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Monoclonal Antibodies to Avian Escherichia coli Iss

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SUMMARY. *Escherichia coli* infections are a major problem for the poultry industry in the United States. Yet, the virulence mechanisms operative in avian *E. coli* are poorly understood. In the present studies, monoclonal antibodies (MAbs) have been generated that may facilitate study of the pathogenesis of avian colibacillosis. These MAbs are directed against the Iss protein because results from our laboratory have shown that the possession of *iss* DNA sequences is strongly correlated with the *E. coli* implicated in avian colibacillosis. As part of an overall effort to explore the role of *iss*/Iss in colibacillosis pathogenesis, Iss protein has been purified, MAbs to Iss have been generated, and the MAbs are being evaluated. B cells from mice immunized with an Iss fusion to glutathione-S-transferase produced antibodies specifically against Iss, and these cells were used to generate the MAbs. These anti-Iss MAbs, when used in western blotting assays, can be used to distinguish *iss*-positive and -negative *E. coli* isolates, suggesting that they may be useful as reagents in the detection and study of virulent avian *E. coli.*

RESUMEN. Anticuerpos monoclonales específicos para la proteína Iss de Escherichia coli.

La infección por *Escherichia coli* es un problema común en la industria avícola de los Estados Unidos. Sin embargo, los mecanismos de virulencia de *E. coli* en las especies aviares no han sido completamente aclarados. En este estudio se generaron anticuerpos monoclonales que pueden facilitar el estudio de la patogénesis de la colibacilosis aviar. Estos anticuerpos monoclonales fueron producidos con especificidad para la proteína Iss porque resultados obtenidos previamente en nuestro laboratorio demuestran que existe una fuerte correlación entre la presencia de secuencias de ADN de *iss,* en cepas de *E. coli* implicadas en casos clínicos de colibacilosis aviar. Como parte de un estudio general encaminado a explorar el papel de la *iss*/Iss en la patogénesis de la colibacilosis, hemos purificado la proteína Iss y hemos generado anticuerpos monoclonales específicos para la proteína, los cuales estas siendo evaluados. Estos anticuerpos fueron producidos con linfocitos B obtenidos a partir de ratones inmunizados con una proteína de fusión entre la Iss y la transferasa de glutationa. Cuando se usaron estos anticuerpos monoclonales específicos contra la Iss en la técnica de transferencia puntual de western, se pudo distinguir entre las cepas de *E. coli* que expresan o no la proteína Iss, lo cual sugiere que los mismos pueden ser una herramienta útil en la detección y estudio de cepas aviares virulentas de *E. coli.*

Key words: avian *Escherichia coli,* avian colibacillosis, Iss, complement resistance, monoclonal antibody development, virulence

Abbreviations: Bio-CaM = biotinylated calmodulin; CBP = calmodulin binding peptide; DMEM = Dulbecco modified Eagle medium; ELISA = enzyme-linked immunosorbent assay; FBS = fetal bovine serum; HAT selection medium = Dulbecco modified Eagle medium, 20% fetal bovine serum, 0.1 mM hypoxanthine, 0.016 mM thymidine, 0.004 mM aminopterin, penicillin (63 μg/ml), streptomycin (0.1 mg/ml), fungizone (0.25 μg/ml), 0.05 mM 2-mercaptoethanol; HT medium = HAT selection medium containing 10% fetal bovine

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serum and no aminopterin; GST = glutathione S-transferase; IFA = incomplete Freund adjuvant; i.p. = intraperitoneal; IPTG = isopropyl-β-D-thiogalactopyranoside; LB = Luria–Bertani; MAb = monoclonal antibody; PEG = polyethylene glycol, molecular weight 1000; PVDF = polyvinylidine difluoride; SAS = saturated ammonium sulfate; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Colibacillosis, caused by *Escherichia coli,* is a major problem for the poultry industry in the United States. The disease most commonly occurs in 5-to-12-wk-old broiler chickens but also may occur in turkey poults and newly hatched chickens (8). *Escherichia coli* infections in avian species occur in many forms, including airsacculitis, coligranuloma, omphalitis, peritonitis, salpingitis, and synovitis, with colisepticemia the most common disease manifestation (8). Altogether, these infections result in multimillion-dollar losses, annually, to the U.S. poultry industry (11). Understanding of avian colibacillosis pathogenesis has been hampered by the lack of identifying markers of virulent avian *E. coli.* However, work in our laboratory has shown that the presence of an *iss* DNA sequence in avian *E. coli* is strongly correlated with an isolate's ability to cause the disease (14). The increased serum survival gene (*iss*) is known for its contribution to the virulence and complement resistance of a mammalian *E. coli* isolate (1). The link between *iss* and disease-causing *E. coli* makes *iss* and the protein it encodes, Iss, candidate markers of avian *E. coli* virulence. As part of an overall effort to explore the potential of *iss*-/Iss-based colibacillosis control strategies and the utility of anti-Iss monoclonal antibodies (MAbs) as reagents to study avian *E. coli,* MAbs to Iss have been generated and characterized. MAbs directed toward Iss may be useful in the detection of virulent *E. coli,* to study *E. coli*'s pathogenic mechanisms, or perhaps, to counteract its pathogenic effects *in vivo.*

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* V-2, isolated from a colisepticemic chicken, and *E. coli* A-7, an avirulent avian strain, which were characterized previously (12,21,22), *E. coli* XL1-Blue (Stratagene, La Jolla, CA), and *E. coli* BL21 (DE3) (Stratagene) (20) were used in this study. *Escherichia coli* V-2 is complement resistant and virulent in chicks and chick embryos (22). All strains were maintained on Luria–Bertani (LB) agar (Difco, Detroit, MI) with ampicillin (100 μg/ml; Amresco, Solon, OH) where appropriate. The plasmids used were pCAL-n, an expression vector (Stratagene), which is designed for production of proteins fused to calmodulin binding peptide (CBP), pGEX-6P-3 (Pharmacia Biotech, Piscataway, NJ), another expression vector designed for production of proteins fused to glutathione-S-transferase (GST), pSF320, and pLN330. pSF320 is composed of pCAL-n with the complete *iss* gene sequence (GenBank accession number AF0422279) inserted into the cloning site. pLN330 is composed of pGEX-6P-3 with the *iss* gene sequence without the coding region for the signal sequence (i.e., the first 72 nucleotides of *iss* are deleted in this construct), as described previously (6). Isolation of plasmid DNA was accomplished by Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI). Plasmid DNA was digested with the appropriate restriction enzymes to confirm insert identity and orientation.

Molecular cloning. The *iss* gene was amplified (with and without the signal sequence) for cloning into pCAL-n with primers designed with Primer Select Program of LaserGene (DNASTAR, Inc., Madison, WI). To amplify the complete *iss* gene for creation of pSF320, the upper primer was 5′ AAAGGGGATCCATGCAGGATAATAAGATGAAAAA 3′, Tm: 73.6 C, and the lower primer used was 5′ CGCCGGAATTCGCAGATGAGCTCCCCATATC 3′, Tm: 82.4 C. Amplitaq (Perkin Elmer, Norwalk, CT) was used to catalyze the amplification of *iss.* The amplification program used was 97 C for 2 min; nine cycles of 97 C for 1 min, 51.8 C for 1 min, 72 C for 1 min; 24 cycles of 95 C for 1 min, 49 C for 1 min, 72 C for 1 min; then, 72 C for 5 min. The amplified *iss* gene was prepared for ligation into pCAL-n as described previously (6). The insert DNA was ligated into pCAL-n according to the manufacturer's protocol (20). Ligations were transformed into XL1-Blue by the "classic" calcium chloride method (17).

DNA sequencing. Clones were sequenced to confirm that the junction points between the expression vector and inserts were "in frame" for proper expression of proteins. The double-stranded plasmid clones were sequenced at the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA). DNA from pSF320 was prepared for sequencing by the manufacturer's protocols (PE Biosystems). The sequencing primer used was the 3′-pCAL-n Sequencing Prim-

er (5′-d[GAATTTCATAGCCGTCTCAGC]-3′ [Sigma-Genosys, The Woodlands, TX]) (20).

Protein expression. Two Iss fusion proteins were used in this project. A GST-Iss fusion was generated as previously described (6), and a CBP-Iss was generated by the following procedure. pSF320 was transformed into the protease-deficient, $CaCl_2$-competent *E. coli* BL21 (DE3) for protein expression (20). The transformed cells were grown overnight on LB plates supplemented with ampicillin (50 μg/ml) (20). Individual colonies were transferred to LB broth containing ampicillin (50 μg/ml), and the cultures were incubated at 37 C with shaking for 3 hr. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM, and the culture was incubated for an additional 5 hr. Protein samples were separated with two 10%–20% Tris-HC1 gradient gels (Bio-Rad, Hercules, CA). At completion, one of the gels was stained with Coomassie blue stain, whereas the other gel was transferred to polyvinylidine difluoride (PVDF) membrane and probed with biotinylated calmodulin (Bio-CaM) (Stratagene) directed to the CBP portion of the fusion protein (CBP-Iss) (19) according to the manufacturer's instructions (19).

Protein purification. Purification of the recombinant protein was carried out with the calmodulin affinity matrix according to the manufacturer's instructions (Stratagene). Purification of the protein was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described above. After positive identification of the eluted fusion protein by molecular size and reaction with Bio-CaM, CBP-Iss, along with GST-Iss, was used for hybridoma generation.

Mouse immunizations. Four BALB/c mice were given an intraperitoneal (i.p.) injection with an emulsion of 100 μg of GST-Iss and complete Freund adjuvant. Each mouse was given two booster injections at 14-day intervals with 100 μg of GST-Iss and incomplete Freund adjuvant (IFA). Then, a final i.p. injection with 100 μg of CBP-Iss and IFA was administered 28 days after the last injection with GST-Iss to stimulate the production of antibodies specific for Iss (13). Fig. 1 outlines the immunization protocol used for the generation of the antibodies. After the second injection of GST-Iss and IFA, mice were tested for seroconversion by enzyme-linked immunosorbent assay (ELISA) with Iss as the coating protein. The ELISA was performed according to published protocols with an alkaline phosphatase-labeled, anti-mouse immunoglobulin (Ig)G and IgM secondary antibody (Pierce) and p-nitrophenyl phosphate detection reagent (17).

Hybridoma generation. Hybridomas were produced by the fusion of splenocytes from a hyperimmune BALB/c mouse and SP2/O mouse myeloma cells (ATCC, Rockville, MD) with the use of polyethylene glycol, molecular weight 1000 (PEG) (Sigma, St. Louis, MO) (7,9). Briefly, the BALB/c mouse with the greatest anti-Iss response was euthanatized by cervical dislocation, its spleen was aseptically harvested, and its splenocytes were collected by syringe perfusion with sterile Dulbecco modified Eagle medium (DMEM) (10). Viable cell counts of the spleen cells and myeloma cells were done, the cells were combined at a 3:1 ratio and centrifuged at 400 × *g* for 8 min at room temperature, and the supernatant fluid was discarded (7). Two milliliters of PEG were slowly added to the pellet, followed by the gradual addition of 6 ml of DMEM with gentle swirling. After additional washes, the hybridoma mixture was suspended in a volume of cloning medium (DMEM, 20% fetal bovine serum [FBS], 0.1 mM hypoxanthine, 0.016 mM thymidine, penicillin [63 μg/ml], streptomycin [0.1 mg/ml], fungizone [0.25 μg/ml], 0.05 mM 2-mercaptoethanol, and 30% myeloma-conditioned medium), such that the fused spleen cells were at a concentration of 2×10^6 cells/ml. Then 0.5 ml of the hybridoma suspension was dispensed into the wells of the 24-well trays containing 0.5 ml of BALB/c feeder cells (1×10^6 cells/ml). On the day after the fusion, 1 ml of HAT selection medium (DMEM, 20% FBS, 0.1 mM hypoxanthine, 0.016 mM thymidine, 0.004 mM aminopterin, penicillin [63 μg/ml], streptomycin [0.1 mg/ml], fungizone [0.25 μg/ml], 0.05 mM 2-mercaptoethanol) was added to each of the wells of the fusion plates to select for hybridoma cells (9). Starting on day 5 and repeating every fifth day, the cells were fed by removing 1 ml of medium from each well and replacing it with fresh medium. The first two feedings were

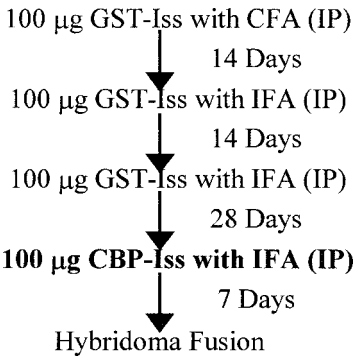

Fig. 1. Overview of the immunization strategy. This flowchart shows the mouse injection protocol used for the generation of anti-Iss MAbs. The first injections were done with the GST-Iss fusion protein. Then, the final injection was done with the CBP-Iss fusion to increase the production of antibodies specific for Iss. The second fusion protein was used in an attempt to shift the proliferation of antibodies away from the larger GST and toward Iss alone.

done with HAT selection medium, then two feedings with HT medium (HAT selection medium containing 10% FBS and no aminopterin), followed by subsequent feeding with growth medium (HT medium without hypoxanthine and thymidine) (9).

Hybridoma screening for anti-Iss antibody product. Once growth of the hybridomas was evident, cell-free supernatant fluid was screened for the presence of antibodies directed at Iss. Three screenings of the hybridoma wells were done at 5-day intervals after growth was observed. The screening was done by an ELISA with GST-Iss as the coating antigen (16).

Dilution cloning and anti-Iss MAb detection. Wells containing hybridomas that reacted positively with GST-Iss in the screening ELISA were serially diluted in 96-well trays to isolate single clones (10). Wells where growth originated from a single colony were screened by ELISA for their ability to recognize Iss. Two sets of ELISAs were used to screen each of the clones; one used GST-Iss as the coating antigen and the other used CBP-Iss as the coating antigen. Monoclones that recognized both GST-Iss and CBP-Iss were frozen, as described below, and recloned to ensure that the apparent clones originated from a single cell. Cell suspensions of the monoclones of interest were subcloned by the dilution method described previously (10).

Isotyping. After screening and expansion of the subclones, the MAbs from the four best responders in the screening ELISA were characterized with the mouse typer sub-isotyping kit (Bio-Rad) according to the manufacturer's instructions. Both GST-Iss and CBP-Iss diluted in coating buffer (20 μg/ml) were used as coating antigens for the isotyping ELISA.

Expansion and freezing of hybridoma cells. Cells from wells demonstrating monoclonal growth were screened as before, and those cells that produced antibodies recognizing both GST-Iss and CBP-Iss were sequentially expanded until a volume of 100 ml was reached. During expansion, the amount of FBS in the growth medium was gradually reduced to 2% FBS. The cell-free supernatant fluid was collected for purification of the MAbs (7,10). A portion of the cells was preserved in 1.0 ml of freezing media (90% FBS, 10% dimethyl sulfoxide) and placed in liquid nitrogen for long-term storage (9).

MAb precipitation. The MAbs were precipitated from the cell-free supernatant fluids by saturated ammonium sulfate (SAS) precipitation, as described elsewhere (10). A final SAS concentration of 45% (v/v) was used for the precipitation (5).

Specificity and sensitivity of MAbs. To test the specificity of the anti-Iss MAbs, lysates of BL21 containing pLN330, BL21 (DE3) containing pSF320, and A-7, an *iss*-negative avian *E. coli* isolate (21), were subjected to SDS-PAGE, blotted to PVDF, and probed with the anti-Iss MAbs (diluted 1:5 in phosphate-buffered saline) as described previously (16). The titer of the anti-Iss MAbs was determined by an ELISA coated with both GST-Iss and CBP-Iss. Anti-Iss MAbs were added to the wells of the microtiter plate after a series of 10-fold dilutions, and the ELISA was carried out as described previously (15).

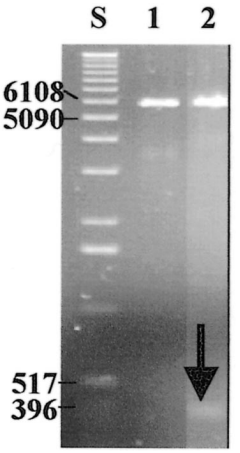

Fig. 2. Cloning of *iss* into the expression vector pCAL-n. This agarose gel represents the electrophoresis products from the cloning experiment indicating that inserts of the appropriate sizes are present in the plasmids. The upper bands in lanes 1 and 2 are at about 5800 base pairs, which correspond to the size of the pCAL-n vector. Lane 1 contains the digested pCAL-n vector without an insert. The arrow in lane 2 points to a band at about 310 base pairs, the size of *iss* gene. Lane 1 contains the digested pCAL-n vector without an insert.

RESULTS

In order to generate MAbs specific for Iss, both GST-Iss and CBP-Iss fusion proteins were generated. To produce CBP-Iss, *iss* was cloned into pCAL-n. Cleavage of pCAL-n containing the *iss* insert generated fragments of 5800 bp and of 310 bp, corresponding in size to the pCAL-n vector and *iss,* respectively, suggesting that *iss* was successfully cloned in pCAL-n (Fig. 2). This construct was termed pSF320. DNA sequencing confirmed that *iss* was ligated in the proper orientation and reading frame for expression in pCAL-n. Crude total protein preparations of protease-deficient *E. coli* BL21 (DE3), carrying the pCAL-n construct, revealed that BL21 (DE3) containing pSF320 expressed a 15-kD protein corresponding in size to the predicted protein product. The identity

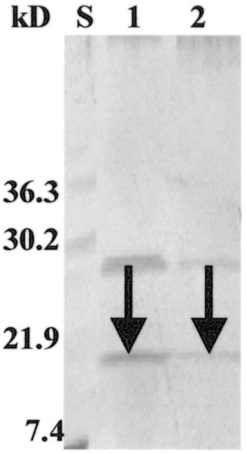

Fig. 3. Immunoblot of CBP-Iss fusion protein. CBP-Iss, expressed from BL21 (DE3) pSF320, was identified by immunoblotting with a biotinylated calmodulin probe followed by the addition of a streptavidin alkaline phosphatase detection reagent. Lane S, a prestained molecular weight standard (BioRad); lane 1, the crude lysate of BL21 (DE3) pSF320 after induction with IPTG; lane 2, the same sample prior to the addition of IPTG. The arrows point to a 15-kD protein detected in lanes 1 and 2 that corresponds in size to that predicted for CBP-Iss. In addition to protein corresponding in size to the CBP-Iss, additional proteins were recognized by the streptavidin detection reagent; these are likely naturally occurring, biotinylated proteins (19). Additionally, there was expression of CBP-Iss prior to the addition of IPTG. This expression did not cause a major problem, for the CBP-Iss fusion protein was successfully purified.

of the CBP-Iss fusion protein was confirmed by immunoblotting (Fig. 3). In addition to the 15-kD fusion protein band, bands of 22.5 kD were recognized; these 22.5–kD bands correspond to a naturally occurring, biotinylated *E. coli* host protein recognized by the streptavidin detection reagent (20). Additionally, there appears to have been expression of the fusion proteins prior to the induction of expression with IPTG (Fig. 3). The CBP-Iss fusion protein was solubilized through lysozyme treatment and sonication and purified by calmodulin affinity resin chromatography, and its identity was confirmed by immunoblotting with the Bio-CaM probe (Fig. 4).

Mice immunized with Iss fusion proteins were able to generate an immune response directed against Iss as compared with controls, which were not. This result was demonstrated by an ELISA with sera from four immunized mice after their second injection with GST-Iss and IFA. Sera from the immunized mice reacted with Iss protein after a dilution of a minimum of 1:1000. No reaction was seen between the serum of the unimmunized mouse and Iss. The mouse that demonstrated the greatest antibody response to Iss (recognized Iss after at least a 1:10,000 dilution) was used to generate the MAbs.

On days 10 and 15 after the fusion, a screening ELISA with GST-Iss as the coating antigen was done. The response to the coating antigen was weak and ambiguous (data not shown). However, on day 20, the ELISA was repeated, and eight wells reacted positively. When growth was observed in the cloning plates, the plates were assayed by a dual screening approach, i.e., the supernatant fluids were screened for reactivity to GST-Iss and CBP-Iss. Of the 56 clones screened, nine reacted to both CBP-Iss and GST-Iss, and these were subcloned. Thirty-one of the 40 wells screened reacted positively to both coating antigens. The 24 clones that reacted most intensely with the coating antigens were expanded to 24-well trays. Of these, the four most intense responders were isotyped with a commercial kit, and each of the monoclones was found to produce IgM antibodies with κ-light chains. Cells from the eight clones that reacted with greatest intensity to both coating antigens in the screening ELISA were stored in liquid nitrogen and also expanded.

The MAbs were evaluated for their ability to recognize Iss through western blotting. The anti-Iss Mabs recognized protein bands corresponding in size to both CBP-Iss and GST-Iss fusions protein through western blotting. The antibody did not appear to recognize any proteins from *E. coli* A-7, an Iss-negative strain (Fig. 5). Additionally, both GST-Iss and CBP-Iss were recognized after a minimum of a 1:10 dilution of the antibody sample in an ELISA to determine titer. Positive reactions were observed between the hyperimmune mouse serum and the coating antigens, and no reaction was seen between the normal mouse serum and the coating antigens.

DISCUSSION AND CONCLUSIONS

A potential marker of virulent avian *E. coli* that may be exploited in the study of colibacil-

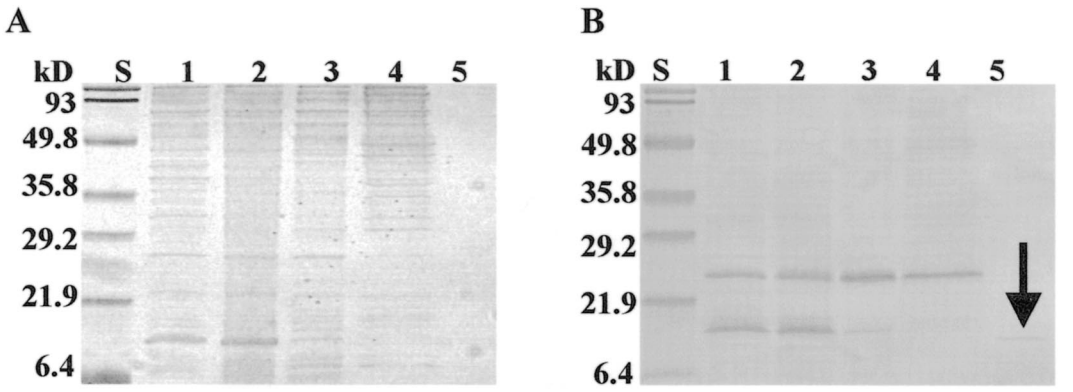

Fig. 4. Solubilization and purification of CBP-Iss from pSF320. (A) SDS-PAGE of the soluble protein (CBP-Iss) obtained after expression from pSF320 in BL21, bacterial lysis by treatment with lysozyme and sonication, and addition of the lysate to the calmodulin affinity resin column. Lane S, molecular weight marker (Bio-Rad); lane 1, crude lysate of culture prior to treatment with lysozyme and sonication; lane 2, soluble protein fraction after treatment with lysozyme and sonication; lane 3, "flow through" collected after the lysate was passed through the calmodulin affinity column; lane 4, calcium chloride binding buffer wash after collection of the column "flow through"; lane 5, = CBP-Iss eluted from the column with CBP elution buffer. (B) Immunoblot of CBP-Iss expressed from pSF320; that is, an immunoblot of the same protein samples as those in panel A. Proteins were transferred to PVDF membrane and probed with biotinylated calmodulin followed by the addition of a streptavidin alkaline phosphatase detection reagent. The probe recognized bands corresponding in size to that predicted for CBP-Iss; this band is indicated by the arrow.

losis is *iss*. *iss* is strongly associated with disease-causing avian *E. coli* but not with isolates from apparently healthy birds (14). For this reason, the present study focused on *iss* and the protein it encodes. The *iss* gene was first described for its role in the serum resistance of an *E. coli* isolated from a human (1). In addition to its role in serum resistance, *iss* has been shown to contribute to *E. coli* virulence (2), and the presence of *iss* is associated with a 20-fold increase in serum survival for *E. coli* (13). Because of its reported role in serum survival of *E. coli* from other host species, *iss,* as well as other genes, was examined as a potential serum-resistance factor in avian *E. coli*. This examination showed that the *E. coli* isolated from birds with colibacillosis are much more likely to contain the *iss* gene than *E. coli* isolated from healthy birds (4). Therefore, *iss*/Iss may be important for complement resistance and virulence in avian *E. coli*. To facilitate the study of Iss in complement resistance and virulence, *iss* was cloned into two different expression vectors, protein expression was induced, Iss and Iss fusion proteins were purified, and MAbs were developed against Iss. These antibodies are currently being evaluated for their utility as reagents in the study of colibacillosis, and they may prove important in colibacillosis diagnostic procedures. Use of these MAbs may lead to further insights into the role that Iss plays in avian disease, which could further aid in development of future control strategies based on knowledge of avian colibacillosis pathogenesis.

Table 1. Anti-Iss antibody titer in mice.

Mouse number	Serum dilution
1	1:1000
2	1:10000
3	1:1000
4	1:1000
Control	No reaction

Because Iss alone was potentially too small to elicit a strong immune response (3), GST was selected as a fusion partner. We thought the large size of GST would help elicit an immune response in mice. However, the size of GST may have contributed to some of the problems with the specificity of the MAbs that were initially generated. That is, because GST was approximately three times larger than Iss, the majority of antibodies produced by the mice were likely directed against GST, not Iss.

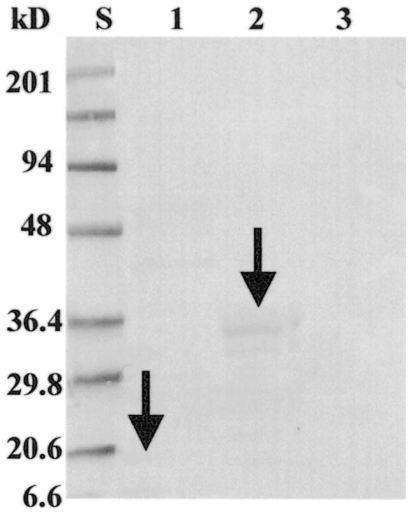

Fig. 5. Western blot to confirm the anti-Iss specificity of the MAb. This western blot demonstrates the specificity of the anti-Iss MAb for the recognition of the Iss fusion proteins. Lane S, the molecular weight standard (Bio-Rad prestained molecular weight standard); lane 1, the crude lysate of BL21 (DE3) pSF320 (the CBP-Iss expression strain after induction); lane 2, the crude lysate of BL21 pLN330 (the GST-Iss expression strain) after induction; lane 3, the crude lysate of *E. coli* A-7, an avian *E. coli* strain that lacks *iss.* In this case, the proteins were blotted to PVDF membrane and probed with anti-Iss MAb. The arrow in lane 1 points to a band that corresponds in size to the CBP-Iss fusion protein, and the arrow in lane 2 points to the GST-Iss fusion protein band. The MAb did not recognize a band in the A-7 lysate.

Also, the protein purification system used with the GST fusion sometimes fails to remove all of the contaminating proteins. Consequently, when Iss was used to coat the screening plates, some GST and/or GST-Iss may have been present, leading to false-positive reactions during screening. To overcome these difficulties, a second fusion was generated with Iss attached to CBP. Because CBP is only 4 kD in size, we reasoned that it would be large enough to make the fusion antigenic but not so large that the majority of MAbs would be directed against CBP rather than Iss. Also, we thought that the small size of CBP would less be likely to have an impact on the function of Iss than GST. Thus, CBP-Iss was ultimately used for the last mouse immunization, with the idea that the B cells that were already directed against Iss would proliferate but that the cells recognizing GST would not be activated. Thus, a "two-fusion protein immunization strategy" was used in this study. Mice were initially given injections with the GST-Iss fusion protein, which had previously been successful in stimulating the production of antibodies specific for Iss. To increase the specificity of the immune response for Iss, a 28-day interval was used between the final GST-Iss injection and the injection with CBP-Iss. Theoretically then, when the CBP-Iss protein was used to inject mice, only plasma cells specific for Iss would proliferate. An unintended consequence of this strategy may have been that the MAbs generated were of the IgM class. Perhaps, the murine immune system recognized CBP-Iss as a new antigen. If this was the case, the 7-day interval between the last injection and fusion, initially chosen to facilitate the selection of IgG antibodies in a secondary immune response, likely coincided with the peak of a primary immune response of which IgM antibodies are a major component, slanting production of antibodies toward the IgM isotype.

This "two-fusion protein approach" was beneficial for the screening of the hybridoma cells. In this case, two plates were coated with antigen, one with GST-Iss and the other with CBP-Iss. MAbs were then screened against both fusion proteins. Hybridomas that recognized both proteins were likely specific for Iss, and these were subcloned, expanded, and frozen for future use.

This "two-fusion protein" immunization and screening strategy appears to have been successful. MAbs that recognize Iss have been generated and are being examined for their potential as reagents for study and diagnosis of avian colibacillosis. Use of MAbs in these ways is well documented. For example, MAbs directed against outer membrane structures have been used in immunofluorescent microscopy to determine the presence of bacterial pathogens (18). An immediate use of these anti-Iss MAbs might be to confirm the location of Iss on the bacterial surface. Further, the MAbs could be used to determine if levels of Iss on the cell surface are related to the degree of virulence or complement resistance of *E. coli,* thus providing insight into the pathogenesis of avian colibacillosis.

In conclusion, avian colibacillosis is a major concern of the poultry industry, yet an understanding of colibacillosis pathogenesis has been hindered by the lack of an identifying characteristic that distinguishes virulent from avirulent avian *E. coli*. On the basis of the work of Pfaff-McDonough *et al.* (14), one such distinguishing characteristic of virulence may be an isolate's possession of *iss*. Because of its potential as a factor to distinguish virulent and avirulent *E. coli, iss* was cloned into an expression vector, the Iss protein was purified, and its identity was confirmed by n-terminal amino acid sequencing. Then, in the present study, MAbs against Iss were generated. The anti-Iss MAbs may prove a useful tool to localize Iss in the bacterium, to detail the interaction of the bacterium and protein with complement, and to facilitate detection and tracking of virulent strains in the production environment.

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