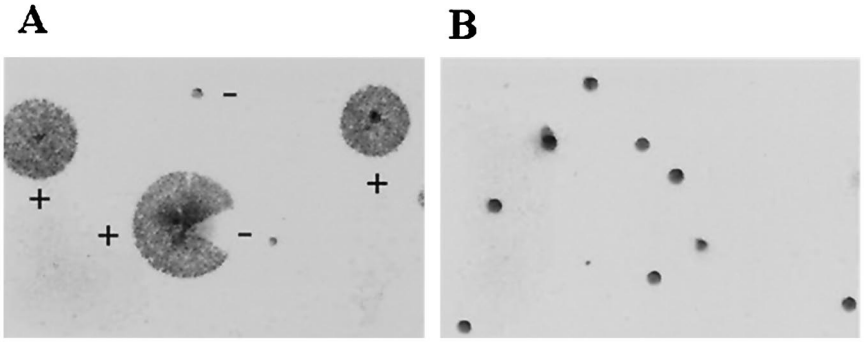

FIG. 1. Binding of erythrocytes to *M. gallisepticum* colonies. Mycoplasma colonies were partially transferred onto nitrocellulose and incubated with erythrocytes as described in Materials and Methods. Hemadsorbing colonies (+) and nonhemadsorbing colonies (−) were observed under a stereomicroscope (magnification, ×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

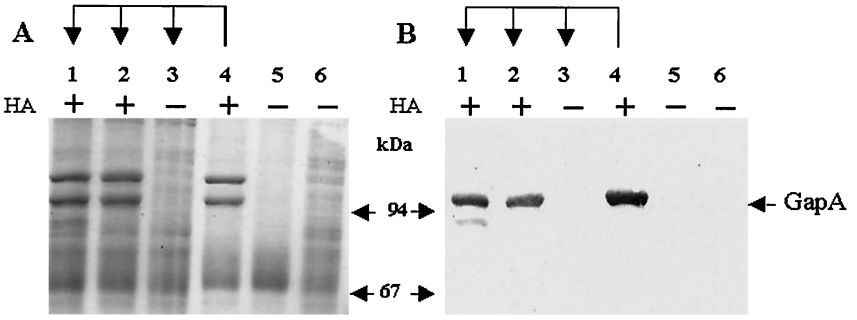

FIG. 2. Identification of proteins involved in HA of *M. gallisepticum* strain R. Whole organisms were subjected to Triton X-114 phase fractionation, and insoluble proteins were analyzed by SDS-PAGE, followed by Coomassie blue staining (A) or Western blot analysis (B), with a rabbit polyclonal antibodies 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.

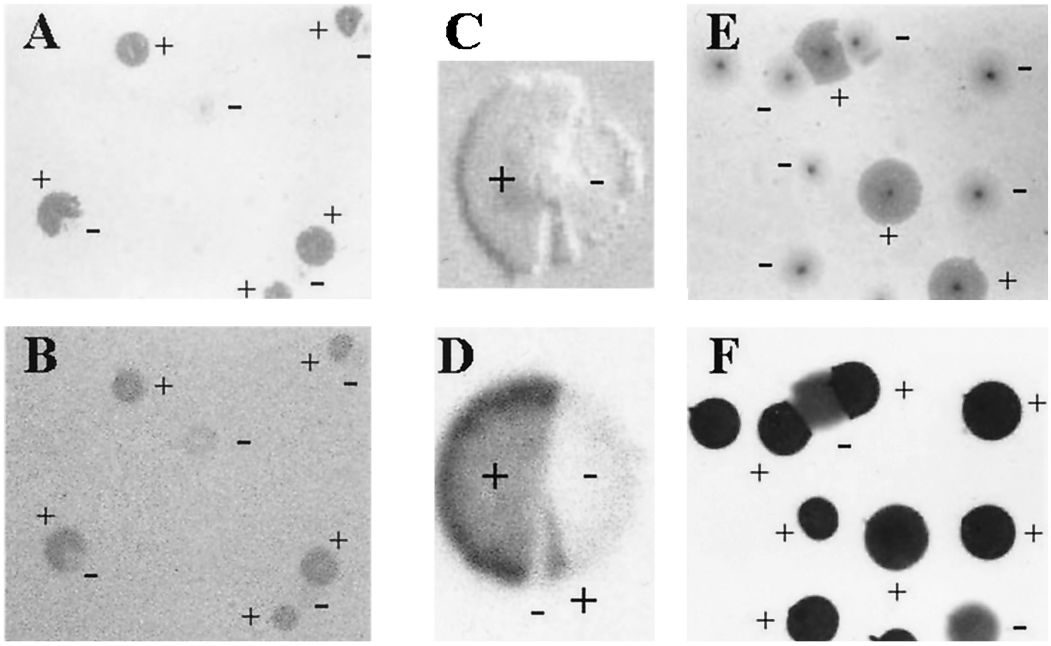

FIG. 3. Correlation between HA of erythrocytes to *M. gallisepticum* colonies and surface expression of GapA. Colonies derived from R_{low} (A, B, E, and F) or from RCL2 (C and D) were partially transferred onto nitrocellulose and then incubated with erythrocytes (A, C, and E), although the corresponding 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

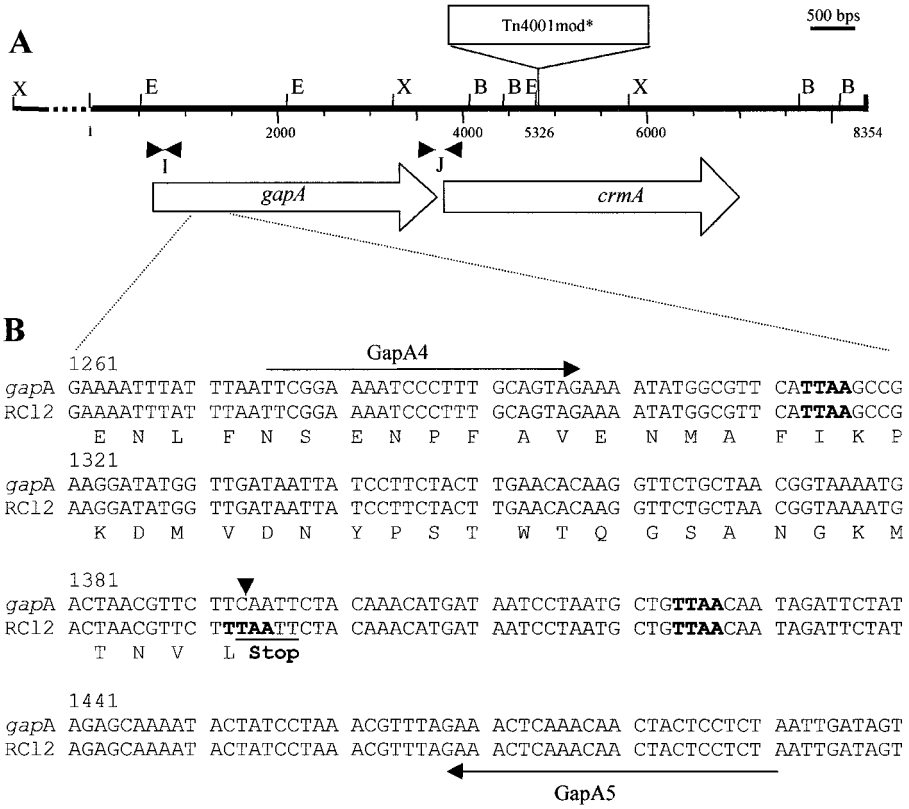

FIG. 4. (A) Schematic representation of the *gapA* and *crmA* gene organization based on the sequence published by Papazisi et al. (24) and localization of the transposon Tn*4001*mod in the mHAD3 mutant. Arrows below the solid line represent the primers used in the present study. (B) The sequence alignment 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

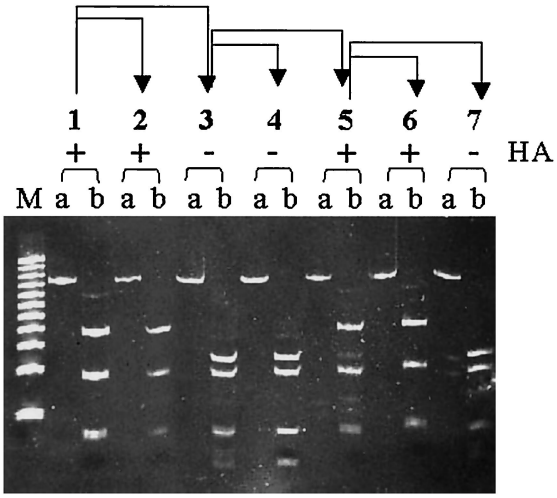

FIG. 5. Detection of an additional *Mse*I restriction site in HA$^-$ clones. The DNA region that encompassed the mutation detected in clone RCL2 was amplified in HA$^+$ and HA$^-$ variants and subjected to *Mse*I digestion. Undigested (lanes a) and digested (lanes b) PCR fragments were analyzed by electrophoresis 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

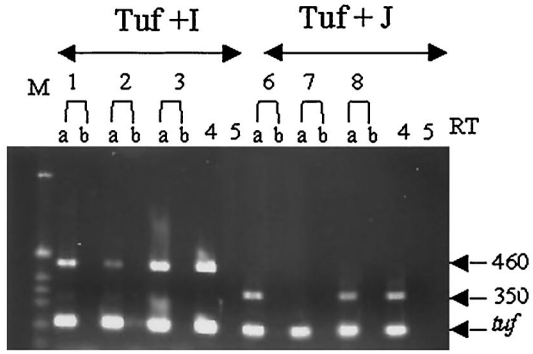

FIG. 6. Analyses of the *gapA* and *crmA* mRNA. A multiplex RT-PCR was applied to total RNA extracted from clones RCL1 (lanes 1 and 6), and RCL2 (lanes 2 and 7), and mutant mHAD3 (lanes 3 and 8) by using the Tuf primers in combination with the I or the J primers as indicated above the gel. Assays were 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.

ACKNOWLEDGMENTS

This work was supported in part by grant P13215-GEN (C.C. and R.R.) from the Fonds zur Förderung der wissenschaftlichen Forschung and by grant 9137 from the Jubiläumsfonds of the Austrian National Bank.

We thank S. J. Geary (University of Connecticut, Storrs, Conn.) for providing the anti-GapA serum and for helpful discussions.

F.W. and I.M. contributed equally to this study.

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Editor: B. B. Finlay