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Research Note—

Iss from a Virulent Avian Escherichia coli

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SUMMARY. No single characteristic of virulent avian Escherichia coli has been identified that can be exploited in colibacillosis detection protocols. Research in our lab suggests a strong association between the presence of an iss DNA sequence with an isolate’s disease-causing ability. The study presented here focuses on the techniques used in the expression, purification, and characterization of avian E. coli Iss protein. In brief, iss was cloned into an expression vector, the construct was transformed into a protease-deficient E. coli, and expression was induced. The protein was expressed as a glutathione-S-transferase (GST) fusion and purified by affinity chromatography. The GST portion was cleaved from Iss, Iss was harvested by affinity chromatography, and the identity of Iss was confirmed by N-terminal sequencing. Currently, purified Iss is being used to prepare hybridomas for production of monoclonal antibodies with the goal of evaluating anti-Iss as a reagent for the detection of virulent avian E. coli.

RESUMEN. Nota de investigación—Proteína Iss de una cepa virulenta de Escherichia coli aviar.

No se ha identificado una característica única para las cepas virulentas de Escherichia coli aviar que pueda utilizarse en protocolos para la detección de colibacillosis. Investigaciones en este laboratorio sugieren una asociación marcada entre la presencia de la secuencia DNA del gen iss con la capacidad de la cepa de causar enfermedad. Este estudio se enfoca en las técnicas utilizadas en la expresión, purificación y caracterización de la proteína Iss de cepas aviares de E. coli. En resumen, se clonó el gen iss dentro de una vector de expresión y se transformó en una cepa de E. coli deficiente en proteasa y se indujo su expresión. La proteína fue expresada como la fusión de glutatión-S-transferasa y purificada por medio de la cromatografía por afinidad. La porción de glutatión-S-transferasa fue separada de la proteína Iss y la proteína Iss fue cosechada mediante la cromatografía por afinidad, confirmándose su identidad mediante la secuencia de N-terminal. Actualmente la protina Iss purificada se está utilizando para la preparación de híbridomas para la producción de anticuerpos monoclonales con la finalidad de evaluar un anticuerpo contra Iss para la detección de cepas virulentas de E. coli.

Key words: avian Escherichia coli, avian colibacillosis, Iss, complement resistance, protein purification, virulence

Abbreviations: DTT = dithiothreitol; EDTA = ethylenediaminetetraacetic acid; GST = glutathione S-transferase; GST-Iss = the fusion protein; IPTG = isopropyl-β-D-thiogalactopyranoside; LB = Luria—Bertani; OD = optical density; PBS = phosphate-buffered saline; PVDF = polyvinylidine difluoride; SDS = sodium dodecyl sulfate; SDS-PAGE = sodium dodecyl sulfate—polyacrylamide gel electrophoresis; TBS = Tris-buffered saline; Tb = melting temperature

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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 pouls and newly hatched chickens. *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 (5). Altogether, these infections result in annual multimillion dollar losses for the U.S. poultry industry (12). One of the difficulties encountered in avian colibacillosis control is that no identifying marker of virulent avian *E. coli* is known. Evidence in the literature suggests that complement resistance may be an important contribution to avian *E. coli* virulence (8,13,22). On the basis of this observation, Pfaff-McDonough and coworkers (14) examined 294 *E. coli* from birds clinically diagnosed with colibacillosis and 75 *E. coli* from apparently healthy birds for their possession of certain factors linked to bacterial complement resistance. Of the factors studied, the occurrence of capsule and the *iss* gene differed significantly across the two groups of birds, and of these two factors, possession of *iss*, the increased serum survival gene, appeared to be the trait of greatest promise for use in distinguishing disease-causing *E. coli* from commensalistic *E. coli*. The *iss* gene is known for its contribution to the virulence and complement resistance of a mammalian *E. coli* isolate (2). The work presented here describes the expression and purification of avian *E. coli* Iss protein for later use in development of *E. coli* detection assays.

**MATERIALS AND METHODS**

**Strains and plasmids.** Strains used were *E. coli* V-2, isolated from a colisepticemias chicken and characterized elsewhere (13,23), *E. coli* XL1-Blue (Stratagene, La Jolla, CA), and *E. coli* BL21 (Pharmacia Biotech, Piscataway, NJ) (20). *Escherichia coli* V-2 has been shown to be virulent in chicks and chick embryos and is complement resistant (23). 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 pGEX-6P-3, the expression vector (Pharmacia), which is designed for production of proteins fused to glutathione S-transferase (GST), pLN320, and pLN330. pLN320 is composed of pGEX-6P-3 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). Isolation of plasmid DNA was accomplished with Wizard Plus Miniprep DNA Purification System (Promega, Madison, WI). Plasmid DNA was digested with restriction enzymes according to the manufacturer's directions (Promega) to confirm insert identity and orientation.

**Cloning.** The *iss* gene was amplified (with and without the signal sequence coding region) for cloning into pGEX-6P-3 with primers designed with Primer Select (DNASTAR, Madison, WI). To amplify the complete *iss* gene for creation of pLN320, the upper primer used was 5'AAAGGGATCCATGCAGGATCTAACAAAGAAAA3', melting temperature (Tm) 73.6 °C; to amplify *iss* without the signal sequence that was used to create pLN330, the upper primer was 5'GACGCGATCCCGA- AAGGTGACTTTGGAAGAA3', Tm 77.8 °C. The lower primer used for both amplifications was 5'CGGCCGAATTCGCGAGAGCTCCCA- TATC3', Tm 82.4 °C. Amplitaq (Perkin Elmer, Nor-

**Fig. 1.** Sequence of the region showing *iss* fused in frame to the carboxyl end of the GST gene in pGEX-6P-3. Arrow = cleavage point for PreScission protease; underlined portion = EcoRI site used to insert the beginning of the *iss* sequences; italicized portion of the DNA sequence = signal sequence found in pLN320 but absent in pLN330. The bold amino acids of the protein were the ones sequenced and found to match the predicted protein sequence.
walk, 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; then, 72°C for 5 min. The amplified iss gene was separated from contaminants in a 0.8% Tris-acetate ethylenediaminetetraacetic acid EDTA (5 Prime → 3 Prime, Boulder, CO) low melting point agarose (FMC, Rockland, ME) gel, excised from the gel, and removed from the agarose with a Wizard PCR PREP DNA purification system (Promega). Amplicons were digested with BamHI for 3 hr at 37°C followed by the addition of EcoRI and its corresponding buffer (Promega), and the reaction mixture was incubated for an additional 3 hr at 37°C. These digests were precipitated overnight at −20°C with 0.1 volumes of sodium acetate and two volumes of absolute ethanol. The precipitated samples were pelleted, the DNA was suspended in sterile water, and the DNA concentrations were determined with DNA dipsticks (Invitrogen, Carlsbad, CA). The insert DNA was ligated into pGEX-6P-3 according to the manufacturer's protocol (15). Liguations were transformed into XL1-Blue by the "classical" 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 by a LI-COR protocol (sequencing bulletin 13; LI-COR, Lincoln, NE) with the Epicentre sequi therm cycle sequencing kit-LC (Epicentre, Madison, WI). IRD800 fluorescent-labeled 5'-pGEX Sequencing Primer (5'-d[GGGCTGGAACGCACTTTGGTG]-3') (15) and 3'-pGEX sequencing primer (5'-d[CCGGGAAGCAGTGTAGGAGG]-3') (15) were used (LI-COR). Sequence data were collected with a LI-COR DNA sequencer model 4000LS and interpreted with BASEIMAGIR software version 2.3 (LI-COR).

**Expression.** The pGEX vectors containing the iss sequences were electrotransformed into the protease-deficient *E. coli* BL21. Electrottransformation was accomplished with the Gene Pulser Transfection Apparatus and Pulse Controller (Bio-Rad Laboratories, Richmond, CA) set at 25 μF, 200 Ω, and 2.5 kV (21). The transformed cells were grown overnight on LB plates supplemented with glucose (20 mM) and ampicillin (100 μg/ml) (15). Individual colonies were transferred to LB broth containing ampicillin (100 μg/ml), and the cultures were held at 37°C with shaking until an optical density (OD) between 0.6 and 1.0 at 600 nm was obtained. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.1 mM. The culture was incubated for an additional 3 hr, and the OD was determined for use in the following step. The cells were pelleted by centrifugation, the supernatant was discarded, and the cells were suspended in 100 μl of 10 mM Tris-HCl, pH 6.7, per absorbance unit at 600 nm. An equal amount of treatment buffer (0.125 M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% glycerol, and 0.02% bromphenol blue) was added to the sample, and the mixture was boiled for 5 min prior to being subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE).
SDS-PAGE and western blotting. After boiling, samples were loaded into wells of 10% polyacrylamide gels for separation of proteins in a Mini-PROTEAN II (Bio-Rad) electrophoresis system according to the method of Laemmli (9). Samples were electrophoresed at 100 V for approximately 1.5 hr. The gels were stained with a solution of 0.025% Coomassie brilliant blue R-250 (Hoeffer Scientific, San Francisco, CA), 40% methanol, and 7% acetic acid (7). Presumptive identification of the bands in the gel was made by molecular size (7), and these were checked through western blotting with monoclonal antibodies directed to the GST portion of the fusion protein (GST-Iss). In the process of western blotting, protein samples were electrophoretically separated as before but were not stained. Proteins in the gel were transferred to polyvinylidine difluoride (PVDF) membrane (Bio-Rad) with the Mini-PROTEAN II blotting apparatus according to the manufacturer's instructions. The membrane was placed into a blocking solution of Tris-buffered saline (TBS) (0.145 M sodium chloride, 0.05 M Tris, pH 7.4) with 3% bovine serum albumin (Sigma, St. Louis, MO) overnight. The next day, the membranes were washed three times with TBS plus 0.02% Tween 20 (Sigma) (16). Next, 10 ml of goat anti-GST (Pharmacia), diluted to the manufacturer's instructions, was added to the membrane, and the membrane was incubated at room temperature for 1 hr with rocking. After incubation, the membrane was washed three times with TBS-Tween 20 before the secondary antibody was added. Ten milliliters of the diluted secondary antibody, rabbit anti-goat immunoglobulin G (Sigma), conjugated to alkaline phosphatase, was added to the membrane and incubated for 1 hr at room temperature with rocking. The membrane was washed with TBS, and Bio-Rad immuno-blot color development reagent (5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolamine in dimethylformamide) was added to the membrane. The reaction was allowed to develop for approximately 10 min and was stopped by washing the membrane in distilled water (16). The antibody incubations and the color development steps were done in plastic bags (Gibco BRL, Gaithersburg, MD).

Lysis and solubilization. Lysis and solubilization of GST-Iss fusion protein was accomplished after expression of protein from pLN330, the construct without the signal sequence, although efforts to solubilize GST-Iss produced from pLN320, which were unsuccessful, also were made. Briefly, the procedures that were successful follow. A 50-ml culture of LB broth, inoculated with BL21 containing pLN330, was incubated 18 hr at 37 C with vigorous shaking. This 50-ml culture was used to inoculate 1.5 liters of LB broth containing 100 µg/ml of ampicillin. Then, this culture was incubated for 3 hr at 37 C with vigorous shaking. After incubation, IPTG was added to the culture to a final concentration of 0.1 mM to induce expression, and the culture was incubated for an additional 3 hr. After the final incubation, the culture was transferred to 250-ml centrifuge tubes and pelleted at 7700 x g for 10 min at 4 C (Beckman JA-14 Rotor; Beckman Instruments, Palo Alto, CA). The supernatants were discarded, and the pelleted cells were suspended in 50 ml of phosphate-buffered saline (PBS) Amresco and placed on ice. Pellets of bacterial cells containing the constructs were lysed by sonication and detergent treatment. Sonication with the Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT), equipped with the macrotip, was done at a power output setting of eight (maximum output of 35%) for 1 min with a 50% duty cycle. During and after each sonication, samples were held in an ice bath to limit denaturation of the proteins by heat. After sonication, samples were treated with the nonionic detergent Triton X-100 (20%) to a final concentration of 1% (Amresco) for 30 min to further lyse the cells and solubilize the fusion protein (15). After incubation, the suspension was centrifuged at 12,000 x g for 10 min at 4 C (Beckman JA-20 Rotor), and the supernatants, containing the soluble fractions, were subjected to further purification techniques.

Purification. Purification was done through use of an affinity matrix. The soluble protein was combined with 1.5 ml of a 50% slurry of glutathione sepharose 4B (Pharmacia) beads, equilibrated with PBS, and incubated for 1 hr at 4 C, as directed by the manufacturer. The matrix mixture was added to a 5-cc column, and the "flow through" was collected for analysis. The column was washed with 5.0 ml of PBS three times, and the GST-Iss fusion protein was eluted with 1.0 ml of glutathione elution buffer (10 mM glutathione, 50 mM Tris, pH 8.0). Fifty microliters of the eluted fusion protein was analyzed by SDS-PAGE and western blotting. After positive identification of the eluted fusion protein by molecular size and reaction with anti-GST, the protein was dialyzed overnight at 4 C against PreScission cleavage buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT]) (15). Then, the dialyzed sample was treated with 80 units of PreScission protease (Pharmacia) to cleave the expression product into GST and Iss. The mixture was combined with 1.0 ml of a 50% slurry of glutathione sepharose 4B and incubated overnight at 4 C with shaking. The next day, the mixture was added to a 5-cc column, and Iss was eluted with the dialysis buffer.

Amino acid sequencing. Iss was prepared for sequencing by concentrating the sample from 1.0 ml to 60 µl using Micron 3 microconcentrators (Amicon, Beverly, MA) according to the manufacturer's directions. The concentrated sample was subjected to SDS-PAGE, and the resulting gel was equilibrated in...
CAPS transfer buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid [Sigma], 0.5 mM DTT, and 10% methanol, pH 11.0) (1). Iss was electroblotted to PVDF membrane at 220 mA for 7 min by the Mini-PROTEAN II (Bio-Rad) electrophoresis system. The membrane was stained with Coomassie brilliant blue R-250. The portion of the membrane containing the bands presumptively identified by size as Iss was cut away from the remainder of the membrane, and the cut membrane containing the bands of interest was submitted to the Biopolymers Service Center of the North Dakota State University Biotechnology Institute for N-terminal amino acid sequencing.

RESULTS AND DISCUSSION

Currently, avian colibacillosis control is hindered by the lack of an identifying marker of virulent avian E. coli. The iss gene is known to contribute to the virulence and complement resistance of a mammalian E. coli isolate (2), and results obtained in our laboratory suggest that the presence of iss may serve as a marker of virulence among avian E. coli. That is, iss is strongly associated with E. coli of birds with colibacillosis but not with E. coli of healthy birds (14). In this study, iss was cloned into an expression vector, expression was induced, and the Iss protein was purified in order to facilitate future exploration of its use in detection of virulent avian E. coli, its role in E. coli virulence, and its potential to elicit immunity against E. coli in birds.

The iss gene, with and without the signal sequence, was cloned successfully into the expression vector, pGEX-6P-3, to create the plasmids pLN320 and pLN330, respectively. The resulting constructs contained iss in the desired reading frame for expression via DNA sequencing (Fig. 1). Crude total protein preparations of a protease-deficient E. coli (BL21) transformed with these constructs revealed that "induced" BL21 containing pLN320 overexpressed a 37-kD protein, BL21 containing pLN330 overexpressed a 34-kD protein, and BL21 containing pGEX-6P-3 (the vector plasmid without an insert) overexpressed a 26-kD protein (Fig. 2). These sizes correspond to those predicted to be produced from these plasmids because GST should be 26 kD (15). Iss with the signal sequence should be 10–11 kD, and Iss without the signal sequence should be 8–9 kD (Gene Inspector; Textco, Inc., West Lebanon, NH). Thus, GST-Iss fusion proteins should be approximately 37 kD with the signal sequence and 34–35 kD without it. These proteins were not overexpressed when the cells were "uninduced" (Fig. 2). In a western blot, proteins of the predicted sizes were recognized by monoclonal antibodies to GST (Fig. 3), indicating the presence of the fusion proteins.
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Iss) with was produced and this and pLN320, from column; column.

Initially, GST-Iss was expressed from pLN320, but after repeated failures to solubilize and purify the fusion protein produced from this construct (results not shown), expression and purification efforts were made with pLN330. Efforts to solubilize GST-Iss produced from pLN320 included varying solubilizing reagents and altering the growth and induction conditions. Changes in growth temperatures, aeration, induction times, and levels of IPTG were used (15,18,19). Also, sonication was done with various power output levels, with different duty cycles, and for various times. Agents such as lysozyme, N-lauroyl sarcosine, urea, DTT, guanidine-HCl, and Triton X-100 were used to treat the postsonicate pellet in efforts to solubilize GST-Iss (4,6,10,11). None of these attempts resulted in a soluble protein that would bind to the glutathione sepharose 4B column.
PpLN330 differs from pLN320 by the absence of the signal sequence, which, we reasoned, might result in a fusion product differing in solubility from pLN320’s product. The 34-kD GST-Iss fusion protein, produced from the pLN330 construct, was successfully solubilized after sonication and Triton X-100 treatment and purified by glutathione sepharose 4B affinity chromatography (Fig. 4). Its identity was confirmed presumptively by size and through western blotting with anti-GST (Fig. 3) and definitively through N-terminal amino acid sequencing. Preparation for sequencing involved cleavage of GST-Iss, separation of Iss from other products via affinity chromatography followed by SDS-PAGE, transfer of Iss to PVDF, and submission of the portion of the membrane containing the 8-kD bands for analysis (Fig. 5). Fifteen N-terminal amino acids were sequenced, and the predicted results from translation of the DNA sequence matched those obtained by amino acid sequencing (Fig. 1). It should be noted that predictions of protein structure based on DNA analysis with Gene Inspector software (Textco) suggest that the removal of the signal sequence will have

Fig. 4. SDS-PAGE of the soluble protein (GST-Iss) obtained after expression of pLN330 in BL21, lysis by sonication, treatment with Triton X-100, and addition of lysate to the glutathione sepharose 4B column. Lane 1 = “flow through” collected after the lysate was passed through the glutathione sepharose column; lane 2 = PBS wash after collection of the column “flow through”; lane 3 = GST-Iss eluted from the column with glutathione elution buffer.

Fig. 5. PVDF membrane stained with Coomassie brilliant blue R 250 containing the purified Iss protein that was subjected to N-terminal amino acid sequencing. Lanes 1, 2 = the sample prepared for amino acid sequencing. The 8-kD bands of interest were cut away from the other bands that copurified, and the presumptive Iss bands were subjected to N-terminal sequencing. The 70-kD protein that copurified with Iss is likely DnaK. Copurification of this protein is a relatively common complication encountered when using this purification system (3,15). Future efforts will be made to eliminate DnaK from the sample.

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little, if any, effect on the antigenicity of Iss. This lack of effect on antigenicity is desirable because we plan to use Iss as an immunogen for developing monoclonal antibodies against Iss and in determining its potential for eliciting immunity in birds.

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