INFECTION AND IMMUNITY, Feb. 1992, p. 529–535
0019-9567/92/020529-07$02.00/0

Vol. 60, No. 2

Cloning and Expression of a *Serpula (Treponema) hyodysenteriae* Hemolysin Gene

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Received 12 August 1991/Accepted 19 November 1991

Serpula (Treponema) hyodysenteriae*, the etiologic agent of swine dysentery, produces a hemolysin which is thought to be an important factor in the pathogenesis of the disease. We report the cloning, sequencing, and expression of a hemolysin gene (*tly*) from *S. hyodysenteriae* B204. A pUC19 gene bank of strain B204 was constructed in the *Escherichia coli* K-12 strain DH5α, and hemolytic recombinants were identified by plating the library on blood agar plates. From the hemolytic recombinants, a 1.5-kb DNA fragment could be isolated that contained information necessary for the production of a hemolysin/cytotoxin in *E. coli*. Nucleotide sequence determination of this 1.5-kb fragment showed that it contained an open reading frame capable of encoding a 26.9-kDa protein. The recombinant hemolysin was easily released from *E. coli* by osmotic shock. As with the native hemolysin, the recombinant hemolysin is EDTA insensitive, thermolabile, and cytotoxic for several eukaryotic cell lines. Southern blot hybridization showed that the cloned *S. hyodysenteriae* hemolysin gene *tly* is present in all pathogenic strains of *S. hyodysenteriae* tested and absent in the nonpathogenic, weakly hemolytic spirochete *S. innocens*.

Serpula (Treponema) hyodysenteriae and *S. innocens* are anaerobic, beta-hemolytic spirochetes found in the porcine large intestine (15, 16). *S. hyodysenteriae* is the etiologic agent of swine dysentery (SD), a highly contagious infectious disease characterized by a mucohemorrhagic diarrhea (7, 9, 31). *S. innocens*, which can be isolated from animals with no apparent disease, is very similar to *S. hyodysenteriae* in both morphology and growth characteristics and has many of the same surface antigens; however, it is nonpathogenic to swine (12).

Spirochetes isolated from the colon of pigs are classified on the basis of their hemolytic activity on blood agar plates. *S. hyodysenteriae* is strongly beta-hemolytic, while the nonpathogenic *S. innocens* is only weakly beta-hemolytic (15). The presence of the strong hemolytic activity is associated with pathogenicity (11, 25, 27). Unless extensively passaged in vitro, *S. hyodysenteriae* isolates that show strong hemolytic activity on blood agar plates are virulent when used to orally infect specific-pathogen-free swine. Isolates possessing weak hemolytic activity are avirulent (17). In spite of the correlation between hemolytic activity and pathogenicity (16, 25–27), the exact role which the hemolysin plays in the pathogenesis of SD is not known.

Virulence factors have not been identified with certainty for *S. hyodysenteriae*, nor have the pathogenic mechanisms used by the spirochete to cause necrosis and lysis of the epithelial cells in the large intestine of infected pigs been elucidated. In vitro, the purified hemolysin has been shown to be cytotoxic for a number of cell types (13). When swine intestinal loops were exposed to purified *S. hyodysenteriae* native hemolysin, lesions similar to those seen in natural cases of SD were observed (22). The hemolysin is a potent cytotoxin for enterocytes and may be an important virulence factor for *S. hyodysenteriae*.

The *S. hyodysenteriae* hemolysin can easily be isolated from culture supernatant. The hemolysin was originally characterized as an oxygen-resistant, proteinase K-sensitive, heat-labile polypeptide (33). Several investigators have reported its isolation and purification; however, the reported size of the purified *S. hyodysenteriae* hemolysin varied from 19 kDa (14) to 68 kDa (17) and 74 kDa (33).

Here we describe the cloning, nucleotide sequence determination, and expression in *Escherichia coli* of a cytotoxin/hemolysin gene (*tly*) from *S. hyodysenteriae* B204. We constructed a genomic library from this virulent *S. hyodysenteriae* strain in the plasmid vector pUC19. *E. coli* K-12 strain DH5α recombinants harboring this library were screened for hemolytic activity on tryptic soy agar (TSA) plates containing 4% sheep erythrocytes (SRBC). From the hemolytic recombinants, a single hemolytic clone (pSML2) was selected for further analysis. Fragments of the insert of pSML2 were subcloned, and the nucleotide sequence of the smallest hemolytic subclone was determined. The sequence revealed the presence of an open reading frame capable of encoding a 26.9-kDa protein.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. hyodysenteriae* strains are classified according to their serotypes, which are

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probably lipopolysaccharide determined. The origins and serotypes of the spirochetes used in this study have been described by M. E. Mapother (24), who kindly supplied *S. hyodysenteriae* strains B234, B204, B169, A-1, B8044, B6933, and Ack 300/8 (serotypes 1, 2, 3, 4, 5, 6, and 7, respectively), *S. hyodysenteriae* strain B204 (serotype 2) attenuated through 124 consecutive passages, and *S. innocens* strain B256. All spirochetes were grown in tryptic soy media (Difco Laboratories, Detroit, Mich.) supplemented with 5% fetal bovine serum (Flow Laboratories, McLean, Va.) as previously described (8). The plasmid pUC19 and the phagemids pBluescript pKS^+ and pSK^+ (Stratagene Cloning Systems, La Jolla, Calif.) were used for all cloning procedures. *E. coli* K-12 strain DH5α (GIBCO BRL, Gaithersburg, Md.) was used as a host for all plasmids.

Preparation of *S. hyodysenteriae* chromosomal DNA. Molecular-grade chemicals and enzymes were from Sigma Chemical Co. (St. Louis, Mo.). Bacteria from 1-liter cultures were collected by centrifugation, and bacterial cell pellets were washed in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and frozen at −70°C until further use. Frozen bacterial cell pellets were thawed in 25 ml of buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM EDTA, 150 mM NaCl, and 10 mg of lysozyme per ml. Following a 1-h incubation at 37°C, 0.5 ml of bovine pancreas RNase A was added to the cells, which were then incubated for an additional 15 min at 70°C. Cell lysis was completed by the addition of 2.5 ml of 30% Sarkosyl, gentle mixing, and incubation at 70°C for 20 min, followed by a 1-h incubation at 37°C. Predigested pronase (22) was added (to a final concentration of 10 mg/ml), and the incubation was continued for 4 h at 37°C. The lysate was transferred to dialysis tubing and dialyzed overnight in TE. The DNA was then extracted once with an equal volume of TE-saturated phenol, followed by an extraction with an equal volume of chloroform-isoamyl alcohol (24:1). The DNA was subsequently dialyzed against TE and then ethanol precipitated. Chromosomal DNA was resuspended in TE at a final concentration of 1 mg/ml. DNA prepared in this manner was used for library construction and Southern blot analysis.

Construction of an *S. hyodysenteriae* genomic library. Restriction enzymes, calf intestinal phosphatase, T4 DNA ligase, bovine pancreas RNase A, and the Klenow fragment of *E. coli* DNA polymerase I were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). All enzymes were used as specified by the manufacturer. Standard cloning protocols (23) were used for all DNA manipulations. *S. hyodysenteriae* DNA was digested with the restriction enzyme *Mbo*I and ligated with T4 DNA ligase to *Bam*HI-restricted, dephosphorylated pUC19 plasmid DNA. *E. coli* K-12 strain DH5α cells were transformed with the ligation mix, and the resulting recombinants were screened for hemolysin production.

Screening for hemolytic clones. Recombinants were plated on tryptic soy agar containing 4% defibrinated SRBC (Colorado Serum Co., Denver, Colo.) and 100 μg of carbenicillin per ml (TSA blood plates). The plates were incubated at 37°C for 24 h to detect hemolytic colonies. A single hemolytic clone, designated pSML2, was chosen for further analysis. From this clone, all other subclones (Fig. 1) were constructed by standard cloning protocols (23).

Southern blotting. Chromosomal DNA from *S. hyodysenteriae* was digested with the restriction enzyme *Eco*RV, separated on a 0.8% agarose gel, and subsequently transferred to a nylon membrane (Amersham, Arlington Heights, Ill.). The 1.5-kb insert from $pJBA^{KS}$, the smallest hemolytic

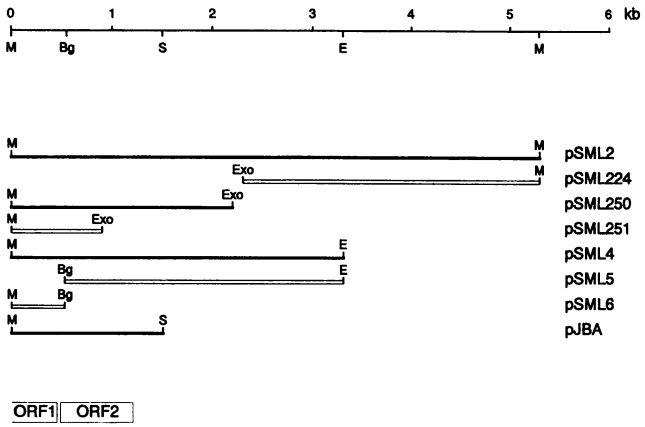

FIG. 1. Recombinant clones. (Top) Positions of the chromosomal restriction sites used to construct the clones used in this study. (Center) Relative positions of the inserts cloned in the indicated recombinants. (Bottom) Relative positions of ORF1 and ORF2 (*tly*). M, *Mbo*I (only the two sites of the partial digest that gave rise to clone pSML2 are indicated); E, *Eco*RI; Bg, *Bgl*II; S, *Sca*I; Exo, Exonuclease III. Hemolytic clones are represented as solid bars; non-hemolytic clones are represented as open bars.

subclone of pSML2, was ^{32}P labeled by random priming (4). Prehybridization, hybridization, and washing of the membrane were done at 60°C, as described before (23). The washed membrane was exposed to X-OMAT AR film (Eastman Kodak Company, Rochester, N.Y.) at −70°C, with a Du Pont (Boston, Mass.) Lightning Plus intensifier screen.

Hemolysin extraction. Hemolysin was extracted from *S. hyodysenteriae* B204 by the RNA core extraction procedure (14). Recombinant hemolysin was extracted from *E. coli* DH5α cells by osmotic shock (10).

Hemolysin assay. The hemolytic activity of both the RNA core hemolysin and the recombinant osmotic shock supernatant was determined as follows. Aliquots were diluted at twofold steps up to 1:640. These dilutions were adjusted to a final concentration of 140 mM NaCl, either directly or after treatment with 50 mM EDTA, heating (5 min, 100°C), or pronase treatment, and added to an equal amount of SRBC which were washed in and resuspended at 10% in 140 mM NaCl. The mixtures were incubated at 37°C for 1 h, and the release of hemoglobin was determined by reading the OD_{540} of the supernatant. The values for 0 and 100% lysis were obtained by the addition of 140 mM NaCl and water, respectively, to SRBC. Endpoint dilutions required to lyse 50% of the SRBC suspension were determined.

Cytotoxicity assay. Osmotic shock supernatant from *E. coli* $DH5\alpha(pJBA^{KS})$, DH5α(pSML5), and DH5α(pUC19) and RNA core hemolysin were filter sterilized and added to cultured eukaryotic cell lines in appropriate dilutions. Cell lines tested included Chinese hamster ovary (CHO), HeLa, and Madin-Darby canine kidney (MDCK) cells. The cells were incubated at 37°C in a CO_2 incubator and examined at various time intervals for cytopathic effect. Cytopathic effect was determined by direct visual inspection of the monolayers at 1, 12, and 24 h following the addition of hemolysin to each well.

DNA sequencing. The insert from clone $pJBA^{KS}$ was subcloned into M13mp18 and M13mp19. Both strands were sequenced by dideoxynucleotide chain termination with a Sequenase kit (United States Biochemical, Cleveland, Ohio). The −40 M13 sequencing primer and internal primers

(synthesized on a Cyclone Plus DNA synthesizer; Millipore Corp., Bedford, Mass.) were used to determine the sequence of the insert.

Nucleotide sequence accession number. The sequence determined has been submitted to the EMBL data base (accession number X61684).

RESULTS

Molecular cloning of the *tly* gene. The plasmid vector pUC19 was used to prepare a library of *S. hyodysenteriae* B204 DNA. The library was plated on TSA blood agar plates and screened for hemolytic colonies. This screening resulted in the identification of several hemolytic clones. A single hemolytic clone, designated pSML2, was chosen for subsequent analysis. Restriction enzyme analysis of the plasmid DNA from clone pSML2 showed that this clone contained an insert of approximately 5.3 kb (Fig. 1).

Deletion analysis of the cloned *tly* gene. In order to determine the region encoding the hemolysin, the enzymes exonuclease III and mung bean nuclease were used to create nested deletions from both ends of the pSML2 insert. All deletions at the left-hand end of the insert of clone pSML2 (clone pSML224, Fig. 1) resulted in the abrogation of hemolytic activity. It was possible to remove 3.1 kb of DNA from the right-hand end of the insert of clone pSML2 and still obtain full hemolytic activity (clone pSML250, Fig. 1). Only extremely large right-hand end deletions of the pSML2 insert abolished all hemolytic activity (clone pSML251, Fig. 1). These data suggest that the *tly* gene is at the left-hand end of clone pSML2.

The results obtained with exonuclease III were confirmed by subcloning restriction enzyme fragments from the left-hand end of pSML2. Subclone pSML4 contains a 3.3-kb *Eco*RI fragment of pSML2 and is as hemolytic as the parent plasmid. To further define the DNA region encoding hemolytic activity, pSML4 was digested with the restriction endonucleases *Bgl*II and *Hin*cII. The staggered *Bgl*II ends of the 2.8-kb *Bgl*II-*Hin*cII fragment were filled in with the large Klenow fragment of DNA polymerase I (23) and then ligated into *Sma*I-digested DNA of the pUC19 vector. The ligation mixture was subsequently transformed into competent *E. coli* K-12 strain DH5α cells. The resultant recombinant clone, pSML5 (Fig. 1), was not hemolytic. Digestion of pSML4 with *Sca*I and *Bam*HI produced a 1.5-kb fragment which, when subcloned into pBluescript phagemid pKS^+ or pSK^+ digested with the restriction enzymes *Eco*RV and *Bam*HI, yielded the plasmids $pJBA^{KS}$ and $pJBA^{SK}$, respectively. Since both the $pJBA^{KS}$ and the $pJBA^{SK}$ plasmids were as hemolytic as pSML2, expression of the *tly* gene is independent of the orientation of the insert in the plasmid; thus, it is possible that the 1.5-kb fragment contains both the complete hemolysin gene and all necessary transcription and translation signals. The insert of clone $pJBA^{KS}$, the smallest hemolytic clone obtained, was used for sequence determination of the *tly* gene.

Copy number of the *tly* gene. To establish the copy number of the cloned hemolysin gene, genomic DNA from *S. hyodysenteriae* was digested with five different restriction enzymes that did not cut within the 1.5-kb insert of clone $pJBA^{KS}$. Only one band was visible when Southern blots of the digested DNA were probed with the 1.5-kb insert of clone $pJBA^{KS}$ (*Eco*RV in Fig. 2; other digests not shown). This indicates that the *tly* gene is present in the chromosome of *S. hyodysenteriae* as a single copy.

Conservation of the *tly* gene within different serotypes.

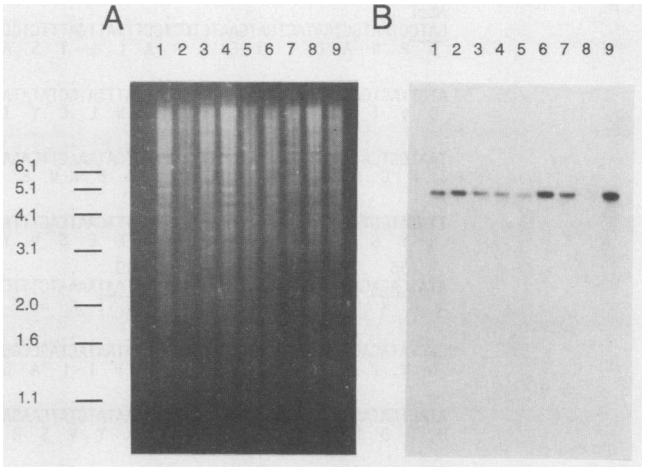

FIG. 2. Presence of the *tly* gene in *S. hyodysenteriae* serotypes. (A) Ethidium bromide-stained agarose gel of *Eco*RV-restricted chromosomal DNA from *S. hyodysenteriae* and the nonpathogenic spirochete *S. innocens*. (B) Southern blot of gel in panel A probed with the 1.5-kb insert of $pJBA^{KS}$. Lane 1, *S. hyodysenteriae* B8044, serotype 5; lane 2, *S. hyodysenteriae* B234, serotype 1; lane 3, *S. hyodysenteriae* B169, serotype 3; lane 4, *S. hyodysenteriae* Ack 300/8, serotype 7; lane 5, *S. hyodysenteriae* B6933, serotype 6; lane 6, *S. hyodysenteriae* B204, serotype 2, attenuated at 124 passages; lane 7, *S. hyodysenteriae* A-1, serotype 4; lane 8, *S. innocens* B256; lane 9, *S. hyodysenteriae* B204, serotype 2. Positions of molecular size markers (GIBCO BRL) are indicated (in kilobases).

Hemolytic *S. hyodysenteriae* strains are classified according to their serotypes, which are probably lipopolysaccharide determined (24). To establish whether the cloned hemolysin gene was present in all *S. hyodysenteriae* serotypes, Southern hybridization of endonuclease *Eco*RV-digested chromosomal DNA of strains belonging to seven different *S. hyodysenteriae* serotypes was performed. In all hemolytic serotypes of *S. hyodysenteriae*, a single 4.8-kb *Eco*RV fragment hybridized with the hemolysin gene containing the 1.5-kb *Sca*I-*Bam*HI fragment of clone $pJBA^{KS}$ (Fig. 2). No hybridization occurred with weakly hemolytic treponemes (only the prototype *S. innocens* B256 is shown in Fig. 2). No hybridization signal above background was detected with chromosomal DNA from hemolytic strains of the enteric pathogens *Salmonella typhimurium* and *E. coli* (not shown).

Sequence of the *tly* gene. The nucleotide sequence of the hemolysin gene containing the 1.5-kb *Sca*I-*Bam*HI fragment of clone $pJBA^{KS}$ was determined and is shown in Fig. 3. The sequence was exceptionally A+T rich (74%), as has been reported for pathogenic and nonpathogenic *Serpula* strains (28). The DNA sequence contained only two large open reading frames (ORFs); both ORFs are located on the same strand. ORF1 spans the first 456 nucleotides of the insert of clone $pJBA^{KS}$; ORF2 runs from residues 471 to 1190 (Fig. 3). Based on our deletion experiments, ORF1 cannot encode the hemolysin, since both clone pSML250 and clone pSML6 contain the complete ORF1 but are nonhemolytic (Fig. 1). Therefore, ORF2 most likely represents the *tly* gene.

The hemolysin-encoding ORF2 spans 720 bp and is capable of encoding a protein of 240 amino acids with a calculated molecular mass of 26.9 kDa. The hemolytic activity of *E. coli* containing the 1.5-kb *Sca*I-*Bam*HI fragment is independent of the orientation of the insert in the vector (Fig. 1), and thus the hemolysin gene may be preceded by promoter signals that function in *E. coli*. Assuming that *S. hyodysenteriae*

FIG. 3. Nucleotide sequence of the *tly* gene of *S. hyodysenteriae*. The deduced amino acid sequence of ORF1 and ORF2 is presented under the DNA sequence in the one-letter code. Asterisks denote the stop codons of ORF1 and ORF2. Relevant restriction enzyme recognition sites (*Mbo*I, *Bgl*II, and *Sca*I, Fig. 1), a potential ribosome binding site (RBS), and putative −10 and −35 promoter regions are underlined. The potential cell attachment site (RGD [32]) is underlined.

promoter sequences are similar to those of *E. coli*, putative −10 and −35 promoter regions could be identified and are indicated in Fig. 3. No extensive sequence homology with the *E. coli* ribosome-binding site was detected. The best fit is the triplet GGA, 18 bp upstream of the initiator codon, ATG.

The DNA sequence of the 1.5-kb insert of pJBA^{KS} and the derived amino acid sequences of ORF1 and ORF2 were compared with the EMBL, GenBank, NBRF, and the Swiss-Prot (releases 27, 67, 28, and 18) sequence data bases, using the FASTA and TFASTA programs (29). This search did not reveal any significant sequence homologies with the sequences present in these data bases.

In order to visualize the hemolysin encoded by the 1.5-kb *Sca*I-*Bam*HI fragment, *E. coli* minicell and maxicell experiments were performed. We could not detect any insert-specific proteins in these cells (data not shown). This suggests that the expression level of the hemolysin is extremely low. Porcine anti-*S. hyodysenteriae* sera from animals in the acute and convalescent stages of swine dysentery were reacted with partially purified native RNA core and recombinant hemolysins on a Western immunoblot. Neither immunoglobulin G (IgG) nor IgM conjugates detected any specific response on these blots.

Characterization of the recombinant hemolysin. Hemolytic activity can easily be detected in the culture supernatant of *S. hyodysenteriae* B204. No detectable hemolytic activity was present in the supernatant of *E. coli* recombinants which contained any of the hemolytic clones. Thus, with these *E. coli* recombinants, excretion of recombinant hemolysin into the medium differs from excretion by the native organism. By using an osmotic shock procedure, supernatant from *E. coli* DH5α(pJBA^{KS}) was found to contain potent hemolytic activity. As with the native RNA core hemolysin, this activity was destroyed when the supernatant was boiled or treated with pronase. Control osmotic shock supernatant from *E. coli* DH5α(pSML5) and DH5α(pUC19) were totally nonhemolytic even when concentrated 40 times with Amicon microconcentrator.

Unlike the hemolysins from *E. coli*, which require calcium for activity (21), the *S. hyodysenteriae* hemolysin activity is calcium independent. The addition of 50 mM EDTA to the native (RNA core) hemolysin and the osmotic shock supernatant of *E. coli* DH5α(pJBA^{KS}) did not affect their ability to lyse SRBC.

Carbohydrates of various molecular dimensions have been used to determine whether various substances (34) or toxins, such as the *E. coli* hemolysin (2), generate transmembrane pores. The sugars physically "plug" pores which may form in cell membranes, preventing subsequent cell lysis. Osmotic shock supernatant from *E. coli* DH5α(pJBA^{KS}) and

DH5α(pKS$^+$) and the RNA core hemolysin were added to SRBC that had been treated with carbohydrates of increasing molecular dimensions: sucrose (0.9 nm), raffinose (1.2 to 1.4 nm), and dextran 5000 (1.75 to 2.3 nm). These sugars would serve as osmoprotectants if the recombinant or native hemolysin was able to lyse erythrocytes by forming pores in the membrane. Induction of SRBC lysis by both the osmotic shock supernatant of DH5α(pJBA^{KS}) and native hemolysin was not influenced by any of the above three sugars.

Cytotoxic activity of the recombinant *tly*. The supernatant from DH5α(pJBA^{KS}) revealed potent cytolytic activity, as CHO, HeLa, and MDCK cells incubated with it were killed within a 1-h incubation period. The native RNA core hemolysin was as effective in killing these cells as was the recombinant hemolysin. Osmotic shock supernatant from DH5α(pSML5) and DH5α(pUC19) had no cytolytic effect (even when concentrated 40 times with an Amicon microconcentrator) on the cells following a 24-h incubation period, nor did heat-inactivated recombinant hemolysin, RNA core extraction buffer, or heat-inactivated RNA core hemolysin.

DISCUSSION

Hemolysin isolated from *S. hyodysenteriae* is cytotoxic for a number of different cell types (13) and causes lesions similar to those occurring in natural cases of SD in isolated swine ileal and colonic loops (22). Cytotoxin/hemolysin production has been shown to be associated with virulence for many bacterial species (1, 3, 5, 6, 18, 37). The pathology of SD also supports the presence and activity of a cytotoxin/hemolysin molecule during the acute phase of the disease. In vitro, both cholesterol and phospholipids are needed for *S. hyodysenteriae* growth in lipid-depleted broth cultures (36), and cholesterol appears to be an essential growth factor for the spirochete (19, 35, 36). The cytotoxin/hemolysin appears to be an important virulence factor. It kills intestinal mucosal cells supplying *S. hyodysenteriae* with vital sterols from lysed membranes, and it causes damage to the lamina propria and submucosa, which renders them susceptible to invasion by the intestinal flora.

In spite of the probably importance of the cytotoxin/hemolysin as a potential virulence factor of *S. hyodysenteriae*, virtually nothing is known about the molecular mechanisms associated with these activities. There is no evidence as to whether the cytotoxic and hemolytic activity are entailed within a single molecule, or whether they represent the action of two or more separate molecules present in the same fraction. The reported molecular masses of semipurified *S. hyodysenteriae* hemolysin vary from 19 kDa (14) to 68 kDa (17) and 74 kDa (33). Although we have no conclusive biochemical data relating ORF2 to hemolytic activity, from the subcloning data, we suggest that ORF2 encodes a recombinant hemolysin of 26.9 kDa. As *S. hyodysenteriae* may produce more than one hemolysin, it is possible that one or more of these other reported values is also correct.

We have cloned a hemolysin gene, *tly*, from *S. hyodysenteriae* and by Southern blotting demonstrated its presence in all tested pathogenic serotypes of *S. hyodysenteriae* and its absence in the weakly hemolytic nonpathogenic organism *S. innocens*. Southern blot hybridization experiments (Fig. 2 and not shown) suggest that only a single copy of the *tly* gene is present in the genome of all hemolytic *S. hyodysenteriae* strains, as when the *tly* gene probe was used, even under low-stringency conditions, only one hybridization signal was observed. This indicates that if other cytotoxin/hemolysin genes are present within hemolytic *S. hyodysenteriae* strains, they are not related to the cloned *tly* gene. The weak hemolytic activity of *S. innocens* is probably the product of a gene(s) unrelated to the cloned *tly* gene.

The recombinant hemolysin, which also possesses cytotoxic activity, behaves like the native RNA core hemolysin in all tested parameters. Both are potent hemolysins and cytotoxins, and their hemolytic activity is sensitive to heat and pronase treatment and insensitive to added sugars and EDTA; thus, it is likely that the cloned *tly* gene represents a cytotoxin/hemolysin present in the RNA core extract of hemolytic *S. hyodysenteriae*.

We cannot provide a solid explanation for the differences between the reported sizes of the hemolytic protein present in the RNA core hemolysin (19, 68, and 74 kDa; references 14, 17, and 33, respectively) and the calculated molecular mass of the cloned hemolysin (26.9 kDa). Posttranslational modification of the *tly*-encoded hemolysin may result in a molecule with the molecular size reported in the articles cited above. One cannot, however, exclude the possibility that *S. hyodysenteriae* possesses more than one hemolysin, and other hemolysins—not homologous to the cloned *tly* gene—may be encoded by *S. hyodysenteriae*.

For unknown reasons, sodium ribonucleate increases the hemolytic activity of *S. hyodysenteriae*, by inducing either its production, release, or activity (20, 30). Hemolytic clones could be identified without sodium ribonucleate core supplementation of the agar, since the recombinant hemolysin was sufficiently active to express the hemolytic phenotype. As hemolysis could be detected regardless of the orientation of the *tly* gene, the hemolysin may be transcribed from its own promoter in *E. coli*. As we have no transcript mapping data, one cannot exclude the possibility that a vector-promoted transcription unit might read into the insert to give expression. However, addition of isopropyl-β-D-thiogalactopyranoside to *E. coli* carrying pJBA^{KS} or pJBA^{SK} did not induce high levels of hemolytic activity. If the hemolysin is being driven from its own promoter in *E. coli*, it is probably not very strong, since it was impossible to label a unique *tly*-encoded protein with [^{35}S]methionine in either *E. coli* minicells or maxicells. Alternatively, the *E. coli* tRNAs may fail to translate the peptide efficiently. In vitro transcription-translation of the cloned DNA did not reveal any unique proteins of between 25 and 30 kDa.

Western blot analysis with sera from infected pigs did not detect the presence of the hemolysin in partially purified hemolysin preparations. This suggests that during actual disease, either the hemolysin does not induce a serologically detectable immune response or hemolysin is produced in minute quantities. If the latter is true, the hemolysin is a molecule with high specific activity, and minute amounts suffice to lyse large numbers of SRBC. Preliminary data on the purification of the recombinant hemolysin by anion-exchange chromatography support this assumption, since no unique protein band was seen after electrophoresis of the fractions containing the hemolytic activity.

Computer searches of both nucleic acid and protein data bases revealed no homologies between the *S. hyodysenteriae* hemolysin and other reported bacterial hemolysins. Furthermore, there were no sequences in any of these data bases which possessed statistically significant homologies with ORF1 or the *tly* gene. Thus, based on its molecular composition, ORF1 and the *tly* gene encode a novel molecule. At nucleotide positions 1020 to 1028, the *tly* gene sequence encodes the tripeptide arginine-glycine-aspartic acid. This RGD tripeptide sequence is also present in fi-

bronectin, fibrinogen, vitronectin, von Willebrand factor, and some slime mold discoidins, in which it has been shown to play a role in cell adhesion (32). The RGD sequence is also found in viral surface proteins and in some collagens, where it may serve a similar function. The *S. hyodysenteriae* hemolysin was shown to adhere to SRBC at 4°C without lysing them (13). With cell adherence and cell lysis being two separate activities of the hemolysin, the RGD sequence may serve in the cell attachment function. How the hemolysin lyses its target cells, via an enzymatic interaction or by pore formation, is not known. If the recombinant *S. hyodysenteriae* hemolysin or the RNA core hemolysin lyse erythrocytes by forming pores, these pores must be smaller than 0.9 nm or larger than 2.3 nm in diameter. Osmotic shock supernatant from *E. coli* DH5α(pSML4) was assayed for sphingomyelinase activity (a hemolytic mechanism used by, e.g. *Leptospira interrogans*); however, no sphingomyelinase activity could be detected.

Hemolysin will probably not be the only important *S. hyodysenteriae* virulence factor, and factors such as lipopolysaccharides and outer envelope carbohydrates could also be involved in the pathogenesis of SD (38). To establish the role of the *tly* gene in virulence and to investigate whether *S. hyodysenteriae* cytolytic and hemolytic activities are due solely to the *tly* gene product, it will be necessary to construct *tly* mutants of *S. hyodysenteriae*. Such mutants are currently under construction.

ACKNOWLEDGMENTS

We thank E. M. Mapother for providing *S. hyodysenteriae* and *S. innocens* strains, M. Halter for Western blot data, and G. Songer for performing phospholipase assays on the cloned hemolysin gene. M. So and B. A. M. van der Zeijst are acknowledged for helpful discussions and review of the preliminary manuscript.

This work was funded in part by U.S. Department of Agriculture Competitive Grant 89-37266-4644 (L.A.J.), Public Health Service grant AI from the National Institutes of Health (F.H.), and a research grant from Duphar, B. V., Weesop, the Netherlands (M.B.H.K. and J.G.K.).

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