INFECTION AND IMMUNITY, Dec. 2002, p. 6839–6845 Vol. 70, No. 12
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.12.6839–6845.2002

GapA and CrmA Coexpression Is Essential for *Mycoplasma gallisepticum* Cytadherence and Virulence

L. Papazisi,[1,2] S. Frasca Jr.,[2] M. Gladd,[1,2] X. Liao,[1,2] D. Yogev,[3] and S. J. Geary[1,2]*

Center of Excellence for Vaccine Research[1] and Department of Pathobiology and Veterinary Science,[2] The University of Connecticut, Storrs, Connecticut 06269, and Department of Membrane and Ultrastructure Research, The Hebrew University Hadassah Medical School, Jerusalem, Israel[3]

Received 1 July 2002/Returned for modification 16 August 2002/Accepted 18 September 2002

It was previously demonstrated that avirulent *Mycoplasma gallisepticum* strain R_{high} (passage 164) is lacking three proteins that are expressed in its virulent progenitor, strain R_{low} (passage 15). These proteins were identified as the cytadhesin molecule GapA, the putative cytadhesin-related molecule CrmA, and a component of a high-affinity transporter system, HatA. Complementation of R_{high} with wild-type *gapA* restored expression in the transformant (GT5) but did not restore the cytadherence phenotype and maintained avirulence in chickens. These results suggested that CrmA might play an essential role in the *M. gallisepticum* cytadherence process. CrmA is encoded by the second gene in the *gapA* operon and shares significant sequence homology to the *ORF6* gene of *Mycoplasma pneumoniae*, which has been shown to play an accessory role in the cytadherence process. Complementation of R_{high} with wild-type *crmA* resulted in the transformant (SDCA) that lacked the cytadherence and virulence phenotype comparable to that found in R_{high} and GT5. In contrast, complementation of R_{high} with the entire wild-type *gapA* operon resulted in the transformant (GCA1) that restored cytadherence to the level found in wild-type R_{low}. In vivo pathogenesis trials revealed that GCA1 had regained virulence, causing airsacculitis in chickens. These results demonstrate that both GapA and CrmA are required for *M. gallisepticum* cytadherence and pathogenesis.

Mycoplasma gallisepticum is one infectious agent initiating the chronic respiratory disease complex in chickens and is the primary agent of infectious sinusitis in turkeys (74). This bacterium has developed a wide array of surface molecules that are involved in cytadherence to host cells (9, 17, 22, 51). GapA is considered the primary cytadhesin. Goh et al. (21) identified its gene in *M. gallisepticum* based on its nucleotide sequence homology to the *Mycoplasma pneumoniae* cytadhesin *ADP1* gene. Subsequent studies showed that anti-GapA Fab fragments were able to significantly inhibit *M. gallisepticum* cytadherence (22). Troy (69) reported that GapA was absent in avirulent *M. gallisepticum*. These data led us to hypothesize that complementation of GapA expression in strain R_{high} via Tn*4001* might restore cytadherence and perhaps virulence. Neither cytadherence nor virulence was restored upon the *gapA*-complemented strain R_{high}, transformant GT5 (56, 57). This indicated that other factors might play an important role in *M. gallisepticum* cytadherence. Protein profile comparison between virulent and avirulent strains showed that, in addition to GapA, two other proteins are absent in R_{high} (69). One of these proteins was found to be encoded by the second gene of the *gapA* operon. Its gene product shows significant sequence homology with the precursor of *M. pneumoniae ORF6* gene products, which are known to play accessory roles in P1 (ADP1)-mediated cytadherence (35, 36, 40, 66). (MGPC is the new GenBank designation for the *M. pneumoniae* ORF6 and *M. genitalium* ORF6 homolog. *M. pneumoniae* MGPC gene encodes a 130-kDa protein which is posttranslationally cleaved into two products of 40 and 90 kDa by a special proteolytic event [accessory proteins B and C]. *M. gallisepticum* CrmA does not undergo such posttranslational modification. We will adhere to the new nomenclature in this paper.) This *M. gallisepticum* protein was designated as cytadherence-related molecule A, or CrmA.

The aim of this study was to evaluate the role of CrmA in *M. gallisepticum* cytadherence and virulence. We demonstrate that coexpression of *gapA* and *crmA* is essential for effective *M. gallisepticum* cytadherence and virulence.

MATERIALS AND METHODS

Strains and media. *M. gallisepticum* strains R_{low} and R_{high} (15 and 164 passages, respectively) were provided by Sharon Levisohn (formerly of the Department of Food Animal and Equine Medicine, North Carolina State University, Raleigh, N.C.). Generation of the GT5 transformant has been described previously (57). All strains were cultured at 37°C in Hayflick's medium (10% serum and 5% yeast extract) or on Hayflick's plates with 1% Agar Noble (Difco). Gentamicin was added to the liquid medium (final concentration, 300 μg/ml) for propagating the GT5, SDCA, and GCA1 transformants.

DNA extractions. *Mycoplasma* total genomic DNA was extracted according to the method of Hempstead (25). Plasmid DNA was extracted by using QIAprep Spin Miniprep (Qiagen, Valencia, Calif.) or minipreparations according to the protocol described by Engelbrecht at al. (16).

Immunoblot analysis. Proteins from 2×10^7 CFU of *M. gallisepticum* R_{low}, R_{high}, and transformants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (38) and then transferred onto a nitrocellulose membrane (Osmonics, Westborough, Mass.) according to the method of Towbin et al. (68). After blocking with 5% bovine serum albumin in phosphate-buffered saline (PBS) for 1 h at room temperature, the membranes were reacted with rabbit anti-GapA or anti-CrmA serum at dilutions of 1:8,000 and 1:50,000, respectively, for 2 h at 4°C with gentle rocking and then washed three times with 0.5% PBS–Tween. Membranes were then incubated with peroxidase-conjugated goat

* Corresponding author. Mailing address: Center of Excellence for Vaccine Research and Department of Pathobiology and Veterinary Science, University of Connecticut, 61 N. Eagleville Rd., U-89, Storrs, CT 06269. Phone: (860) 486-0835. Fax: (860) 486-2794. E-mail: geary@uconnvm.uconn.edu.

TABLE 1. Primers used to amplify *crmA* and the entire *gapA* operon, the probe for the Southern hybridization, and the gentamicin resistance gene

Primer	Sequence[a] (direction)	Gentamicin resistance gene
SG322	5′ GGGGGATCCAGACCAAACTTCCCTAAC 3′ (forward)	*gapA*
SG721	5′ GGGGGATCCCCTTATCGTAGAGAAGGGAGGT 3′ (reverse)	*crmA*
SG768	5′ AAGGGGATCCGCTCCAGCACCAACTAAGAAAATTGA 3′ (forward)	*crmA*
SG804	5′ GGCAATTATGATCATCTTAGGA 3′ (forward)	3′ *crmA*
SG805	5′ TAGAGAAGGGAGGTTATTTT 3′ (reverse)	3′ *crmA*
SG643	5′ ACACAGGAGTCTGGACTTGACTGA 3′ (forward)	GmR[b]
SG665	5′ TTACACAGGAGTCTGGACTTGACTCA 3′ (reverse)	GmR

[a] *Bam*HI restriction endonuclease sites are underlined.
[b] Gentamicin resistance gene from the Tn*4001*mod transposon.

anti-rabbit immunoglobulin G (Sigma, St. Louis, Mo.) at a final concentration of 1:15,000 for 1 h at 4°C with gentle rocking, followed by three consecutive washings with PBS-Tween. Reactions were visualized by the addition of 4-chloro-1-naphthol and hydrogen peroxide.

PCR. PCRs were performed in a total volume of 50 μl containing 50 ng of template. Primers (Table 1) were synthesized at the University of Connecticut Biotechnology Center. The *gapA* operon and *crmA* were amplified with primer pairs SG322-SG721 and SG768-SG721, respectively. The *gapA* operon was amplified with the same forward primer (SG322), which was used previously to amplify *gapA* alone to obtain GT5 (57). *Bam*HI sites were incorporated at the 5′ ends of each primer to facilitate cloning into the *Bam*HI site of Tn*4001*mod in the vector pISM2062 (33) (Fig. 1). The *gapA* operon and *crmA* were amplified by using the High-Fidelity Expand PCR kit (Roche Biochemicals, Indianapolis, Ind.) according to the manufacturer's instructions. The AmpliTaq kit (Applied Biosystems, Perkin Elmer, Norwalk, Conn.) was used for other PCRs as previously described (57). Briefly, the amplifications were performed under the following conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and 72°C for 2 min, followed by 1 cycle at 72°C for 10 min. A total of 2.5 U of AmpliTaq was used in each reaction.

Cloning and transformation. PCR products (*gapA* operon and *crmA*) were initially cloned into the pCR 2.1 TOPO vector according to the manufacturer's instructions and maintained in Top 10 *Escherichia coli* cells (Invitrogen, Carlsbad, Calif.). Extracted plasmids were digested with *Bam*HI (Roche Biochemicals), and the inserts were separated by electrophoresis in a 1% agarose gel. The bands were eluted from the agarose gel and purified with DNA Clean Concentrator 5 (Zymo Research, Orange, Calif.). Fragments were cloned into the *Bam*HI site of the transposon Tn*4001*mod in the plasmid pISM2062 (33) by using a ligation kit (Epicentre Technologies, Madison, Wis.). The constructs containing the *gapA* operon and *crmA* were designated GCA1 and SDCA, respectively (Fig. 1). R_{high} (from the same clonal isolate stock used to generate GT5 [57]) was transformed following the electroporation method of Minion and Kapke (52). Following transformation, single colonies were picked and propagated in Hayflick's medium containing gentamicin. Clones were initially screened by PCR for the gentamicin resistance gene. Positive clones were analyzed by immunoblotting with anti-GapA and anti-CrmA sera (Fig. 2A) and by Southern blot hybridization with a 402-bp ^{32}P-labeled portion of the 5′ end of *crmA* as a probe (primer pair

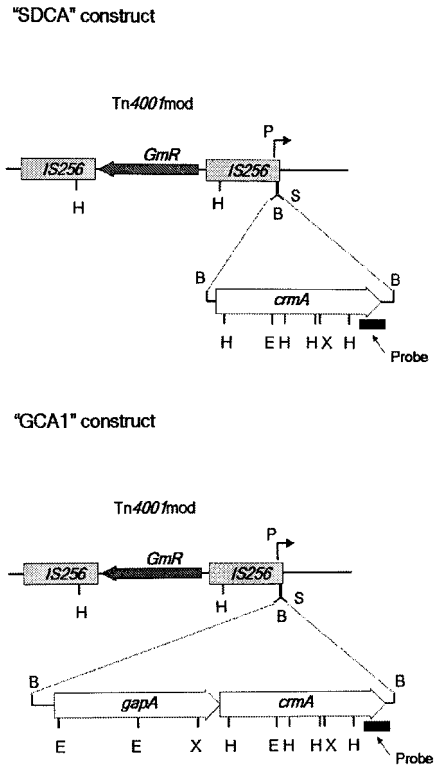

FIG. 1. Tn*4001*mod constructs containing the wild-type *gapA* and *crmA* gene from strain R_{low}. Schematic representation map of Tn*4001*mod constructs containing wild-type *crmA* alone (SDCA) or both *gapA* and *crmA* (GCA1, i.e., the whole *gapA* operon). *GmR* is the gentamicin resistance gene. P is the outward promoter. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I.

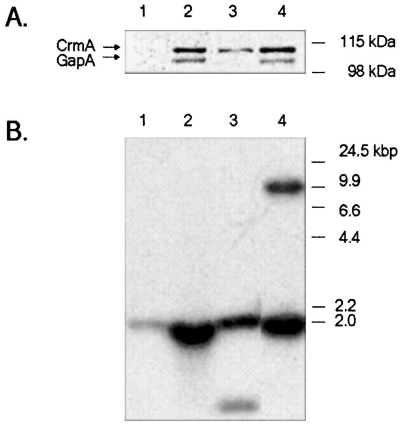

FIG. 2. Characterization of the *M. gallisepticum* R_{high} transformants SDCA and GCA1 by immunoblotting and DNA hybridization analysis. Lane 1, R_{high}; lane 2, R_{low}; lane 3, $SDCA_{c4}$; lane 4, $GCA1_{c5}$. (A) Immunoblots developed with mixed anti-GapA and anti-CrmA sera. (B) Southern blot of *Hin*dIII-digested total genomic DNAs probed with a ^{32}P-labeled portion of *crmA*. Lanes in this digitized image have been reordered by using Adobe PhotoShop.

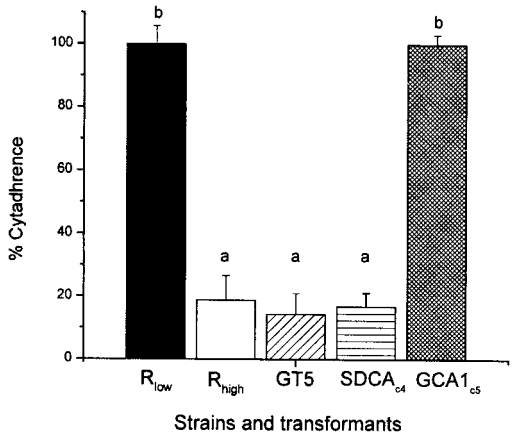

FIG. 3. Cytadherence assessment of *M. gallisepticum* transformants (data presented relative to the R_{low} cytadherence level of 100%). Bars labeled with the same letter are not significantly different ($P > 0.05$). Error bars represent standard deviations.

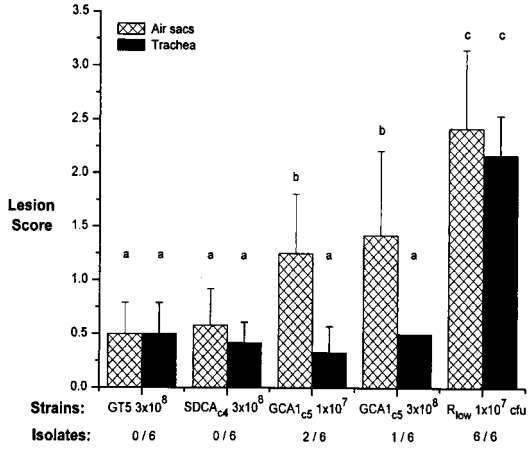

FIG. 4. Evaluation of the pathogenicities of different *M. gallisepticum* R_{high} transformants in the lower respiratory tracts of chickens. Bars labeled with the same letter are not significantly different ($P > 0.05$). Error bars represent standard deviations.

SG804-SG805) (Table 1 and Fig. 2B). SDCA clone 4 ($SDCA_{c4}$) and GCA1 clone 5 ($GCA1_{c5}$) were used for all of the experiments.

Cytadherence assays. MRC-5 cell culture cytadherence analysis was performed as described previously (17–19, 57) with tritium-labeled *M. gallisepticum* R_{low}, R_{high}, GT5, SDCA, and GCA1. These assays were performed twice using four replicates per strain or transformant.

Animals. Four-week-old, specific-pathogen-free White Leghorn chickens (SPAFAS, North Franklin, Conn.) were used for challenge experiments. Upon arrival, the birds were tagged, placed in HEPA filtered isolators (Controlled Isolator Systems, Pittsburgh, Pa.), and allowed to acclimate for 1 week in accordance with the approved Institutional Animal Care and Use Committee protocol. Nonmedicated feed (Blue Seal, Waltham, Mass.) and water were provided ad libitum.

Preparation challenge strains. Stocks were prepared as follows: 10-ml aliquots of mid-log-phase cultures were centrifuged at 10,000 × *g* and 4°C for 15 min and then resuspended in 100 μl of fresh medium and stored at −70°C. Four randomly chosen aliquots from each batch were thawed, and serial 10-fold dilution plate counts were performed to determine total CFU. On the day of administration, 100-μl aliquots were thawed, 900 μl of fresh medium was added to each tube, and the cultures were incubated for 5 h at 37°C. The titers of the organisms were established prior to administration to determine the dose administered to ensure consistency among all challenge experiments.

Challenge. Chickens were divided into five groups of six birds each. Birds were challenged on days 0 and 2 by intratracheal instillation of 100 μl of *M. gallisepticum* with a micropipette and disposable 200-μl pipette tips (Fisher Scientific, Fairlawn, N.J.). Birds in groups 1, 2, and 4 received 3×10^8 CFU of GT5, $SDCA_{c4}$, or $GCA1_{c5}$, respectively. Group 3 and 5 birds received 10^7 CFU of $GCA1_{c4}$ or R_{low}, respectively. Two weeks postchallenge (day 16), chickens were sacrificed, tracheal swabs were performed, and trachea and air sacs were prepared for histological examination. A previous study (56) demonstrated that 10^7 CFU of R_{low} delivered intratracheally caused significant gross, as well as histopathological, lesions in the lower respiratory tracts of birds, whereas 3×10^8 CFU of GT5 was avirulent. Therefore, groups of birds challenged with 3×10^8 CFU of GT5 and 10^7 CFU of R_{low} were used as negative and positive controls, respectively.

Gross and histopathological examination. Necropsy was performed as previously described (56). In addition to the collection of samples from tracheas, tissue samples from the cranial and caudal thoracic and abdominal air sacs were collected. Tissue samples were routinely processed, embedded in paraffin blocks, sectioned at 4 μm, stained with hematoxylin and eosin, and evaluated by light microscopy.

All gross necropsy examinations and evaluations of histological sections of trachea and air sacs were performed in a blind fashion. Evaluation of histopathological changes was based on the criteria of Nunoya et al. (54) and modified for the assessment of air sac lesions. Briefly, we implemented the following scoring system similar to that used to evaluate tracheal lesions (54, 56) as follows: 0, no significant findings; 0.5, minimal multifocal lymphocytic infiltrates with one to three discrete, small, widely separated foci; 1, mild multifocal lymphocytic or lymphofollicular infiltrates amounting to four or more discrete foci without stromal edema or heterophils; 2, moderate, multifocal lymphocytic or lymphofollicular infiltrates and loose interstitial infiltrates of lymphocytes, histiocytes, and heterophils with edema, intraepithelial heterophils, and occasional luminal exudates; 3, severe multifocal lymphofollicular aggregates to diffuse infiltrates of lymphocytes, histiocytes, and heterophils with edema, epithelial attenuation and hyperplasia, and luminal exudates.

Reisolation and quantitation of mycoplasmas. The mucosa of 5- to 7-cm-long segments of trachea was abraded with a cotton swab. Each swab was inoculated into 3 ml of Hayflick's medium in 15-ml sterile culture tubes and then gently vortexed to release attached mycoplasmas into the medium. The samples were filtered through 0.45-μm-pore size filters (Millipore, Bedford, Mass.) and then serially diluted (10-fold). Twenty microliters of each sample dilution was plated onto Hayflick's agar for CFU determination. All cultures were incubated at 37°C for 4 weeks and observed for color change (acid shift) or colony growth on plates.

Statistical analysis. SAS software version 8.01 (SAS Institute, Cary, N.C.) was used for statistical analysis. Attachment levels (percentages) and lesion scores were initially subjected to the arcsinus of the percentage square root and rank transformation, respectively, because they violated the normal distribution assumption required for parametric statistical analysis (75). Analysis of variance was used to determine the significant differences between groups. When differences were found, a mean separation analysis using Duncan's multiple range test was performed. Cytadherence levels of SDCA and GCA1 were compared to those of R_{high}. Lesion scores of the groups challenged with $SDCA_{c4}$ and $GCA1_{c5}$ were compared to those of groups challenged with GT5. Fisher's exact test was used to compare differences regarding the number of isolates between groups.

Sequence analysis. TMHMM (37, 53), PSIPRED (30), SAPS (10), and SSpro (3) were used to predict protein secondary structure. Sequence homology searches were performed on the BLOCKS, PROSITE, and Pfam databases (4, 11, 26; http://motif.genome.ad.jp/). FastA (58) and Patternmatch (http://workbench.sdsc.edu, University of San Diego Supercomputer Center, San Diego, Calif.) searches were performed with sequences from well-characterized binding domains. Heparin sulfate- and lectin-binding domains were obtained from published reports (27, 48, 49, 64, 65, 67, 70). Amino acid sequence homologies were considered significant if they possessed a ≥30% identity or similarity and the indicated functional motifs. Structural homology searches were performed using three-dimensional predicted protein secondary structure matrices (3D-PSSM) (5), GenTHREADER (29), and 123D+ (1). Vector NTI (Informax, Bethesda, Md.) was used for visualization of the domains in the protein sequence.

RESULTS

Complementation of R_{high} with wild-type *crmA* and the *gapA* operon. Immunoblot analysis indicated that all of the SDCA transformants expressed CrmA (Fig. 2A) and that GCA1

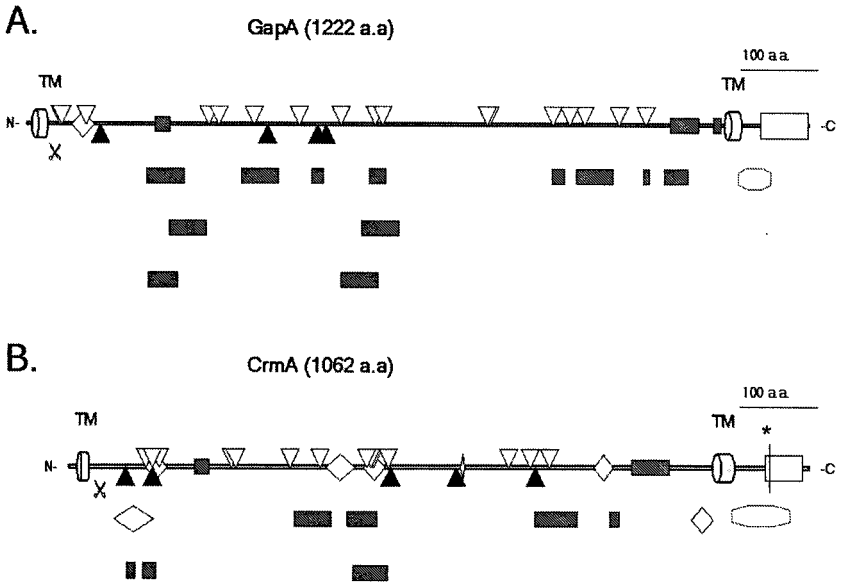

FIG. 5. Putative binding and interactive domains of *M. gallisepticum* GapA (A) and CrmA (B). [▯], transmembrane region (TM); [✕], putative signal peptidase cleavage site; [■], domains shared by carbohydrate binding proteins (determined by performing a BLOCKS database search [26]); [▽], heparin sulfate binding-like domains (determined by performing a FastA search [58] with data from references 27, 48, 64, 65, and 70); [▲], lectin-like recognition domains (determined by performing a Patternmatch search with data from references 49 and 67); [◇], other domains shared by some adhesion molecules (determined by performing a RPS-BLAST search on the Conserved Domain database [2]); [□], interaction domains shared proteins involved in the signal transduction-MARCKS family (determined by performing a BLOCKS database search [26]); [⬭], histone H1, HMG14/HMG17 signature (GapA), H2B and H5 signature, HMG14/HMG17 family, DNA polymerase family X, and AlgR homology (CrmA) (determined by performing a BLOCKS database search [26]). [*], ATP/GTP-binding site motif A (P-loop) (determined by performing a PROSITE motif search [11]).

transformants expressed both GapA and CrmA (Fig. 2A). Southern blot hybridization confirmed the presence of the wild-type genes. As shown in Fig. 2B, all of the SDCA and GCA1 transformants possessed two copies of the genes. The common band represents the genomic *crmA*, and the second band represents the inserted wild-type gene.

Cytadherence assessment of SDCA and GCA1 transformants. As shown in Fig. 3, $SDCA_{c4}$ adhered to the MRC-5 cell at the same level as both R_{high} and GT5 ($P > 0.05$). In contrast, cytadherence of $GCA1_{c5}$ was significantly high relative to that of R_{high} ($P < 0.0001$) and comparable to wild-type R_{low} levels ($P > 0.05$).

SDCA and GCA1 challenge experiments. Histopathological evaluations of the respiratory tract lesions are shown in Fig. 4. Birds challenged with 3×10^8 CFU of GT5 had histopathological changes in air sacs that ranged from no significant lesions (score = 0) to mild multifocal lymphocytic infiltrates (score = 1), with most having minimal multifocal stromal expansion by a few, small, widely scattered lymphocytic or lymphofollicular infiltrates (score = 0.5). The same was found in birds challenged with 3×10^8 CFU of $SDCA_{c4}$. Birds challenged with either 10^7 CFU or 3×10^8 CFU of $GCA1_{c5}$ had mild to moderate airsacculitis. Histopathologic lesions in birds challenged with GCA1 ranged from mild multifocal lymphocytic infiltrates (score = 1) to moderate multifocal lymphocytic or lymphofollicular infiltrates and loose interstitial infiltrates of lymphocytes, histiocytes, and heterophils with edema (score = 2). Occasional luminal exudates composed of necrotic and degranulated heterophils and macrophages, along with protein and necrotic epithelial cell debris, were present. In comparison to birds from the GT5 and SDCA challenge groups, GCA1 challenges had increased numbers of lymphofollicular aggregates; interstitial infiltrates of lymphocytes, histiocytes, and heterophils accompanied by edematous separation of stromal collagen and intraepithelial heterophils; and luminal exudates of necrotic cellular debris. Birds challenged with 10^7 CFU of strain R_{low} had the most severe airsacculitis, characterized by marked-to-severe stromal expansion by numerous, densely cellular, follicular lymphocytic aggregates accompanied by multifocal-to-diffuse interstitial infiltrates of lymphocytes, histiocytes, and heterophils. R_{low}-challenged birds also had segmental lesions to the epithelium ranging from loss and attenuation to stacking of epithelial cells along short stretches. The most cellular and extensive luminal exudates of necrotic and viable heterophils and macrophages were present in R_{low}-challenged birds.

Tracheal lesions in all groups except those challenged with R_{low} were absent (score = 0) or consisted of a few, widely separated, minimal, lymphocytic foci (score = 0.5). Birds challenged with R_{low} had tracheitis characterized by marked to severe mucosal thickening, diffuse lymphocytic infiltrates with heterophilic stromal and intraepithelial infiltrates, and luminal exudates of necrotic cellular debris.

No significant difference was found between birds challenged with $SDCA_{c4}$ and those challenged with GT5 ($P > 0.05$). Birds challenged with $GCA1_{c5}$ (regardless of the dose) developed airsacculitis ($P < 0.05$). Three isolates were obtained from birds challenged with $GCA1_{c5}$ but none from $SDCA_{c4}$- or GT5-challenged birds. The presence of the transposon carrying the *gapA* operon was confirmed by PCR in all

of the GCA1 isolates (data not shown). None of the groups challenged with GT5, $SDCA_{c4}$, or $GCA1_{c5}$ exhibited significant tracheal lesions ($P > 0.05$).

Sequence analysis. Potential carbohydrate moiety- and heparin sulfate-binding-like domains (confined to regions of 150 to 300 amino acids) were found in the predicted extracellular portion of these cytadhesins (Fig. 5). The predicted secondary structure of these putative domains indicates that they are mostly coils with some partial beta sheets and that their residues are exposed, which emphasizes their potential role in binding (15, 31, 47, 49, 71, 73). Structural homology searches revealed that the GapA and CrmA regions containing predicted binding domains appear similar to the already resolved three-dimensional structures of lectins and other binding proteins in the databases (data not shown). Both the GapA- and CrmA-predicted cytoplasmic tails share significant sequence homology with HMG14/MMG17, MARCKS, histone 2B (H2B), H1, AlgR, DNA polymerase, and CAP protein family motifs (7, 8, 12, 14, 32, 50). Members of these families are well characterized and known to be involved in DNA binding and/or signal transduction.

DISCUSSION

Levisohn et al. (46) reported that *M. gallisepticum* strain R lost virulence after 165 successive passages in vitro. Molecular analysis (69) revealed that three proteins that are present in *M. gallisepticum* R_{low} are absent in R_{high}. One of these, GapA, is the ADP1 homologue of *M. pneumoniae* and *Mycoplasma genitalium*. Functional characterization of ADP1 proteins in vitro has shown that they mediate cytadherence for these mucosal pathogens (34, 36, 59, 63). Goh at al. (22) demonstrated that GapA not only shares significant sequence homology with these two ADP1 proteins but that incubation of *M. gallisepticum* with anti-GapA Fab fragments reduced cytadherence by 64%. The fact that GapA was absent in the avirulent R_{high} strain indicated that it might have also affected *M. gallisepticum* pathogenicity. To test this hypothesis, we reconstituted R_{high} with wild-type *gapA* and then determined if cytadherence and virulence were restored. Neither cytadherence nor virulence was enhanced upon restoration of GapA expression in R_{high} (57). The fact that GapA alone did not restore *M. gallisepticum* cytadherence suggested that at least one of the other two proteins absent in R_{high} might play a role in this process. Sequence analysis of the *gapA* operon revealed that *crmA* is the second open reading frame following *gapA* (formerly called ORF3 by Goh et al. [22]). CrmA shares significant sequence homology with the MGPC gene product(s) of *M. pneumoniae* and *M. genitalium*. CrmA and the two MGPCs also share sequence homology with the GapA and ADP1 proteins of *M. pneumoniae*, *M. genitalium*, and *Mycoplasma pirum* (57). Together, these proteins constitute a unique mycoplasma adhesin family, the ADP1 family (57, 63). The physical organization of those operons encoding the major cytadhesins and their cytadherence-related molecules in *M. pneumoniae*, *M. genitalium*, and *M. gallisepticum* is conserved.

Layh-Schmitt et al. (6, 39–45, 66) showed that MGPC gene products (accessory proteins B and C) are essential for *M. pneumoniae* cytadherence. MGPC gene products are found in close proximity to ADP1 as well as to other cytadherence-related molecules, such as HMW1, HMW3, p65, and p30 in *M. pneumoniae* (42, 45, 66). The *M. pneumoniae* mutant M5 expresses ADP1 but not the MGPC gene products, and it was shown to be hemadsorption negative and deficient in formation of the tip structure (23, 24, 36, 40). MGPC gene products have not been found in the noncytadsorbing and avirulent *M. pneumoniae* B176 strain (62, 72), implying that they play a role in virulence as well.

Our data demonstrate that neither GapA nor CrmA alone is sufficient to mediate *M. gallisepticum* cytadherence and that their coexpression is necessary for efficient cytadherence and virulence. This is consistent with the interactive role of ADP1 and MGPC of *M. pneumoniae*.

The finding that $GCA1_{c5}$ caused significant lesions in the air sacs of chickens and that organisms were recovered from challenged birds suggests that GapA-CrmA-mediated cytadherence enabled *M. gallisepticum* to attach and multiply in the lower respiratory tract of the birds. Surprisingly, $GCA1_{c5}$ challenge did not result in tracheal lesions, suggesting that GapA and CrmA might effect *M. gallisepticum* tissue tropism in the host respiratory tract. Taken together, these findings indicate that other factors might be responsible for *M. gallisepticum* virulence.

Mycoplasma cytadhesins have been shown to bind to sialo- and asialo-glucoconjugates as well as sulfated glycolipids (19, 60, 63). The present belief is that ADP1 molecules are primarily responsible for mycoplasma cytadherence and that MGPC gene product(s) play an accessory role in this process. Dallo et al. (13), Jacobs et al. (28), Gerstenecker and Jacobs (20), and Opitz and Jacobs (55) identified binding domains of *M. genitalium* and *M. pneumoniae* ADP1 by immuno-inhibition employing monoclonal antibodies. Binding domains of other mycoplasma ADP1 family adhesins have not been determined, and their three-dimensional structures have not been resolved. Predicted secondary structure of the seven ADP1 family members indicates that they share two transmembrane domains adjacent to their N and C termini and an extracellular portion between the transmembrane domains and a cytoplasmic tail of 68 to 104 amino acids. The extracellular portion of the ADP1 family members represents approximately 90 to 95% of that for each of the proteins, implying that this portion might contain functional cytadherence domains. In addition to GapA and CrmA, a similar modular organization of binding domains has been observed in ADP1 of *M. pneumoniae* and *M. genitalium* (13, 20, 28, 55). Lectins have been shown to form different multimeric structures, which accounts for their ligand binding specificity (15, 31, 47, 49, 71, 73). The observed necessity for the coexpression and potential tissue tropism of GapA and CrmA (based on air sac versus tracheal lesions) might be explained by the lectin-like properties of the extracellular portions of mycoplasma cytadherence molecules (61, 62). Sequence analysis of GapA and CrmA cytoplasmic tails revealed features that suggest that they may interact with each other at this intracellular location also. We found that both the GapA and CrmA cytoplasmic tails share significant sequence as well as structural homology with the proteins and protein family motifs involved in DNA binding and protein-protein interactions (Fig. 5.). This suggests that, in addition to cytadherence, these molecules might function as DNA-binding proteins and may play a role in the regulation of gene expression. This

hypothesis, if proven, may help to explain mycoplasmal gene regulation in light of the paucity of classic two-component regulatory systems in the genomes of *M. pneumoniae*, *M. genitalium*, and *M. gallisepticum*.

ACKNOWLEDGMENTS

We thank Matthew Mikoleit for necropsy technical assistance, Ione Jackman and Sallyann Gemme for tissue processing and histologic preparations, Paul Hudson and Rekha Rangarajan for assistance with reisolation and analysis, and Lawrence Silbart and Timothy S. Gorton for their critical review of the manuscript.

This work was supported by USDA grant 58-1940-0-007 to S.J.G. and a United States-Israel Binational Science Foundation (BSF) grant to S.J.G. and D.Y. This work was supported by the Center of Excellence for Vaccine Research (CEVR #81) and by ag. exp. #2123, Storrs Agricultural Experiment Station, Storrs, Conn.

REFERENCES

1. **Alexandrov, N. N., R. Nussinov, and R. M. Zimmer.** 1996. Fast protein fold recognition via sequence to structure alignment and contact capacity potentials, p. 53–72. *In* L. Hunter and T. E. Klein (ed.), Pacific symposium on biocomputing '96, World Scientific Publishing Co., Singapore.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Baldi, P., S. Brunak, P. Frasconi, G. Soda, and G. Pollastri.** 1999. Exploiting the past and the future in protein secondary structure prediction. Bioinformatics **15:**937–946.
4. **Bateman, A., E. Birney, R. Durbin, S. R. Eddy, K. L. Howe, and E. L. Sonnhammer.** 2000. The Pfam protein families database. Nucleic Acids Res. **28:**263–266.
5. **Bates, P. A., L. A. Kelley, R. M. MacCallum, and M. J. Sternberg.** 2001. Enhancement of protein modeling by human intervention in applying the automatic programs 3D-JIGSAW and 3D-PSSM. Proteins **45:**39–46.
6. **Baum, H., A. Strubel, J. Nollert, and G. Layh-Schmitt.** 2000. Two cases of fulminant *Mycoplasma pneumoniae* pneumonia within 4 months. Infection **28:**180–183.
7. **Baxevanis, A. D., and D. Landsman.** 1997. Histone and histone fold sequences and structures: a database. Nucleic Acids Res. **25:**272–273.
8. **Blackshear, P. J.** 1993. The MARCKS family of cellular protein kinase C substrates. J. Biol. Chem. **268:**1501–1504.
9. **Boguslavsky, S., D. Menaker, I. Lysnyansky, T. Liu, S. Levisohn, R. Rosengarten, M. García, and D. Yogev.** 2000. Molecular characterization of the *Mycoplasma gallisepticum pvpA* gene which encodes a putative variable cytadhesin protein. Infect. Immun. **68:**3956–3964.
10. **Brendel, V., P. Bucher, I. R. Nourbakhsh, B. E. Blaisdell, and S. Karlin.** 1992. Methods and algorithms for statistical analysis of protein sequences. Proc. Natl. Acad. Sci. USA **89:**2002–2006.
11. **Bucher, P., and A. Bairoch.** 1994. A generalized profile syntax for biomolecular sequences motifs and its function in automatic sequence interpretation, p. 53–61. In R. Altman, D. Brutlag, P. Karp, R. Lathrop, and D. Searls (ed.), Proceedings of the 2nd International Conference on Intelligent Systems for Molecular Biology 1994. AAAI Press, Menlo Park, Calif.
12. **Bustin, M., and R. Reeves.** 1996. High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. Prog. Nucleic Acid Res. Mol. Biol. **54:**35–100.
13. **Dallo, S. F., C. J. Su, J. R. Horton, and J. B. Baseman.** 1988. Identification of P1 gene domain containing epitope(s) mediating *Mycoplasma pneumoniae* cytoadherence. J. Exp. Med. **167:**718–723.
14. **Deretic, V., and W. M. Konyecsni.** 1990. A procaryotic regulatory factor with a histone H1-like carboxy-terminal domain: clonal variation of repeats within *algP*, a gene involved in regulation of mucoidy in *Pseudomonas aeruginosa*. J. Bacteriol. **172:**5544–5554.
15. **Elgavish, S., and B. Shaanan.** 2001. Chemical characteristics of dimer interfaces in the legume lectin family. Protein Sci. **10:**753–761.
16. **Engebrecht, J., R. Brent, and M. A. Kaderbhai.** 1987. Mini preps of plasmid DNA, p. 1.6.4–1.6.5. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. StruhI (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
17. **Forsyth, M. H., M. E. Tourtellotte, and S. J. Geary.** 1992. Localization of an immunodominant 64 kDa lipoprotein (LP 64) in the membrane of *Mycoplasma gallisepticum* and its role in cytadherence. Mol. Microbiol. **6:**2099–2106.
18. **Geary, S. J., M. G. Gabridge, and M. F. Gladd.** 1988. Utilization of a human lung fibroblast receptor site to identify a 32 kD *Mycoplasma pneumoniae* antigen involved in attachment. *In* Proceedings of the 7th Congress of the International Organization for Mycoplasmology. Gustav Fischer Verlag, Stuttgart, Germany.
19. **Geary, S. J., M. G. Gabridge, R. Intres, D. L. Draper, and M. F. Gladd.** 1989. Identification of Mycoplasma binding proteins utilizing a 100 kilodalton lung fibroblast receptor. J. Recept. Res. **9:**465–478.
20. **Gerstenecker, B., and E. Jacobs.** 1990. Topological mapping of the P1-adhesin of *Mycoplasma pneumoniae* with adherence-inhibiting monoclonal antibodies. J. Gen. Microbiol. **136:**471–476.
21. **Goh, M. S., M. H. Forsyth, T. S. Gorton, and S. J. Geary.** 1994. Cloning and sequence analysis of a Mycoplama gallisepticum cytadhesin gene, p. 669–670. *In* Proceedings of the 10th International Congress of the International Organization for Mycoplasmology. International Organization for Mycoplasmology, Bordeaux, France.
22. **Goh, M. S., T. S. Gorton, M. H. Forsyth, K. E. Troy, and S. J. Geary.** 1998. Molecular and biochemical analysis of a 105 kDa *Mycoplasma gallisepticum* cytadhesin (GapA). Microbiology **144:**2971–2978.
23. **Hansen, E. J., R. M. Wilson, and J. B. Baseman.** 1979. Isolation of mutants of *Mycoplasma pneumoniae* defective in hemadsorption. Infect. Immun. **23:**903–906.
24. **Hansen, E. J., R. M. Wilson, W. A. Clyde, Jr., and J. B. Baseman.** 1981. Characterization of hemadsorption-negative mutants of *Mycoplasma pneumoniae*. Infect. Immun. **32:**127–136.
25. **Hempstead, P. G.** 1990. An improved method for the rapid isolation of chromosomal DNA from Mycoplasma spp. Can. J. Microbiol. **36:**59–61.
26. **Henikoff, S., J. G. Henikoff, and S. Pietrokovski.** 1999. Blocks+: a non-redundant database of protein alignment blocks derived from multiple compilations. Bioinformatics **15:**471–479.
27. **Herrera, E. M., M. Ming, E. Ortega-Barria, and M. E. Pereira.** 1994. Mediation of *Trypanosoma cruzi* invasion by heparin sulfate receptors on host cells and penetrin counter-receptors on the trypanosomes. Mol. Biochem. Parasitol. **65:**73–83.
28. **Jacobs, E., B. Gerstenecker, B. Mader, C. H. Huang, P. C. Hu, R. Halter, and W. Bredt.** 1989. Binding sites of attachment-inhibiting monoclonal antibodies and antibodies from patients on peptide fragments of the *Mycoplasma pneumoniae* adhesin. Infect. Immun. **57:**685–688.
29. **Jones, D. T.** 1999. GenTHREADER: an efficient and reliable protein fold recognition method for genomic sequences. J. Mol. Biol. **287:**797–815.
30. **Jones, D. T.** 1999. Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. **292:**195–202.
31. **Karlsson, K. A.** 1995. Microbial recognition of target-cell glycoconjugates. Curr. Opin. Struct. Biol. **5:**622–635.
32. **Kawamukai, M., J. Gerst, J. Field, M. Riggs, L. Rodgers, M. Wigler, and D. Young.** 1992. Genetic and biochemical analysis of the adenylyl cyclase-associated protein, cap, in *Schizosaccharomyces pombe*. Mol. Biol. Cell **3:**167–180.
33. **Knudtson, K. L., and F. C. Minion.** 1993. Construction of Tn4001lac derivatives to be used as promoter probe vectors in mycoplasmas. Gene **137:**217–222.
34. **Krause, D. C.** 1996. *Mycoplasma pneumoniae* cytadherence: unravelling the tie that binds. Mol. Microbiol. **20:**247–253.
35. **Krause, D. C., and M. F. Balish.** 2001. Structure, function, and assembly of the terminal organelle of *Mycoplasma pneumoniae*. FEMS Microbiol. Lett. **198:**1–7.
36. **Krause, D. C., D. K. Leith, R. M. Wilson, and J. B. Baseman.** 1982. Identification of *Mycoplasma pneumoniae* proteins associated with hemadsorption and virulence. Infect. Immun. **35:**809–817.
37. **Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer.** 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. **305:**567–580.
38. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
39. **Layh-Schmitt, G.** 1993. The ORF6 gene product of the P1 operon of *Mycoplasma pneumoniae*. Zentbl. Bakteriol. **278:**287–295.
40. **Layh-Schmitt, G., and M. Harkenthal.** 1999. The 40- and 90-kDa membrane proteins (ORF6 gene product) of *Mycoplasma pneumoniae* are responsible for the tip structure formation and P1 (adhesin) association with the Triton shell. FEMS Microbiol. Lett. **174:**143–149.
41. **Layh-Schmitt, G., and R. Herrmann.** 1992. Localization and biochemical characterization of the ORF6 gene product of the *Mycoplasma pneumoniae* P1 operon. Infect. Immun. **60:**2906–2913.
42. **Layh-Schmitt, G., and R. Herrmann.** 1994. Spatial arrangement of gene products of the P1 operon in the membrane of *Mycoplasma pneumoniae*. Infect. Immun. **62:**974–979.
43. **Layh-Schmitt, G., H. Hilbert, and E. Pirkl.** 1995. A spontaneous hemadsorption-negative mutant of *Mycoplasma pneumoniae* exhibits a truncated adhesin-related 30-kilodalton protein and lacks the cytadherence-accessory protein HMW1. J. Bacteriol. **177:**843–846.
44. **Layh-Schmitt, G., R. Himmelreich, and U. Leibfried.** 1997. The adhesin related 30-kDa protein of *Mycoplasma pneumoniae* exhibits size and antigen variability. FEMS Microbiol. Lett. **152:**101–108.
45. **Layh-Schmitt, G., A. Podtelejnikov, and M. Mann.** 2000. Proteins complexed to the P1 adhesin of Mycoplasma pneumoniae. Microbiology **146:**741–747.
46. **Levisohn, S., M. J. Dykstra, M. Y. Lin, and S. H. Kleven.** 1986. Comparison

of in vivo and in vitro methods for a pathogenicity evaluation for *Mycoplasma gallisepticum* in respiratory infection. Avian Pathol. **15:**233–246.
47. **Lis, H., and N. Sharon.** 1998. Lectins: carbohydrate-specific proteins that mediate cellular recognition. Chem. Rev. **98:**637–674.
48. **Locht, C., P. Bertin, F. D. Menozzi, and G. Renauld.** 1993. The filamentous haemagglutinin, a multifaceted adhesion produced by virulent *Bordetella* spp. Mol. Microbiol. **9:**653–660.
49. **Loris, R., T. W. Hamelryck, J. Bouckaert, and L. Wyns.** 1998. Legume lectin structure. Biochim. Biophys. Acta **1383:**9–36.
50. **Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond.** 1997. Crystal structure of the nucleosome core particle at 2.8 Å resolution. Nature **389:**251–260.
51. **Markham, P. F., M. D. Glew, J. E. Sykes, T. R. Bowden, T. D. Pollocks, G. F. Browning, K. G. Whithear, and I. D. Walker.** 1994. The organisation of the multigene family which encodes the major cell surface protein, pMGA, of *Mycoplasma gallisepticum*. FEBS Lett. **352:**347–352.
52. **Minion, F. C., and P. A. Kapke.** 1998. Transformation of Mycoplasmas, p. 227–234. *In* R. J. Miles and R. A. J. Nicholas (ed.), Mycoplasma protocols, vol. 104. Humana Press Inc., Totowa, N.J.
53. **Moller, S., M. D. Croning, and R. Apweiler.** 2001. Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics **17:**646–653.
54. **Nunoya, T., M. Tajima, T. Yagihashi, and S. Sannai.** 1987. Evaluation of respiratory lesions in chickens induced by *Mycoplasma gallisepticum*. Jpn. J. Vet. Sci. **49:**621–629.
55. **Opitz, O., and E. Jacobs.** 1992. Adherence epitopes of *Mycoplasma genitalium* adhesin. J. Gen. Microbiol. **138:**1785–1790.
56. **Papazisi, L., L. K. Silbart, S. Frasca Jr., D. Rood, X. Liao, M. Gladd, M. A. Javed, and S. J. Geary.** Modified live *Mycoplasma gallisepticum* vaccine to protect chickens from respiratory disease, in press.
57. **Papazisi, L., K. E. Troy, T. S. Gorton, X. Liao, and S. J. Geary.** 2000. Analysis of cytadherence-deficient, GapA-negative *Mycoplasma gallisepticum* strain R. Infect. Immun. **68:**6643–6649.
58. **Pearson, W. R.** 1999. Flexible similarity searching with the FASTA3 program package, p. 185–219. Bioinformatics methods and protocols. Humana Press, Totowa, N.J.
59. **Razin, S.** 1999. Adherence of pathogenic mycoplasmas to host cells. Biosci. Rep. **19:**367–372.
60. **Razin, S.** 1985. Mycoplasma adherence, p. 161–202. *In* S. Razin and M. F. Barile (ed.), The Mycoplasmas, vol. 4. Academic Press, Orlando, Fla.
61. **Razin, S.** 1986. Mycoplasmal adhesins and lectins, p. 217–235. *In* D. Mirelman (ed.), Microbial lectins and agglutinins: properties and biological activity. Wiley Interscience, New York, N.Y.
62. **Razin, S., and E. Jacobs.** 1992. Mycoplasma adhesion. J. Gen. Microbiol. **138:**407–422.
63. **Razin, S., D. Yogev, and Y. Naot.** 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiol. Mol. Biol. Rev. **62:**1094–1156.
64. **Roderiquez, G., T. Oravecz, M. Yanagishita, D. C. Bou-Habib, H. Mostowski, and M. A. Norcross.** 1995. Mediation of human immunodeficiency virus type 1 binding by interaction of cell surface heparan sulfate proteoglycans with the V3 region of envelope gp120-gp41. J. Virol. **69:**2233–2239.
65. **Rostand, K. S., and J. D. Esko.** 1997. Microbial adherence to and invasion through proteoglycans. Infect. Immun. **65:**1–8.
66. **Seto, S., G. Layh-Schmitt, T. Kenri, and M. Miyata.** 2001. Visualization of the attachment organelle and cytadherence proteins of *Mycoplasma pneumoniae* by immunofluorescence microscopy. J. Bacteriol. **183:**1621–1630.
67. **Sharma, V., and A. Surolia.** 1997. Analyses of carbohydrate recognition by legume lectins: size of the combining site loops and their primary specificity. J. Mol. Biol. **267:**433–445.
68. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
69. **Troy, K. E.** 1998. M.S. thesis. The University of Connecticut, Storrs, Conn.
70. **Trybala, E., T. Bergstrom, B. Svennerholm, S. Jeansson, J. C. Glorioso, and S. Olofsson.** 1994. Localization of a functional site on herpes simplex virus type 1 glycoprotein C involved in binding to cell surface heparin sulphate. J. Gen. Virol. **75:**743–752.
71. **Vijayan, M., and N. Chandra.** 1999. Lectins. Curr. Opin. Struct. Biol. **9:**707–714.
72. **Vu, A. C., H. M. Foy, F. D. Cartwright, and G. E. Kenny.** 1987. The principal protein antigens of isolates of *Mycoplasma pneumoniae* as measured by levels of immunoglobulin G in human serum are stable in strains collected over a 10-year period. Infect. Immun. **55:**1830–1836.
73. **Weis, W. I.** 1997. Cell-surface carbohydrate recognition by animal and viral lectins. Curr. Opin. Struct. Biol. **7:**624–630.
74. **Yoder, H. W. J.** 1978. *Mycoplasma gallisepticum* infection, p. 236–250. *In* Diseases of poultry, 7th ed. Iowa State University Press, Ames, Iowa.
75. **Zar, J. H.** 1999. Biostatistical analysis, 4th ed. Prentice Hall, Upper Saddle River, N.J.

Editor: J. T. Barbieri