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THE HEMOLYSINS PRODUCED BY SERPULINA HYODYSENTERIAE AS PUTATIVE VIRULENCE DETERMINANTS

by

Doreene Rose Hyatt

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

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Janet Decker	Date 12-22-95
Harris Bernstein	Date 12-22-95
	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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Dissertation Director
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DEDICATION

This dissertation and the work that it involves must be dedicated to the people who have actually brought me to this point, my Mother and Father, Irene and William H. Hyatt. Without your help and support I know that I would not have achieved yet another goal, my doctorate. I wish that you will always be there for me throughout all of the goals yet to be dreamed and attained. Thank you both, I love you.

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ABSTRACT

Serpulina hyodysenteriae is the etiological agent of swine dysentery (SD). The hemolysin produced by this spirochete is believed to be a virulence factor of the bacteria. Muir et al. (78) have recently cloned a gene (*tlyA*) from *S. hyodysenteriae* responsible for the expression of a hemolysin protein. The B204 RNA-core extract and recombinant hemolysins were active at temperatures ranging from 27-42 ° C and pH ranges of three to nine. There was no inhibitory effect by calcium, magnesium, BSA, sucrose or 50 mM EDTA while both zinc and copper were inhibitory. The activity of the two hemolysins could be partially blocked by components with a diameter of 2.0-2.3 nm. Neutralization of the hemolysins by newborn but not fetal bovine serum was observed. Cholesterol and cholesterol derivatives were able to partially block the hemolytic activity.

To test the mode of activity of the hemolysins, sheep erythrocytes were internally labeled with ^{86}rubidium. The release of both hemoglobin and ^{86}rubidium from the erythrocytes were compared after exposure to the B204 RNA-core hemolysin. It was found that ^{86}rubidium was released after 2 minute exposure to the hemolysin. Hemoglobin was released at 4 minutes of exposure, indicating that the hemolysin lyses the erythrocytes using a porin mechanism.

TlyA-minus mutants of two *S. hyodysenteriae* strains (B204 and C5) were tested for virulence in pigs. None of the animals inoculated with the mutant strains developed SD. Inoculation of pigs with the wild-type B204 or C5 strain produced SD in 100% and 60% respectively. Pigs infected with the mutant strains were further tested for susceptibility to challenge with the wild-type strain B204. After challenge, 50% of the pigs previously

inoculated with the B204 *tlyA*-minus mutant were protected, whereas all of the control pigs contracted SD. None of the pigs previously inoculated with the C5 *tlyA*-minus mutant, developed SD upon challenge with the B204 wild-type strain, whereas 40% of the control pigs developed SD in this experiment. Thus the *tlyA* encoded hemolysin of *S. hyodysenteriae* appears to be an important virulence factor in SD and previous colonization with *tlyA*-minus mutants provides partial protection to challenge with a virulent strain.

CHAPTER I. BACKGROUND

A. SWINE DYSENTERY

Swine dysentery (SD) is a mucohemorrhagic diarrheal disease caused by an anaerobic spirochete, *Serpulina hyodysenteriae* (32, 33, 74, 75, 76, 84, 99, 109). This disease was initially characterized and the agent isolated in 1972 and continues to have world-wide significance to the pork industry (113). Despite the fact that SD has existed for many years, very little is known about the exact mechanism of pathogenesis of the disease. Since prophylactics for any disease are usually determined by virulence factors associated with the etiological agent, pork producers are faced with a serious problem when trying to control or eradicated SD from their herds. In 1987 the Food and Drug Administration ruled against extra-label use of Ipronidizole and Dimetridizole, two very effective drugs for the control of SD. Pork producers now resort to using less effective and more expensive antibiotics and drugs with an inability to prevent increased time to market even while preventing death loss. Clearly, studies on the pathogenesis of SD are needed to develop more efficient means of controlling this highly infectious disease both for the development of vaccines and the understanding of the disease process.

B. LESIONS OF SWINE DYSENTERY

Acute swine dysentery is the most common form of the disease and is characterized initially by depression, a slight lack of appetite, and solid feces with mucus strands (29). Diarrhea follows with feces becoming yellow and soft to watery. In most cases frank blood and mucus are passed with the stool. As the disease progresses, the animals become anorexic,

gaunt, further dehydrated and weak, with death ensuing due to acidosis and hyperkalemia (29, 34). Morbidity rates are close to 90% with mortality averaging between 50 to 100% (70). Lesions are limited to the large intestine of both pigs and CF-1 mice (a laboratory model) and are characterized by extensive but superficial necrosis of the mucosal luminal surface and of the crypts of Lieberkuhn; there is no evidence of invasion or necrosis beyond the lamina propria (1, 2, 29).

Argenzio et al. (4) and Schmall et al. (94) demonstrated that the diarrhea which is seen in infected pigs is due primarily to colonic malabsorption rather than an active efflux of fluid. The authors observed that the transport of sodium and chlorine from the lumen to the blood was not active, thereby leading to the diarrhea. They also hypothesized that the observation that the levels of cAMP (cyclic adenosine monophosphate) and cGMP (cyclic guanosine monophosphate) in the colon of the infected piglet were normal demonstrates that enterotoxins are not responsible or involved in the production of the diarrhea.

C. IMMUNITY TO SWINE DYSENTERY

Immunity to the organism was first reported by Olson in 1974 (83) and has been studied extensively (23, 35, 41). His studies seemed to indicate acquired immunity to the disease and Joens et al. (41) demonstrated that pigs previously exposed to the bacteria were resistant to SD up to 17 weeks after initial inoculation and even when not shedding the bacteria. It has also been demonstrated that shedding of the bacteria from asymptomatic pigs can occur for at least 89 days after recovery and can cause disease in non-immune pigs (97). This has led others (41) to theorize that there is a low level infection in the colon which is

constantly stimulating the immune system and thereby conferring the acquired immunity displayed. The immunity that is observed is also partially serotype specific (43, 79, 112).

The immunity that is seen against swine dysentery has not been fully elucidated. The antibody response against *Serpulina hyodysenteriae* has been studied and although there is data on the antibody response and serum components which have been found in the colon of pigs which display acute swine dysentery (29), none of the data indicates that there was a correlation between the antibody response and protection from infection (39, 87). During this study, Rees et al. (87) also found that the serum IgG titers were more associated with the duration of the clinical signs observed. The same year, Wright et al. (112) demonstrated that naturally infected pigs which were showing clinical signs had lower serum antibody titers than infected pigs without clinical signs.

There is indication that the antibody response does have an effect on the bacteria *in vitro*. Joens and Neussen (46) demonstrated that when normal swine sera was added to pathogenic and avirulent *S. hyodysenteriae* strains, the avirulent strains were killed by the serum and the pathogenic strains were not affected. However, incubation of the bacteria with normal swine serum including hyper-immune sera against the *Serpulina hyodysenteriae*, resulted in bactericidal activity. Joens et al. (43, 45) also demonstrated the protection, by serum from convalescent pigs, of ligated swine colonic loops against dysentery-like lesions. These data seem to indicate that there is some role for antibody production in the protection against the *Serpulina hyodysenteriae* bacterial cell.

Joens et al. (44) suggested that the colonic antibodies could be responsible for

protection against rechallenge. Previously, Rees et al. (87) had suggested that gut IgA levels may be related to recent exposures rather than immunity, in that the secretory IgA antibody titers were higher after the first infection than after following challenges. Rees et al. (88) later demonstrated that there was an increase in the amount of specific IgA compared to specific IgG titers after infection. Vaccination with killed bacteria produced a rise in both antibody titers, but no specific increase in the relative amounts of the antibodies.

All of the studies done thus far have seemed to indicate the importance of serum antibodies in immunity to swine dysentery while still not giving the exact mechanism by which the protection is obtained. Cell mediated immune responses have not been studied and data has not been obtained on its role in the immunity to swine dysentery.

D. SERPULINA HYODYSENTERIAE

S. hyodysenteriae is a Gram-negative anaerobic spirochete which is 6-8.5 μm in length and 320-380 nm in diameter. It is oxygen-tolerant, loosely coiled and displays corkscrew-type motility. The organism contains 7-9 periplasmic fibrils which are inserted at each end of the cell and overlap at the middle of each protoplasmic cylinder (29, 32). There are currently 9 serotypes identified of *Serpulina hyodysenteriae*.

Serpulina hyodysenteriae is a fastidious organism in its nutritional requirements. As mentioned, it grows anaerobically, preferring media with a redox potential not greater than 125 mV and an initial pH of 6.9 (64). It also requires nutritionally rich media. This is usually accomplished by the addition of 10% erythrocytes to solid media or 5-7% fetal bovine serum to broth cultures (62). Stanton et al. (98) have shown that erythrocytes are a source of

cholesterol and phospholipid for the spirochete and that the bacteria could grow to high numbers when cholesterol was the only supplement. Stanton (98) also demonstrated that the bacteria could use sterol in other forms besides cholesterol. This requirement for cholesterol seems to be related to the membrane formation of the bacteria and is incorporated into the membrane in the form of cholestanol (98). Bacteria grow best between 36 and 42 °C and population doubling time is 5.2 hours in broth media at 37 °C (64).

E. VIRULENCE FACTORS OF SERPULINA HYODYSENTERIAE

1. Flagella

As mentioned previously, the spirochetes demonstrate motility and contain periplasmic flagella. Their motility is due to 7-9 periplasmic flagella (PF) which are inserted into the end of the cell and overlap at the middle of the protoplasmic cylinder. Reports on the number and size of *S. hyodysenteriae* PF have been determined largely by SDS-PAGE. The data from these reports vary, with the PF composed of five to seven major proteins with approximate molecular masses of 29 through 44 kDa. Koopman et al. (61) found five major proteins which are 32, 34, 35, 37 and 44 kDa respectively. They found that the 35 and 44 kDa proteins are a part of the sheath of the flagellum and the others are part of the core. N-terminus sequence data was also determined and the 44 kDa protein was found to have some homology with the sheath proteins of other spirochetes. The 35 kDa had no homology to the sheath proteins of other spirochetes, but was present in other strains of *Serpulina hyodysenteriae* as demonstrated by immunoblotting. The core proteins also had N-terminal sequence homology to other core proteins of spirochetes as well as being highly similar to

each other. The authors concluded that this data indicates that the polypeptides produced are similar, but not identical, to each other. Koopman et al. (59) have cloned the gene (*fla*B2) which encodes for the 31.1 kDa core protein and expressed it in *E. coli*. Southern blot analysis of the gene demonstrated that there was a possibility of two genes related to the *fla*B2 gene present in *S. hyodysenteriae,* which confirms their earlier immunoblotting work. Also, the sequence showed significant homology to core flagellin proteins from other spirochetes, again confirming earlier N-terminal sequencing data.

It has been suggested that motility along with chemo-attraction is the mechanism that the *S. hyodysenteriae* bacteria uses to colonize the colon of pigs (49). It is known that *S. hyodysenteriae* is highly motile in intestinal mucus and strain B204, grown *in vivo,* is chemotactic to 1% hog gastric mucin (49). Milner and Sellwood (77) found that virulent strains were chemotactic towards hog gastric mucin and porcine colonic mucin but not bovine submaxillary mucin. They indicated that this chemotactic response could be responsible for the ability of the bacteria to colonize the mucosal surface and cause disease.

Since the flagella seem to be an important mechanism in virulence and since studies of sera from protected pigs demonstrate that the predominant humoral response is to flagellar proteins (15, 16, 52), other studies had been undertaken to see if the flagellar proteins are protective against experimental challenge. Many projects are involved in the cloning of genes which encode for flagellar proteins (10, 26, 60). Boyden et al. (10) and Koopman et al. (60) have used cloned and purified flagellar proteins and tested for protection in the mouse model. Boyden et al. (10) found that serum from mice which had been injected with a crude extract

of a flagellar clone was bactericidal *in vitro* for virulent *S. hyodysenteriae*. They also found that the partially purified antigen from the clone was able to protect mice against homologous and heterologous challenge. However, the crude extracts were limitedly protective in that only one in five mice injected with one of the extracts was protected against challenge with virulent *Serpulina hyodysenteriae*. The other clone was a little more protective in that three of the five mice were protected but this was when they used double the amount of protein. Koopman (59) used recombinant sheath flagellin (FlaA) and fused it in frame to the glutathion-S-transferase (*Gst*) gene. The gene product was purified and used to immunize mice intra-peritoneally. After challenge with the virulent *S. hyodysenteriae* strain, the authors observed partial protection from lesion severity and production of specific IgG and IgA antibodies. The severity of lesions was measured by mean cecal scores. The cecal lesions for each mouse were given a number from 0 to 3, with 0 being no lesions and 3 being severe lesions. The mean cecal score of the group receiving two intraperitoneal injections of the GST fused flagella protein and challenged with *S. hyodysenteriae* was 1.6 and the control group had a mean cecal score of 2.4. Neither study, while having high levels of protection, were able to completely abrogate disease. This seems to indicate that, although the flagellar proteins are important in the disease process, they are not the only virulence factor that needs to be considered.

2. Attachment

Kennedy et al. (49) have previously demonstrated no appreciable attachment of *Serpulina hyodysenteriae* to epithelial cells *in vivo*. The frequency of adherence has been

shown to be dependent upon on motility and viability. As expected, heat, cold and formalin treatments significantly reduced adherence (11, 56, 111). In 1989, Bowden et al. (11) demonstrated the attachment of the bacteria to Henle intestinal epithelial (HEI) cells *in vitro*. The authors observed that adherence of the spirochete to HEI cells was end-on-end by light microscopy and random on the cell surface by electron microscopy. They found that maximum adherence occurred at 90 min. with an average of 20 bacteria for every HEI cell. Using the same criteria, they found that the non-pathogenic *S. innocens* attached at a lower frequency of 10 or less bacteria per HEI cell. The authors found that treatment with either rabbit hyper-immune or swine convalescent antisera was able to inhibit attachment of the bacteria to HIE cells. They also demonstrated that the treatment of the spirochete with neuraminidase, N-acetylneuraminic acid, D-glucuronic acid or fetuin was also able to inhibit attachment. The authors concluded that sialic acid is probably involved with the receptor-binding ligand of the bacteria to epithelial cells due to both the inhibition of adherence after neuraminidase treatment which cleaves sialic residues and the observation that unwashed bacterial cells and spent culture supernatant fractions contained higher concentrations of sialic acid than washed cells and uninoculated media. Whether by chemo-attraction or by specific receptor binding, it is generally believed that adherence of the bacteria to cells does occur *in vivo*.

3. Invasion

Although adherence of the bacteria has been demonstrated, invasion does not seem necessary for lesion production (29) and has not been clearly demonstrated.

Researchers have suggested the invasion of the bacteria into goblet cells (86) and Kang and Olander (48) found *S. hyodysenteriae* in the intercellular gaps between intestinal cells with possible penetration of the basal cytoplasmic membrane of the epithelial cells, but the significance of these findings has still not been elucidated.

4. Lipopolysaccharide

The lipopolysaccharide (LPS) of the bacteria has also been suggested to be an important virulence factor. The LPS has been found to cause lesions typical of SD in C3HeB/FeJ mice but not in the related lipopolysaccharide resistant C3H/HeJ mouse strain (81). The lesions of the mice when inoculated with whole bacteria correlated strongly with these results. This study also demonstrated that the LPS was chemotactic for murine macrophages from the C3H/HeJ mice but not for those from the C3HeB/FeJ mice. Also, the LPS has been demonstrated to be similar to the LPS of other Gram-negative bacteria, in that it is able to act as a mitogen for splenocytes with 5 to 10 μg/ml, generate a chemotaxin for leukocytes in fresh swine serum and is cytotoxic for murine peritoneal macrophages at concentrations as low as 15 μg/ml (80). Also, during this same study, Neussen et al. observed that analysis by spectrophotometry, acid hydrolysis, extraction with $CHCL_3$ and gas liquid chromatography demonstrated that the LPS is composed of approximately 2% lipid which are primarily hydroxylated C_{16} fatty acids and nonhydroxylated C_{15} fatty acids.

Halter and Joens (31) extracted LPS from *Serpulina hyodysenteriae* serotypes 1-7 and analyzed them by silver staining and immunoblot. They found the presence of two bands that were between 18 and 24 kDa in size and were serotype specific for *Serpulina hyodysenteriae*.

They also used these procedures on attenuated pathogenic strains and *Serpulina innocens*. The attenuated strains had lost the higher molecular weight band and the *S. innocens* strains had six bands between 17 and 26.9 kDa.

Despite these findings, Greer and Wannemuehler (30) have demonstrated that the differences in the pathogenicity between *Serpulina hyodysenteriae* and *Serpulina innocens* could not be related to the difference in biological activity of the LPS or endotoxin extracted from the bacterial cells. *S. hyodysenteriae* and *S. innocens* were extracted using phenol/water to isolate LPS or butanol/water to isolate an endotoxin preparation. Comparisons were made between the extraction methods and bacterial cultures. The authors found that there were differences in the concentration of hexoses in the two extractions and the LPS contained the greater amount. The endotoxin extract of *S. hyodysenteriae* had larger amounts of thiobarbituric acid-reactive compound, and protein content than the LPS extract. They also used the endotoxin samples in the Limulus amebocyte lysate assay and found that the endotoxin activity between the two bacterial isolates were approximately equal when *E. coli* LPS was used as the standard. The endotoxin from both isolates were found to induce IL-1 and TNF from murine peritoneal exudate cells, increase NK cell killing. The preparations were also able to increase natural killer cell activity. Both of them were poor adjuvants, not pyrogenic and could not cause a dermal Shwartzman reaction. More of the LPS than the endotoxin was needed to reach a 50% lethal dose in Balb/cByJ mice. In contrast to the earlier work by Nuessen et al. (80), Greer and Wannemuehler (30) did not observe the mitogenicity of their LPS preparation for splenocytes, whereas the endotoxin preparations were mitogenic.

These studies indicate that although the LPS is important in both the serotyping and the virulence of the bacteria, it cannot be considered the only mechanism by which the bacteria cause disease. They also concluded that the endotoxin extracts contained more biological activity than the LPS extracts from the two bacteria.

5. Hemolysins

As mentioned above, the virulence factors of *S. hyodysenteriae* have been an area of scientific interest and thus far the primary factors are lipopolysaccharide (LPS), flagellar proteins and also hemolysins. Although LPS seems to play an important role, pathogenicity of the organism appears to be related to the presence of hemolysins since the non-pathogenic *S. innocens* lacks one or more of the *S. hyodysenteriae* hemolysins (54, 74, 75, 76, 84, 91, 105).

The native RNA-core extracted hemolysin produced by *Serpulina hyodysenteriae* has been shown to be very similar in both mode of action and characterization to streptolysin S (SLS) produced by *Streptococcus pneumoniae* (69). One of the most important similarities which led into the study comparing the two is the finding that SLS also requires a carrier molecule for the expression of hemolytic activity (7, 20, 28). Also, SLS is cytotoxic for leukocytes, lymphocytes, platelets, cell cultures and erythrocytes (38). SLS is also an oxygen stable, heat-sensitive, protease-sensitive, EDTA -insensitive protein (3, 7, 20). Both SLS and the *Serpulina hyodysenteriae* hemolysins are also inhibited by trypan blue (92).

Saheb et al. (89) first used a procedure of yeast RNA-core extractions to free the hemolysin from the cell wall of the pathogenic *Serpulina hyodysenteriae*. This procedure was

based on observations by Picard et al. (85) that *S. hyodysenteriae* produces more hemolysin after the addition of sodium-RNA core. Lemcke et al. (65) demonstrated that a carrier molecule was necessary for hemolysin production as shown by the inability to extract hemolysin from blood-cultures with PBS or from semi-solid cultures by freezing and thawing. Lemcke also demonstrated that RNA-core was more effective than sodium RNA-core, BSA fraction V or Tween 80, strengthening the findings of Picard and Saheb (85, 89).

The hemolysin of *S. hyodysenteriae* was originally characterized as an oxygen and pH resistant lipoprotein of 68-74 kDa which is proteinase K sensitive and heat-labile (51, 57, 65, 89, 90, 91, 92). Other reports, utilizing native gels to separate the protein have reported the hemolysin to be 19 kDA (51). The size discrepancies which have been reported are probably due to the variations in methods of extraction and separation.

The RNA-core extracted hemolysin produced by *Serpulina hyodysenteriae* has also been shown to lyse the erythrocyte cells by some other mechanism than either lipolytic or proteolytic activity. Saheb et al. (89, 91) used both the egg yolk method and the lecithin method to determine if there was any phospholipase activity as well as casein and rabbit erythrocyte membranes to assay for proteolytic activity. Saheb et al. (90) demonstrated that the lysis of erythrocytes induced by the hemolysin was generally associated with a swelling of the erythrocytes. They found that the rate of swelling is dose-dependent and swelling occurs rapidly at high concentrations of hemolysin. One conclusion made after this study was that the mechanism of action of the hemolysin may be similar to that of the action of saponin which would be interaction with membrane lipids. There were differences between the two,

in that the mechanism of action of the RNA-core hemolysin was temperature dependent and could be blocked by sucrose.

Kent (50) demonstrated the hemolysin to be cytotoxic *in vitro* for a number of cell types. In an earlier report, rat ileal loops exposed to *S. hyodysenteriae* hemolysin developed lesions of hemorrhage, epithelial cell erosion, and the infiltration of inflammatory cells in the lamina propria (89). Recently, Lysons et al. (71) demonstrated the presence of microscopic lesions in small and large intestinal loops of germ-free pigs inoculated with the hemolysin from *S. hyodysenteriae.* These two reports demonstrate the RNA-core extracted hemolysin to be a potent cytotoxin for enterocytes and a probable virulence determinant for *S. hyodysenteriae.*

F. HEMOLYSIN GENE CLONING

Muir et al. (78) have cloned the gene (*tlyA*) encoding a *S. hyodysenteriae* hemolysin and have determined that this gene is present in a single copy in all tested pathogenic strains of *S. hyodysenteriae* and is absent in the non-pathogenic strains. The 1.5 kb DNA fragment was cloned into pUC19 and transformed into the non-hemolytic *E. coli* K12 strain DH5α. Two open reading frames were found in the 1.5 kb fragment and the second open reading frame encodes a 26.9 kDa protein. No homology has been found between the cloned hemolysin (*tlyA*) sequence and any other known bacterial hemolysins in any nucleic acid and protein data bases. Based on the cloned hemolysin's activity, they have also suggested that this hemolysin is the one extracted using an RNA-core extraction on native bacteria.

A partial sequence of the hemolysin gene (*tly*A) was then used to produce a fusion

protein. In that the native hemolysin is non-immunogenic, ter Huurne et al. (100) used twenty base pairs from the *tly*A gene and cloned and expressed it in plasmid pGEX-3X in frame with the glutathione-S-transferase gene. This plasmid was transformed into *E. coli* strain DH5α and the protein expressed. The protein was purified by affinity chromatography and used to vaccinate OF-1 mice to see if the protein was protective in the laboratory model. The results from the mice which were inoculated twice with the protein intra-peritoneally seemed to indicate that there was protection which was associated with specific antibody production. The protection was not complete which indicates that the hemolysin is not entirely responsible for the protection seen in the field cases.

Recently ter Huurne et al. (102) have described the cloning of two other hemolysin genes encoded by pathogenic strains of *S. hyodysenteriae* which are non-homologous with the previously cloned hemolysin gene. The new genes (*tly*B, *tly*C) are distinct from each other and are present in a single copy in all *Serpulina hyodysenteriae* strains tested and seem to be related, but not identical to a hemolysin gene(s) in *Serpulina innocens*. Gene *tly*B was cloned into pBluescript (pBS) pKS+ and pSK+, using *E. coli* K12 strain DH5α as a host. The working clone of gene *tly*B was named pSMURV and was approximately 1.6 kb and contained one large open reading frame which had a number of start codons. It is unknown at this time the size of the protein encoded by the gene because the exact start codon is not presently known. *tly*C was cloned into the cosmid vector pLAFR2 and the same DH5α host was used. The working clone containing the gene *tly*C, pMVH2, was 2.2 kb in length, had 4 open reading frames (ORF 3 represents the *tly*C gene), and encoded a protein of 268 amino

acids and 30.8 kDa. Data base searches found no exact matches for either gene, but *tly*B did have significant homology at its 5' end of the ORF with a subunit of the Clp ATP-dependent protease. The proteins encoded by these clones have not been described to the same extent as the proteins encoded by the first clone. The two proteins encoded by the cloned genes are probably not those which are extracted from the native bacteria and are probably not involved in the pathogenesis of *Serpulina hyodysenteriae* in that the genes are related to genes found in the non-pathogenic *Serpulina innocens*. More than likely, they function as proteases for the organisms.

G. HEMOLYSIN GENE (*TLY*A) INACTIVATION

ter Huurne et al. (101) have recently inactivated the hemolysin gene (*tlyA*) by homologous recombination. Using the pJBA^{KS} plasmid, the 1.3 kb Kanamycin Resistance GenBlock (Pharmacia) from transposon Tn903 was digested with *Bam*HI and inserted into a unique *Bgl*II site present in the hemolysin gene. The derivative, named pTly-, was electroporated into the *S. hyodysenteriae* strain C5 and plated on trypticase soy broth plates supplemented with 5% sheep red blood cells, 400 μg of spectinomycin per ml and 150 μg/ml kanamycin and checked for diminished hemolysis. Colonies with diminished hemolysis were then checked for the presence of the kanamycin cassette by PCR. Five clones with diminished hemolysis were found and upon PCR only four contained the kanamycin cassette and were counted as hemolysin-minus mutants.

Using the hemolysin mutant that they had just produced, mouse studies were carried out. Mice were inoculated with 10^6 or 10^8 of either wild type bacteria (C5) or hemolysin

negative mutant strain (MutI) and checked for shedding of bacteria (culture positive), lesion production and severity of lesions. The groups of mice were given a mean cecal score based on macroscopic cecal lesions as follows: severe lesions, 3; moderate lesions, 2; mild lesions, 1; no lesions, 0. Of the seven mice inoculated with 10^8 of the wild-type strain, all seven were lesion and culture positive and had a mean cecal score of 2.4. Of the seven mice inoculated with 10^8 MutI (hemolysin negative *tlyA*- mutant strain), five of the seven were lesion positive and all seven were culture positive. Also, the mice had a decreased mean cecal score (1.3) compared to the native strain. The authors concluded that although the differences in the number of mice which were lesion negative was not significant, the decrease in the mean cecal score demonstrated the hemolysin-minus mutant strain to be less virulent than the wild-type strain.

Recombinant DNA technology allows for a most effective means to study, isolate and characterize the gene or gene families responsible for *S. hyodysenteriae* hemolysin production. However, these studies have little significance without parallel studies on the native protein and a means of ascertaining the pathogenicity of the hemolysin in swine. Because the pig is the natural host, and because of the interesting results obtained from the mouse studies, it was decided that the next logical step of study to prove that the hemolysin is an important virulence factor in the pathogenicity of *S. hyodysenteriae* was to test the mutant strain in pigs.

H. SPECIFIC AIMS

The objectives of this work were to determine the characteristics of both the RNA-core and recombinant hemolysin proteins produced by *Serpulina hyodysenteriae*. Also, using a previously produced strain, negative in hemolysin production, the last objective was to determine the importance of the hemolysin (TlyA) in the pathogenesis of *Serpulina hyodysenteriae* and in the production of immunity. The hypothesis of the project was that the hemolysin is a unique protein which could be characterized and shown to be an important factor in the virulence of *Serpulina hyodysenteriae*.

CHAPTER II. ANALYSIS OF THE LYTIC ACTIVITY OF THE SERPULINA HYODYSENTERIAE HEMOLYSIN

A. INTRODUCTION

Swine dysentery (SD) is a disease caused by an anaerobic spirochete, *Serpulina hyodysenteriae* (32, 33, 99, 105, 109) and characterized by a mucohemorrhagic diarrhea. Acute SD is characterized initially by depression, a slight lack of appetite, and solid feces with mucus strands (29). Diarrhea follows with feces becoming yellow and soft to watery. Usually, frank blood and mucus are passed with the stool. Animals become anorexic, gaunt, further dehydrated and weak, with death ensuing due to acidosis and hyperkalemia (29, 34). Lesions are limited to the large intestine of both pigs and mice, used as a laboratory model, and are characterized by extensive but superficial necrosis of the mucosal luminal surface and of the crypts of Lieberkuhn; there is no evidence of invasion or necrosis beyond the lamina propria (1, 29).

Spirochetes isolated from the colon of pigs are identified on the basis of their hemolytic activity on blood agar with pathogenic *Serpulina hyodysenteriae* producing a complete hemolytic zone and nonpathogenic *S. innocens* producing an incomplete hemolysis (1, 50). The hemolysin of *S. hyodysenteriae* was originally characterized as an oxygen resistant, proteinase K-sensitive, heat-labile, pH resistant lipoprotein of 68-74kDa (50, 57, 90, 91, 92) which is toxic for several cell types (50). Recent work by Muir et al. identified a hemolysin gene (*tly*) of *S. hyodysenteriae* which encodes for a 26-27 kDa protein (78).

Since the RNA-core hemolysin is a putative virulence factor for *S. hyodysenteriae*,

we undertook this study to further characterize the hemolysin and help clarify the mode of action of the hemolysin. Also, the recombinant protein (TlyA) was used in the same studies to determine the characteristics of the cloned hemolysin. This information was then used to determine if the gene (*tlyA*) product is the primary protein that is released during extraction of the RNA-core hemolysin.

The hemolysin was subjected to various temperatures, pH ranges and protease inhibitors in this study. Previous unpublished black lipid experiments (Joens et al.) demonstrated that the hemolysin produces a pore in oxidized cholesterols. To confirm this previous work, two studies were undertaken. One study analyzed the ability of carbohydrates and compounds of specific diameter to inhibit the activity of the hemolysin. Another study was fashioned after one used by Duncan et al. (19) to describe the lysis of erythrocytes by streptolysin O. The release of ^{86}Rb, through the potassium channels, from labeled erythrocytes can occur independently of hemoglobin release, therefore, it is possible to predict how the cell is being lysed by determining which compound is released first.

B. MATERIALS AND METHODS

1. Cultures

Serpulina hyodysenteriae strain B204 (serotype 2) was originally isolated from a pig showing clinical signs of swine dysentery and is pathogenic when inoculated orally into mice and pigs (73). The isolate was originally obtained from J. M. Kinyon, Iowa State University, Ames, IA. The isolate has been maintained in our laboratory at less than 25 passages and is stored frozen at -70°C.

E. coli strain DH5α (pJBA) was originally obtained from S. Muir (78), University of Utrecht, the Netherlands. The isolate has been maintained in our laboratory and is stored in agar stabs at room temperature.

2. Media and culture conditions

The medium used to culture *S. hyodysenteriae* consisted of reduced Trypticase Soy Broth (TSB) supplemented with FBS (10%). Cultures were incubated at 37 °C under a gas mixture of 50:50 CO_2:H_2 for 16 h in a stoppered round bottom flask and then viewed on a phase contrast microscope to assess purity.

E. coli DH5α was cultured in 2x TY media supplemented with 100 μg/ml ampicillin and 0.5% citrated (10%) sheep red blood cells. Cells were plated for isolation and incubated aerobically at 37 °C for 24 h, at which time a single hemolytic colony was inoculated into 2x TY and ampicillin broth and incubated overnight. Cultures were viewed by phase contrast microscopy to assess purity.

3. Hemolysin extraction

Cultures of *Serpulina hyodysenteriae* were harvested by centrifugation (J14 Beckman rotor) at 6500 x g for 25 min. at 4 °C. Native hemolysin extraction consisted of repeated incubation at 37 °C and centrifugation with phosphate buffered saline containing 0.05% yeast RNA-core as a carrier molecule for the removal of the hemolysin from the bacterial cell (90). The supernatant was then filtered through a 0.2 μm filter to remove any extraneous bacteria, lyophilized and then stored at -70 °C.

The recombinant protein was extracted using a modified version of an osmotic shock procedure introduced by Heppel et al. (36). After growth, the cells were spun down at 6,500 x g for 10 min. at 4 °C in a Beckman centrifuge (J14 rotor). The supernatant fluid was removed and the cell pellet resuspended in a 30 mM Tris (pH 7.2) 20% sucrose solution and incubated on ice for 10 min. The cells were centrifuged and resuspended in 5 ml HPLC water and incubated on ice for 10 min. The cells were centrifuged, and the water phase collected and assayed for hemolytic activity. This was then lyophilized and stored at -20 °C for later use.

4. Hemolysin titration

Sheep red blood cells in 10% citrate were washed with PBS and resuspended in barbital buffered saline (BBS) (14) to a concentration of 0.5%. Two fold dilutions of the RNA-core supernatant fluids were made in BBS and an equal amount of the erythrocyte suspension was added to give a final erythrocyte concentration of 0.25%.The mixture was incubated at 37 °C for either 2 h for the native RNA-core hemolysin or overnight for the recombinant and centrifuged at 450 x g for 10 min. Hemoglobin release was measured at 540

nm wavelength against a buffer control containing SRBC and no hemolysin. The endpoint of the dilution was the highest dilution with 50% lysis. A hemolytic unit (HU) was defined as the amount of hemolysin needed to lyse 50% of the 0.25% erythrocyte suspension. Unless otherwise indicated, RNA-core hemolysin and recombinant hemolysins were used at 30HU/ml for testing in subsequent experiments.

5. Temperature dependence

Native hemolysin (30 HU/ml) or 30 HU/ml of recombinant was added to an equal volume of 0.5% SRBC and incubated with agitation at 4, 10, 27, 37 or 40 °C for either 2 h for the native RNA-core hemolysin or overnight for the recombinant hemolysin. The assays were spun down in a tabletop centrifuge at 450 x g for 10 min. and hemoglobin release was measured at 540 nm against an incubated and centrifuged BBS blank containing SRBC and no hemolysin. The effect of washing and temperature on the hemolysins and SRBC interaction was also assayed. Both native and recombinant hemolysin (30 HU/ml) were incubated with SRBC for 1 h at 4 °C with agitation and then divided into 2 aliquots. One was washed with phosphate buffered saline (PBS) and then resuspended in BBS to original volume. Both aliquots were then incubated at 37 °C, centrifuged at 450 x g for 10 min. and assayed for hemoglobin release against an incubated and centrifuged buffer control containing SRBC and no hemolysin.

6. pH dependence

Native RNA-core hemolysin (30 HU/ml) or 30 HU/ml of recombinant was placed in saline of pH 3, 5, 7 or 9. Equal amounts of 0.5% SRBC in 4x BBS was added and incubated

with agitation at 37 °C. Mixtures were centrifuged at 450 x g for 10 min. and hemoglobin release was measured at 540 nm against a buffer control containing SRBC and no hemolysin.

7. Effect of protease inhibitors

Leupeptin (Sigma Chemical Co., St. Louis, MO.) (final concentration of 10 μM and 100 μM), phenylmethylsulfonyl fluoride (PMSF; final concentration of 100 nM), EDTA (100 mM), bestatin (40 μg/ml) and pepstatin (0.7 μg/ml) were tested against 30 HU/ml of B204 RNA-core extracted hemolysin or 30 HU/ml of recombinant hemolysin. Microtiter assays were done to test for the diminution of hemolytic units, as were tube assays to test for diminished hemoglobin release. The protease inhibitors and EDTA were allowed to interact with the hemolysin for 10 min. before an equal amount of 0.5% erythrocytes were added and assayed for hemolysin activity.

8. Blocking of activity by carbohydrates

PEG 1000 and PEG 1450 were used at a concentration of 0.6 M and added to 30 HU/ml of RNA-core hemolysin or 30 HU/ml of recombinant hemolysin and then incubated for 2 hrs at 37 °C. The mixtures were then centrifuged at 450 x g for 10 min. and the supernatant fluids were measured for hemoglobin release against a buffer control containing SRBC and no hemolysin.

Dextran 1500 was isolated using size exclusion chromatography. Colored Vit B_{12} (approximately 1357 mw) from the Pharmacia LKB size exclusion dye kit was used as a molecular weight marker. Dextran 8800 (Sigma Chemical Co., St. Louis, MO.) was digested for one hour with dextranase (Sigma Chemical Co., St. Louis, MO.) (approx 100 U) and put

on a 9 mm x 30 cm sephadex G25 column and eluted with BBS. The Vit B_{12} was removed by elution over activated charcoal. The solution was then lyophilized and resuspended in one-twentieth the original volume. This was also then assayed for hexose concentration via the sulfuric acid/phenol method of Bligh and Dyer (9).

Dextran 1500 (2.3 nm) at 20 μM, arabinose (0.62 nm), glucose (0.72 nm), sucrose (0.92 nm), raffinose (1.14 nm), and dextran 8800 (4 nm) at 30 μM were tested individually or as a pool as an inhibitor of hemolytic activity (93).The mixture with added dextran 1500 and without dextran 1500 was added to 70 HU of RNA-core hemolysin or 30 HU/ml of recombinant and incubated at 37 °C with an equal part of the 0.5% erythrocyte suspension. The mixtures were then centrifuged at 450 x g for ten minutes and assayed at 540 nm for hemoglobin release against an incubated and centrifuged buffer control containing SRBC and no hemolysin. All assays were compared to controls containing hemolysin and SRBC with no inhibitors added.

9. Release of ^{86}Rubidium from erythrocytes

Three protease inhibitors were allowed to react with the hemolysins (either RNA-core or recombinant) for 10 min. before use in the hemolysin assay. Leupeptin at a final concentration of 100 μM, PMSF at 100 nM and EDTA at 100 mM were added to the hemolysins to inactivate extraneous proteolytic enzymes.

Sheep red blood cells in 10% citrate were washed multiple times in PBS in a tabletop centrifuge at 450 x g until no further hemoglobin release was observed. The cells were then

resuspended in PBS + 0.5% glucose to a final concentration of 0.5%.

The assay was performed using a modified technique of Duncan (19) whereby fresh sheep red blood cells are washed with PBS and resuspended in PBS containing 0.5% glucose. For each experiment, 40 ml was removed, centrifuged and resuspended in 1.9 ml of PBS + 0.5% glucose. ^{86}Rubidium (100 μCi (0.01 ml $^{86}Rb^{+}$ in 0.9 ml buffer); New England Nuclear Corp., Boston, MA) was added to the erythrocytes and the mixture incubated for two hours at 37 ^{0}C. The cells were washed once and resuspended in 40 ml of PBS + 0.5%. Two ml portions were placed into 13 x 100 glass serological tubes and placed into a 37°C water bath. After 0, 2, 4 and 5 min., 2 ml of 32 HU/ml hemolysin (16 HU/ml final concentration), control buffer or 0.2 ml of 1% saponin were added to individual tubes. After 7 min. the tubes were placed in an ice bath for 5 min., centrifuged and 0.1 ml of the supernatant was placed into scintillation vials. The tubes were then read on a spectrophotometer (Bousch and Lomb Spectrophotomer 20) at 540 nm for hemoglobin release. The scintillation vials were read in a Beckman LS 1800 scintillation counter (Beckman Instruments, Inc. Fullerton, Ca.) for both gamma and beta release. Values were compared to buffer controls. Total intracellular hemoglobin and ^{86}Rubidium were determined with lysis by saponin.

The recombinant hemolysin was also tested using this protocol except that the time was expanded from 12 min. to 2 hours. Recombinant hemolysin was added every half hour and at two hours, readings were taken.

10. Statistical procedure

One way randomized ANOVA test was run using the CoStat program to analyze the

values obtained for hemoglobin release at a 540 nm wavelength.

C. RESULTS

In order to further define the biochemical properties associated with the hemolysin of *S. hyodysenteriae* a variety of tests were done. Before assays were performed, hemolysin was extracted from the bacterial cell wall by incubation with a yeast RNA-core buffer and subsequent centrifugation.

Typical dilution endpoints after native RNA-core extractions were 1:256 per ml of extract (300 ml total). Specific activity of the RNA-core extracted hemolysin was 4000 (HU mg^{-1}).

Typical dilution endpoints after recombinant osmotic shock extractions and lyophilization were 1:12 per ml of extract (20 ml total). Specific activity of the osmotic shock extracted protein encoded by the pJBA plasmid was 125 (HU mg^{-1}).

1. Temperature dependence of hemolysin activity.

The hemoglobin release at 27, 37, and 42 °C was not significantly different than positive controls when either RNA-core or recombinant hemolysins were used. Even after two hours, 4 and 10 °C incubations showed no hemoglobin release. To determine the effect of the cold temperature on the hemolysins, an assay was run with 30 HU/ml of either native RNA-core or recombinant hemolysin at 4 °C for 10 min. The assay tube was then split into two aliquots and one was washed with PBS, resuspended to original volume and then assayed at 37 °C. The other was left unwashed and also assayed at 37 °C. After incubation, the washed portion showed no hemoglobin release while the unwashed portion showed hemolysis comparable to that of the positive control containing hemolysin.

2. pH dependence of hemolysin activity.

Total hemolysis was apparent at all pH units tested (pH 3, 5, 7, 9) and no pH tested was significantly different than the positive control of the hemolysin in saline for either hemolysin.

3. Inactivation by protease inhibitors

After addition of leupeptin, PMSF, bestatin, pepstatin and EDTA to the hemolysins, no significant decrease in hemolytic activity occurred (Table 2). The hemolysin, in each case, was able to totally lyse all erythrocytes after one hour of incubation. Control wells containing the protease inhibitors without hemolysin did not display any hemoglobin release.

4. Blockage of activity by carbohydrates

Attempts were made to block the activity of the hemolysin using specifically sized molecules. PEG 1000, which has a diameter of 2.0 nm, was tested at a concentration of 6 % and was able to block the hemoglobin release by the RNA-core hemolysin by almost 50%. Dextran 1500 with a diameter of 2.3 nm was also able to partially block the activity of the RNA-core hemolysin and almost totally blocked hemoglobin release by the recombinant hemolysin. Sugars less than 2.0 nm and larger than 2.3 nm (arabinose, raffinose, sucrose, glucose and dextran 8800) and PEG 1450 were unable to block hemoglobin release (see Table 1). None of the sugars tested individually were able to significantly block hemoglobin release.

5. ^{86}Rubidium release from erythrocytes

^{86}Rubidium release from erythrocytes was assayed using 16 HU/ml of B204 RNA-

core hemolysin and checked at times ranging from 2 minutes to 12 minutes. After reaction with the ^{86}Rubidium labeled bovine erythrocytes, it was found that at 3 min. there was significant release of ^{86}Rubidium and no significant release of hemoglobin. The ^{86}Rubidium release was comparable to that of total lysis of the cells after the addition of 1% saponin (See Table 3).

For the recombinant hemolysin, the same procedures were used except that the time of exposure was increased to a total of 2 h instead of 12 minutes to allow time for the hemolysin to lyse the cells. Release of ^{86}Rubidium from controls was equal to that of the release after addition of recombinant and no significant values were obtained.

D. DISCUSSION

Current studies were undertaken in order to further define the biochemical and physical characteristics of the *S. hyodysenteriae* native and recombinant hemolysins. The study was approached from two different objectives. The first objective was to further characterize the biochemical activity of the RNA-core and recombinant hemolysins. The second objective was to determine if the hemolysins were lysing cells via the action of pore formation.

Our results and those of previous studies concur (90, 91, 92), in that the native RNA-core hemolysin was not sensitive to any pH value tested and was only active from 27-42 °C ($p<0.01$). The recombinant hemolysin was also active at all pH values and was active from 27 to 42 °C. We also tested the effects of washing the hemolysin from the SRBC during temperature testing. With either hemolysin, after incubation at four °C and washing with PBS there was no hemoglobin release, indicating that the hemolysins were not bound, or were bound with low affinity to the membrane and could not be reactivated at a higher temperature after washing.

It was also vital to show that the hemolysins were not lysing cells via the action of a protease. The addition of leupeptin, PMSF, bestatin, pepstatin and EDTA to an assay would inhibit the action of any serine, thiol, cysteine or aspartic proteases, aminopeptidases and metalloproteases, (trypsin, chymotrypsin, plasmin, papain, cathepsin B, protease, acetyl-cholinesterase and phospholipase C). In this case, the inhibitors, either individually or collectively, were unable to prevent the lysis of erythrocytes by the native RNA-core

hemolysin or the recombinant hemolysin. There was no decrease in the titer of either of the hemolysins after addition of the protease inhibitors. These results demonstrate that the hemolysins are not lysing cells via a protease activity.

Our next interest was related to some previous data (not shown) that we had generated pertaining to the mechanism of action of the RNA-core hemolysin in artificial black lipid bilayers. These data indicated that the hemolysin had a high activity in oxidized cholesterol membranes and created a pore. In order to further demonstrate the possibility of pore formation and to further characterize the pore size, sugars and glycols of known molecular size were added to the hemolytic assay. As the previous data had indicated, molecules such as PEG1000 (2.0 nm) and dextran 1500 (2.3 nm) were able to block hemolysis of the RNA-core hemolysin to a significant degree ($p<0.01$). The results with the recombinant were not as clear, in that PEG 1450 was also able to block the activity and the PEG 1000 was not as efficient at blocking. It is possible that the hemolysin was bound to the PEG or that the PEG was inhibiting the hemoglobin release by disrupting the erythrocyte membrane and this was the reason for the results which we obtained. However, the dextran 1500 was able to almost totally block the activity of the recombinant hemolysin. In the case of the RNA-core hemolysin, the lack of total blockage by the dextran 1500 could be due to the presence of other hemolysins which do not use the same mechanism of action or produce a different sized pore.

Results of our experiments suggest the RNA-core hemolysin secreted by the spirochete is a membrane-active cytolysin which lyses erythrocytes osmotically through the

formation of pores and that the recombinant hemolysin may act in a similar manner. In order to further prove this theory, we adopted a study used by Duncan et al.(19, 20) to test the lysis of erythrocytes by streptolysin O (and streptolysin S). In this study, Duncan et al. used a protocol developed by Madoff et al. (72) for use with staphylococcal alpha-toxin. In these experiments, $^{86}Rb^{+}$ was used via the K+ transportation system in the erythrocytes (96, 110). Therefore, it is possible to tell which is released from the cell first, the small $^{86}Rb^{+}$ or the larger hemoglobin molecule. If the cell is lysed by pore formation, it would be expected that the $^{86}Rb^{+}$ would be released to a significant degree, while the hemoglobin would not be released until the cell lysed. In the case of the *S. hyodysenteriae* RNA-core hemolysin, $^{86}Rb^{+}$ was released from the cell before hemoglobin and these results further confirm that the hemolysin lyses the cells using a pore type of mechanism. The recombinant hemolysin, due to the length of time necessary for lysis, was not analyzed by this method. There was not enough time for the hemolysin to lyse the erythrocytes before the leakage of $^{86}Rb^{+}$ from the potassium channels occurred.

Our results indicate that the RNA-core and recombinant hemolysins produced by *S. hyodysenteriae* are active at a temperature range of 27-42 °C and are unaffected by leupeptin, PMSF, bestatin, pepstatin and EDTA. The hemolysin lyses cells via pore formation as demonstrated by the $^{86}Rb^{+}$ release assays in the case of the RNA-core hemolysin and the inhibition of the RNA-core and recombinant hemolysins by molecules with a diameter of 2.0-2.3 nm.

Table 1. The effects of agents with particular diameter on the *S. hyodysenteriae* RNA-core and recombinant hemolysins.

	RNA-core Native Hemolysin		Recombinant Hemolysin	
Blocking Agent	**A_{540} (mean +/- SD)[a]**	**Percent Blockage**	**A_{540} (mean +/- SD)[a]**	**Percent Blockage**
Positive Control	0.61 ± 0.1068		0.63 ± 0.06	
PEG 1000	0.36 ± 0.0648	40.98%	0.4 ± 0.04	36.5%
PEG 1450	0.553 ± 0.0957	9.34%	0.44 ± 0.04	30.2%

Blockage by Sugars	**RNA-core Native Hemolysin**		**Recombinant Hemolysin**	
Positive Control	0.31 ± 0.0455		0.396 ± 0.031	
Dextran 1500	0.09 ± 0.0356	65.22%	0.016 ± 0.012	95.9%
Carbohydrate + Dextran 1500	0.07 ± 0.0386	62.30%	0.046 ± 0.05	88.4%
Carbohydrate - Dextran 1500	0.277 ± 0.0566	0	0.42 ± 0.04	0

a Data were pooled from three separate experiments.

Table 2. The effect of protease inhibitors on the RNA-core and recombinant hemolysin.

	RNA-core Native Hemolysin		Recombinant Hemolysin	
Inhibitor	A_{540} (mean +/- SD)[a]	Percent Inhibition	A_{540} (mean +/- SD)[a]	Percent Inhibition
Pos. Control	0.455 ± 0.005	-	0.55 ± 0.03	-
PMSF	0.44 ± 0.008	3.3%	0.53 ± 0.008	3.6%
Leupeptin	0.44 ± 0.014	3.3%	0.53 ± 0.009	3.6%
EDTA	0.527 ± 0.009	-	0.53 ± 0.022	3.6%
Bestatin	0.467 ± 0.019	-	0.54 ± 0.016	1.8%
Pepstatin	0.467 ± 0.009	-	0.543 ± 0.009	1.3%

[a]Data were pooled from three separate experiments.

Table 3. 86rubidium release from erythrocytes after exposure to 16HU/ml B204 RNA-core hemolysin.

Time of exposure	A_{540} (mean +/- SD)[a]	Scintillation reading
2 minutes	0.01	1934 ± 223.2 CPM
3 minutes[b]	0.05 ± 0.014	3820.7 ± 314.2 CPM
5 minutes	0.27 ± 0.014	4270.3 ± 323.1 CPM
7 minutes	0.33 ± 0.012	4395 ± 147.4 CPM
Positive Saponin control	0.345 ± 0.015	4165.5 ± 26.5 CPM
Negative buffer control	0.0075 ± 0.013	591.5 ± 32 CPM

[a] Data were pooled from three separate experiments.
[b] CPM values not significantly different than positive control; 540 nm reading significant versus positive control.

CHAPTER III. BLOCKAGE OF SERPULINA HYODYSENTERIAE RNA-CORE AND RECOMBINANT HEMOLYSIN ACTIVITY WITH CATIONS AND SERUM COMPONENTS

A. INTRODUCTION

Serpulina hyodysenteriae is an anaerobic spirochete which is the etiological agent of swine dysentery (SD) (32, 33, 99, 105, 109). Acute SD, characterized by depression, anorexia and diarrhea with mucus strands, is the most common form of the disease observed and in most cases blood as well as mucus are passed with the stool (29). Dehydration, weakness and lethargy follow, usually ending in death due to acidosis and hyperkalemia (29, 34). The lesions usually consist of extensive but superficial necrosis of the mucosal luminal surface and of the crypts of Lieberkuhn and are limited to the large intestine of both pigs and the laboratory model, mice. Edema and hyperemia are also usually present. Invasion or necrosis beyond the lamina propria has not been demonstrated (1, 29).

Although other methods are available, spirochetes isolated from the colon of pigs are identified on the basis of their hemolytic activity on blood agar with pathogenic *Serpulina hyodysenteriae* producing a complete hemolytic zone and nonpathogenic *S. innocens* producing an incomplete hemolysis (1, 50). The hemolysin produced by *S. hyodysenteriae* is generally accepted to be a virulence factor of the spirochete. Because of this fact, many studies are underway to characterize the RNA-core hemolysin. Muir et al. (78) have recently isolated and cloned a gene responsible for hemolysin production in *S. hyodysenteriae* and have demonstrated an open reading frame in the hemolysin gene (*tlyA*) which encodes for a

26-27 kDa protein. This gene is present in a single copy in all pathogenic strains tested, and not in any of the non-pathogenic strains. Since its production, the protein has been fused to glutathione-S-transferase and the fusion protein has been studied to see if it is able to protect mice against inoculation with a virulent *S. hyodysenteriae* strain (100). In these studies, it was demonstrated that the protein is able to partially protect the mice against the challenge inoculum and therefore seems to have a role in the virulence of the native bacteria.

Both the native RNA-core hemolysin and the recombinant hemolysin were tested against various bivalent cations as well as serum components and cholesterol derivatives in order to assay the decrease in hemolytic activity of the hemolysin after their addition. The protein encoded by the *tly*A gene was compared to the RNA-core extracted hemolysin to determine if the recombinant protein is the primary protein extracted from the organism after addition of the RNA-core.

B. MATERIALS AND METHODS

1. Cultures

Serpulina hyodysenteriae strain B204 (serotype 2) was originally isolated from a pig showing clinical signs of swine dysentery and is pathogenic when inoculated orally into mice and pigs. The isolate was originally obtained from J. M. Kinyon, Iowa State University, Ames, IA. The isolate has been maintained in our laboratory at less than 25 passages and is stored frozen at -70 °C.

E. coli strain DH5α (pJBA) was originally obtained from S. Muir (78), University of Utrecht, the Netherlands. The isolate has been maintained in our laboratory and is stored in agar stabs at room temperature.

2. Media and culture conditions

The medium used to culture *S. hyodysenteriae* consisted of reduced Trypticase Soy Broth (TSB) supplemented with FBS (10%). Cultures were incubated at 37 °C under a gas mixture of 50:50 CO_2:H_2 for 16 h. in a stoppered round bottom flask and then viewed on a phase microscope to assess purity.

E. coli DH5α was cultured in 2x TY media supplemented with 100 μg/ml ampicillin and 0.5% citrated (10%) sheep red blood cells. Cells were plated for isolation and at 24 hours, hemolytic clones were picked and inoculated into 2x TY and ampicillin broth. Cultures were incubated aerobically at 37 °C for 24 h with agitation in a screw-cap Erlenmeyer flask and then viewed on a phase microscope to assess purity.

3. Hemolysin extraction

Cultures were harvested by centrifugation (J14 Beckman rotor) at 6500 x g for 25 min at 4 °C. Hemolysin was extracted using a yeast RNA-core extraction procedure as described by Saheb et al. (89). Repeated incubation at 37 °C with the yeast RNA-core buffer and centrifugation yielded a supernatant rich with hemolysin. The supernatant was then filtered through a 0.2 μm filter to remove any residual *S. hyodysenteriae* and stored at -70 °C.

The recombinant protein was extracted using a modified version of an osmotic shock procedure first introduced by Heppel et al. (36). After growth, the cells were harvested at 6,500 x g for 10 min. at 4 °C in a Beckman centrifuge (J14 rotor). The supernatant fluid was removed and the cell pellet resuspended in a 30 mM Tris (pH 7.2) 20% sucrose solution and incubated on ice for ten minutes. The cells were centrifuged again and resuspended in 5 ml HPLC water and incubated on ice for ten minutes. The cells were centrifuged again, and the water phase was collected and assayed for hemolytic activity. This was then lyophilized and stored at -20 °C for later use.

4. Hemolysin titration

Sheep red blood cells in 10% citrate were washed multiple times with PBS in a tabletop centrifuge at 450 x g. The cells were then resuspended in barbital buffered saline (BBS) (14) at a concentration of 0.5%. Two fold dilutions of the RNA-core supernatant fluids were made in BBS and an equal amount of the erythrocyte suspension was added to give a final erythrocyte concentration of 0.25%. The mixture was incubated for 2 h at 37 °C and then centrifuged at 450 x g for 10 min. Hemoglobin release was measured at 540 nm wavelength against a buffer control containing SRBC and no hemolysin. The endpoint of the

dilution was the highest dilution with 50% lysis. A hemolytic unit (HU) was defined as the amount of hemolysin needed to lyse 50% of the 0.25% erythrocyte suspension. Unless otherwise indicated, RNA-core hemolysin was used at 80 HU/ml and recombinant hemolysin was used at 30 HU/ml for testing in subsequent experiments.

5. Cation dependence

Ca^{++}, Cu^{++}, Zn^{++} and Mg^{++} were suspended in saline at a 5 mM final concentration. 80 HU/ml of RNA-core hemolysin or 30 HU/ml of recombinant hemolysin in the cation solutions were added to an equal volume of 0.5% SRBC and incubated for 2 hr at 37 °C, centrifuged at 450 x g for 10 min. and measured for hemoglobin release. The control consisted of SRBC in saline and no hemolysin. Bovine serum albumin (Sigma Co.) at a final concentration of 0.5%, sucrose at 0.3 M and 50 mM EDTA were also tested.

6. Neutralization of hemolysin by serum components

Fetal bovine serum, newborn bovine serum and commercially prepared HDL-cholesterol (approx. 200 μg/dL) were added to 30 HU/ml of RNA-core hemolysin or 30 HU/ml of recombinant hemolysin in a 1:10 dilution. The test was incubated for one hour at 37 °C and then an equal amount of 0.5% SRBC was added and incubated for two hours at 37 °C. The assay tube was centrifuged at 450 x g for 10 min. and hemoglobin release was read at 540 nm wavelength. The readings were then compared to a control containing hemolysin and SRBC without the presence of inhibitors.

7. Neutralization of hemolysin by cholesterol derivatives

The cholesterol derivatives used were purchased from Sigma Chemical Co. They

include the following; dihydrocholesterol, beta-sitosterol, ergosterol, stigmasterol, cholesteryl proprionate, cholesteryl oleate, cholesteryl palmitate, cholesteryl laurate, 4-cholesten-3-one, 5-cholesten-3β-ol-7-one and cholesterol. One mg/ml of the cholesterol derivatives were suspended in 100% ethanol. This suspension was then diluted 1:10 in PBS for the assay. This mixture was added to 40 HU/ml of RNA-core extracted native hemolysin or 30 HU/ml of recombinant hemolysin with an equal volume of a 0.5% solution of erythrocytes and incubated for two hours at 37 °C with agitation. Cells were centrifuged at 450 x g for 10 min. and the supernatant fluid was filtered through a 0.2 μm filter. Supernatant fluids were then assayed for hemoglobin release at 540 nm. The readings were compared to an incubated and centrifuged control containing hemolysin and SRBC in the presence of alcohol.

8. Statistical procedure

One way randomized ANOVA test was run using the CoStat program to analyze the values obtained for hemoglobin release at a 540 nm wavelength.

C. RESULTS

In order to further define the biochemical properties associated with the recombinant hemolysin of *Serpulina hyodysenteriae* a variety of tests were done. Before assays could be performed, hemolysin had to be extracted from the bacterial cytoplasm of the *E. coli* cell. The method used involved osmotic shock extraction of the cytoplasmic contents.

Typical dilution endpoints after RNA-core extractions were 1:256 per ml of extract (300 ml total). Specific activity of the RNA-core extracted hemolysin was 4000 (HU mg^{-1}).

Typical dilution endpoints after osmotic shock extractions and lyophilization were 1:12 per ml of extract (20 ml total). Specific activity of the osmotic shock extracted protein encoded by the pJBA plasmid was 125 (HU mg^{-1}).

1. The effect of certain cations was tested using calcium, magnesium, copper and zinc.

Of all of the cations tested only the copper and zinc ions inhibited the native and recombinant hemolysins activity totally, while calcium was able to partially inhibit the activity. Also tested at this time were BSA and sucrose and only BSA could partially inhibit (not significantly) either hemolysin at the concentrations tested (see Table 4).

2. Neutralization by serum components.

The hemolysins were also subjected to normal serum components. Fetal bovine serum (FBS) which has a low concentration of serum lipids was unable to block the activity of the hemolysin. Newborn bovine serum which contains much more lipid than FBS was able to completely block the release of hemoglobin after addition to the RNA-core hemolysin.

Newborn bovine serum was also able to significantly block the hemoglobin release of the recombinant hemolysin. In addition, a commercially prepared HDL-cholesterol compound was tested and was able to partially neutralize the RNA-core hemolysin activity and almost completely block the recombinant activity (see Table 5).

3. Neutralization by cholesterol derivatives.

Hemolysin activity varied after exposure to cholesterol derivatives. All of the cholesteryl derivatives were able to partially block the RNA-core hemolysin, with a range from 20 to 87 percent blockage. The other cholesterol derivatives varied in their blocking activity from 3% to 87%. The recombinant hemolysin was completely inhibited by dihydrocholesterol and stigmasterol, partially inhibited by b-sitosterol, cholesterol and cholesteryl oleate and unaffected by the others tested (see Table 6).

D. DISCUSSION

Current studies were undertaken in order to further define the biochemical and physical characteristics of the RNA-core and recombinant hemolysins derived from *S. hyodysenteriae*. Since it is unknown at this time if the recombinant protein produced by Muir et al. (78) from *S. hyodysenteriae* is the same as the protein extracted from the native bacteria with sodium RNA-core (89), these studies were undertaken in order to confirm their similarity.

We used streptolysin S (SLS) as our model system (69) due to the similarities between the two that have previously been demonstrated. These include the necessity for a carrier molecule for extraction from the bacterial cell wall, oxygen sensitivities, and sensitivity to trypsin (57, 65, 85, 89, 90, 91, 92). Using SLS studies as a basis, we tried to compare the similarities between the RNA-core and recombinant hemolysins of *S. hyodysenteriae.* Differences between the *Serpulina hyodysenteriae* hemolysins were noted following addition to certain compounds. The hemolysins were tested against bivalent cations to ascertain if these molecules were necessary for activity. Recent work by Dupont et al. (21) has shown that when the native hemolysin is extracted in the presence of divalent cations, zinc and copper are able to completely inhibit hemolytic activity after three cycles of hemolysin extraction. The authors concluded that the effect of the zinc was directly on the spirochetes and the synthesis of hemolysin. However, the study failed to directly address the effect of cations on the hemolytic activity of the free hemolysin. Our results supported those of Dupont, in that the RNA-core and recombinant *S. hyodysenteriae* hemolysins were sensitive

to zinc and unaffected by sucrose. These results were opposite to that of SLS. Our results seemed to indicate that the cations tested were not necessary for the activity of the hemolysins ($p<0.01$). In fact, the hemolysins were inhibited in the presence of some of the cations tested.

Our results and those of others (3, 90) show the recombinant and RNA-core hemolysins from *S. hyodysenteriae* are similar (but not identical) to streptolysin S from group A *Streptococcus* in biochemical and biological activity. Both hemolysins are inactivated by endoproteinases, pH resistant, oxygen resistant, temperature dependent, inhibited by copper, not activated by thiols, require a carrier for removal from the cell, have no phospholipase or enzymatic activity, are cytotoxic to eukaryotic cells, and are non-immunogenic substances (3, 20, 22, 65, 69, 89, 90).

Serum neutralization studies with RNA core-hemolysin demonstrated that normal serum from rabbits, mice and pigs, when added to the assay buffer, inhibited the lysis of erythrocytes (104). Also, streptolysin S is inhibited by β-lipoprotein in human serum (28). Further analysis of the inhibitory component was the goal of the next question. Previous work in our lab has demonstrated that IgG and IgM from bovine serum was unable to block the RNA-core toxin. Takahashi (104) also confirmed the IgG findings and demonstrated that both LDL and HDL from human and swine sera can inhibit the RNA-core hemolysin. They were unable to show that either was predominant in its blockage. To determine if the inhibitory component in serum was low, high density lipids, or cholesterol; fetal bovine serum, which has low concentrations of these lipids and newborn serum, which contains a normal concentration of these lipids, were added to the hemolytic assay. Fetal bovine serum had no

effect on either assay, whereas newborn serum significantly ($p<0.01$) inhibited the lytic effect of the RNA core-hemolysin and the recombinant protein on the erythrocytes. A commercially prepared HDL-cholesterol sample was also tested and it was able to significantly ($p<0.01$) block the hemolytic activity of both the RNA-core and the recombinant hemolysin. While it is unknown at the present if the deduced binding of membrane lipids or serum lipoproteins to the hemolysin is a receptor-mediated response (conformational) or non-specific (hydrophobic or sponge-like), the inability of the RNA-core and recombinant hemolysin to form a stable relationship with erythrocytes at four degrees suggest the absence of specific receptors. These results concur with the membrane fluidity model of red blood cells in that at lower temperatures the fluidity of the membrane is decreased and this impedes the insertion of the hemolysin into the membrane. Further study will be needed to address whether or not specific SRBC receptors for the hemolysin are present.

Preliminary experiments with HDL indicates that the hemolysin binds to the lipid portion of the serum component. Saheb et al. (92) demonstrated that phospholipids and cholesterol are not inhibitory to the lytic effect of the RNA-core hemolysin, as compared to their inhibition of streptolysin S (28). Additionally, the black lipid experiments demonstrated the formation of pores possessing permeability-selective cation channel activity in bilayers composed of oxidized cholesterol (unpublished data). These data suggested that sterols which have a higher solubility factor are a target membrane lipid for the RNA-core hemolysin. These findings were the basis of the asking of the final question. In order to determine if this binding is specific, competitive inhibition studies were undertaken as first described by Saheb (92).

Cholesterol and cholesteryl derivatives were tested against the RNA-core and recombinant hemolysins to ascertain if they were possibly the primary blocking agent in the serum solutions. Eleven cholesterol and cholesteryl derivatives were added to the assay and the blockage seen varied greatly between the samples. The values ranged from about three percent blockage to eighty seven percent for the RNA-core hemolysin. Few of the cholesterol derivatives were active against the recombinant hemolysin. Those that were active were more active against the recombinant hemolysin than against the RNA-core hemolysin in almost all of the cases. These findings seem to indicate the importance of the oxidation state of the cholesterol when reacting to the hemolysin. Whether or not these molecules form all or part of the actual binding sites on the erythrocyte is yet to be elucidated.

Our results indicate that the RNA-core hemolysin produced by *S. hyodysenteriae* is inhibited by zinc and copper, partially inhibited by calcium, unaffected by magnesium, BSA and sucrose, inhibited by NBS and HDL, but not FBS, and inhibited by some cholesterol derivatives.

Table 4. Cations effects on *S. hyodysenteriae* RNA-core and recombinant hemolysins.

	RNA-core hemolysin		Recombinant Hemolysin	
Cations and Substances	**A_{540} (mean +/- SD)[a]**	**Percent Blockage**	**A_{540} (mean +/- SD)[a]**	**Percent Blockage**
Positive Control	0.19 +/- 0.005		0.63 +/- 0.06	
Bovine Serum Albumin	0.153 +/- 0.012	19.5%	0.58 +/- 0.025	7.94%
Sucrose (0.3M)	0.1967 +/- 0.012	0	0.61 +/- 0.009	3%
Calcium[b]	0.103 +/- 0.005	45.79%	0.53 +/- 0.037	15.9%
Calcium + EDTA	0.0467 +/- 0.0047	75.42%	ND	
Copper	0	100%	0.067 +/- 0.012	89.4%
Zinc	0.02 +/- 0.008	89.47%	0.02 +/- 0.008	96.8%
Magnesium	0.18 +/- 0.016	5.26%	0.55 +/- 0.045	12.7%

a Data were pooled from three separate experiments.
b Data were pooled from six separate experiments.
ND Not done

Table 5. The neutralization of the *S. hyodysenteriae* RNA-core and recombinant hemolysins by serum components.

	RNA-core hemolysin		Recombinant Hemolysin	
Serum Component	**A_{540} (mean +/- SD)[a]**	**Percent Blockage**	**A_{540} (mean +/- SD)[a]**	**Percent Blockage**
Positive Control	0.536 +/- 0.0273		0.63 +/- 0.06	
Fetal bovine serum	0.5133 +/- 0.0573	2.41%	0.62 +/- 0.05	1.1%
Newborn bovine serum	0.0267 +/- 0.0170	94.92%	0.24 +/- 0.05	62%
Sigma-HDL	0.0233 +/- 0.0047	95.57%	0.06 +/- 0.008	90.5%

a Data were pooled from three separate experiments.

Table 6. The effects of cholesterol and its derivatives on the *S. hyodysenteriae* RNA-core and recombinant hemolysins.

	RNA-core hemolysin		Recombinant Hemolysin	
Cholesterol Derivative	**A_{540} (mean +/- SD)[a]**	**Percent Blockage**	**A_{540} (mean +/- SD)[a]**	**Percent Blockage**
Positive Control	0.3 +/- 0.04		0.175 +/- 0.015	
Dihydrocholesterol	0.12 +/- 0.05	60%	0	100%
β-Sitosterol	0.23 +/- 0.09	23%	0.11 +/- 0.049	37%
Ergosterol	0.185 +/- 0.126	38%	0.213 +/- 0.009	0
Stigmasterol	0.23 +/- 0.11	23%	0	100%
Cholesteryl Proprionate	0.24 +/- 0.03	20%	0.24 +/- 0.029	0
Cholesteryl Oleate	0.15 +/- 0.03	50%	0.063 +/- 0.048	64%
Cholesteryl Palmitate	0.04 +/- 0.04	87%	0.28 +/- 0.043	0
Cholesteryl Laurate	0.21 +/- 0.09	30%	0.216 +/- 0.078	0
4-Cholesten-3-one	0.24 +/- 0.05	20%	0.0176 +/- 0.077	0
5-Cholesten-3β-ol-7-one	0.04 +/- 0.04	87%	0.256 +/- 0.049	0
Cholesterol	0.29 +/- 0.13	3.3%	0.106 +/- 0.151	39%

a Data were pooled from three separate experiments.

CHAPTER IV. REDUCED VIRULENCE IN PIGS OF SERPULINA HYODYSENTERIAE HEMOLYSIN-MINUS MUTANTS AND THEIR POTENTIAL TO PROTECT PIGS AGAINST CHALLENGE WITH A VIRULENT STRAIN.

A. INTRODUCTION

Swine dysentery (SD) is a mucohemorrhagic diarrheal disease caused by *Serpulina hyodysenteriae*, an anaerobic spirochete (32, 33, 105). *S. hyodysenteriae* differs from the closely related non-pathogenic *Serpulina innocens* by strong beta hemolytic activity on blood agar plates and by its enteropathogenicity in pigs and mice (35, 55).

The *S. hyodysenteriae* hemolysin activity is believed to be a major virulence factor (50, 51, 57, 78, 89, 101). Purified *S. hyodysenteriae* hemolysin is cytotoxic for a number of cell types in vitro (50). Swine intestinal loops exposed to purified *S. hyodysenteriae* hemolysin develop microscopic lesions similar to those seen in natural cases of SD (71).

A gene, *tlyA* (previously named *tly*) encoding a *S. hyodysenteriae* hemolysin has recently been cloned and sequenced (78). The *tlyA* gene encodes a protein with a molecular mass of 26.9 kDa. This protein is hemolytic for erythrocytes and is cytotoxic for several eukaryotic cell lines. A *tlyA*-minus mutant of the Dutch *S. hyodysenteriae* field-strain, C5, was then created by homologous recombination (101). Virulence tests in mice with this mutant indicated that the *tlyA* encoded hemolysin is important as a virulence factor, but after inactivation of this gene residual virulence remained. Other *S. hyodysenteriae* hemolysin genes, *tlyB* and *tlyC* (100), and lipopolysaccharide (LPS) (81) may also play a role in the pathogenesis of SD and contribute to the residual virulence

observed in mice.

Since the *tlyA* gene has been found in all of the strains tested, another mutant strain was also used which was produced in a more virulent wild type strain, B204, which is of a different serotype than the first strain tested, C5.

The virulence of the *S. hyodysenteriae tlyA*-minus mutant strains were tested in pigs. In addition, the potential of these mutant strains to protect pigs against heterologous and homologous challenge with a highly virulent *S. hyodysenteriae* wild-type strain was also tested.

B. MATERIALS AND METHODS

1. Bacterial strains and culture conditions.

Serpulina hyodysenteriae strains B204 and C5 (a Dutch field strain) have been described before (73, 103). The construction of the *tlyA*-minus mutant of strain C5 has been described (101). The *tlyA*-minus mutant of strain B204 was constructed in the same way (A.A.H.M. ter Huurne et al., unpublished results). The isolates were stored at -70°C. The medium used to culture *S. hyodysenteriae*, consisted of pre-reduced Trypticase Soy Broth (TSB) supplemented with 5 to 7% fetal bovine serum (FBS). Cultures were incubated at 37°C in a gas mixture of 50:50 CO_2/H_2 for 16 h. All isolates used to challenge pigs had undergone less than 25 *in vitro* passages.

2. Animals.

All piglets used were conventional Yorkshire crosses, obtained from the Valley Hog Farm in Willcox, Arizona. In study A (inoculation with strain C5), the pigs were 4 to 5 weeks old, while for the protection study extra control pigs of 8 to 9 weeks were obtained. The piglets used in study B (inoculation with strain B204) were 8 weeks old and were challenged after another 30 days (at the age of 12 to 13 weeks). Pigs used as controls (12 to 13 weeks old) were obtained from the same farm. The groups were equalized according to size and weight, kept in strict isolation and fed with antibiotic-free grower feed throughout the studies. Animals were checked for serum antibodies to *Serpulina hyodysenteriae* and were tested for the presence of the bacteria in rectal swabs. Animals inoculated with wild-type *S. hyodysenteriae* strains were necropsied after 30

days, whereas pigs inoculated with mutant strains were challenged with the B204 wild-type strain and necropsied 30 days later.

3. Inoculation.

Each pig was deprived of food for 24 h before inoculation and inoculated intragastrically with 100 mls of broth culture, containing 10^9 to 10^{10} colony forming units (CFU).

4. Observation of clinical signs and fecal sampling.

Daily observations were made of each pen. Pens were assessed for the presence of diarrhea, mucus, blood, and depression or anorexia. Clinical symptoms of individual pigs were assessed during rectal swabbing. Rectal swabs were taken three times a week and plated on blood agar plates containing spectinomycin, spiramycin, rifampicin, colistin, and vancomycin (ABAP plates) (63). Selection for the *tlyA*-minus mutant was done on the ABAP plates supplemented with 150 µg/ml kanamycin. Plates were incubated anaerobically for 48 to 72 h and checked for growth of *S. hyodysenteriae*. In study B plates were re-checked after 5 days for growth of the *tlyA*-minus mutant when it was found that it sometimes takes more than 72 h for the mutant to grow on plates. After growth on plates, confirmation that the growth on plates was the *tlyA*-minus mutant strain was shown by the polymerase chain reaction (PCR) as described (101).

5. Necropsy.

Colonic lesions were scored blind and the macroscopic signs of *S. hyodysenteriae* infection in the colon were evaluated by the presence of hemorrhage, fibrin, mucus,

edema, necrosis and hyperemia. Colonic contents were cultured to assay colonization by the *S. hyodysenteriae* wild-type or mutant strains.

6. Data analysis.

Pigs were scored positive for clinical signs of SD if they had blood in their stools. However if they showed diarrhea and depression without blood in their stools, they were scored positive if they had colonic lesions typical for SD. Lesions were considered typical of SD, if hemorrhage and fibrin and/or necrosis were observed in the colon.

7. Statistical analysis.

One way randomized ANOVA test was run using the CoStat program.

C. RESULTS

1. Virulence of the *S. hyodysenteriae tlyA*-minus mutants.

A. Evaluation of clinical signs and colonic lesions.

In both studies, pigs inoculated with the wild-type strains, were necropsied for confirmation of SD. The pigs inoculated with the mutant strains were re-challenged with the B204 wild-type strain.

In study A, pigs inoculated with either the C5 wild-type strain (n=5) or the *tlyA*-minus strain (n=5) were compared (Table 7). The pigs inoculated with the *tlyA*-minus mutant did not show signs of SD during the study period. Only 3 out of 5 pigs inoculated with the C5 wild-type strain, showed depression and diarrhea at an average onset of 19 days post-infection (PI). However, none of these pigs had blood in their stools. All 3 sick pigs died after 1 day of clinical symptoms and SD was confirmed by necropsy, since hemorrhage and mucus and/or fibrin were found in the colon. The other two pigs had either no colonic lesions or only a slight mucosal hyperemia and were considered negative for SD.

In study B, pigs were inoculated with either the *S. hyodysenteriae* B204 wild-type strain (n=6) or the B204 *tlyA*-minus mutant strain (n=6) (Table 7). In this study, pigs inoculated with the *tlyA*-minus mutant also remained healthy until the end of the study. However, all pigs inoculated with the B204 wild-type strain, showed diarrhea, depression, mucus and blood in their stools. The onset of the clinical signs varied from day 7 PI to day

21 PI with an average of 14 days PI. The duration of clinical signs varied from 3 to 13 days. Two pigs died of SD after having had symptoms for 3 or 5 days. All pigs inoculated with the B204 wild-type strain had severe colonic lesions, e.g. frank hemorrhage, mucus, fibrin and/or necrosis, typical of SD.

B. Colonization of *S. hyodysenteriae* as detected by fecal shedding or presence of bacteria in the colon (Table 7).

Direct presence in the colon could not be assayed for the pigs inoculated with the *tlyA*-minus mutant, as they were not necropsied after 30 days. In the group inoculated with the C5 wild-type strain, 4 pigs were shedding *S. hyodysenteriae* with an average onset of 17 days PI. Colonization of the colon occurred in 3 pigs only, of which one had not been shedding. The colonization as detected by fecal shedding and/or presence in the colon is indicated in Table 7. Plates from rectal swabs of pigs inoculated with the C5 *tlyA*-minus mutant were checked for growth after 48 to 72 hours of incubation and found negative.

When inoculated with the *S. hyodysenteriae* B204 wild-type strain (study B), all pigs were shedding until death or necropsy with an average onset of 5 days PI. *S. hyodysenteriae* was also re-isolated from the colons of all pigs. Pigs inoculated with the B204 mutant also shed Serpulina. However, the average onset of shedding in this group was 15 days PI, while the mean duration was 5 days.

2. Evaluation of protection obtained by previous inoculation with *S. hyodysenteriae tlyA*-minus mutants against challenge with the *S. hyodysenteriae* B204 wild-type strain.

A. Evaluation of clinical signs and colonic lesions.

All pigs were necropsied for confirmation of SD. In study A, 5 control pigs and 5 pigs previously inoculated with the *S. hyodysenteriae* C5 *tlyA*-minus mutant, were challenged with the B204 wild-type strain. Two control pigs responded with clinical signs of SD, e.g. diarrhea and mucus and blood in their stools (Table 8). One of these pigs died of SD. Clinical signs were observed from day 15 and day 29 PI respectively. At necropsy, lesions were observed only in the pigs that had shown clinical signs of SD. None of the pigs previously inoculated with C5 *tlyA*-minus mutant responded with clinical signs of SD nor were colonic lesions observed in any of these pigs. Due to a low number of control pigs contracting SD, the values for this study were not significant.

Six control pigs and 6 pigs previously inoculated with the B204 *tlyA*-minus mutant were used in study B. When challenged with the B204 wild-type strain, 5 out of 6 control pigs showed depression, diarrhea and blood in their stools, whereas one pig only showed diarrhea and depression (Table 8). The average onset of clinical signs of the 5 pigs was 14 days PI. Two pigs died of SD 2 or 7 days after the onset of clinical signs, whereas the average duration of signs of the other pigs was 8 days. All control pigs, including the pig which had not demonstrated blood, had lesions of SD, e.g. hemorrhage, bloody watery contents, mucus and/or fibrin. The incidence of clinical signs in this study was found to be

significantly different ($p<0.05$) between the parent strain and *tlyA*-minus mutant strain groups.

In the group previously inoculated with the B204 *tlyA*-minus mutant, 3 pigs showed diarrhea and depression, whereas two of these also had blood in their feces (Table 8). The average onset of clinical signs of these 3 pigs was 9 days PI. The pig that had symptoms without blood, died at day 9 PI and SD was confirmed by necropsy. The duration of signs of SD in the other 2 pigs was 4 days. Of the 2 pigs that had shown blood in their stools, only one had colonic lesions at necropsy, indicating that one pig had already recovered. The lesions in the mutant pigs were less severe than in the control pigs. However 3 pigs in this group were still assessed to have developed SD. The incidence of swine dysentery lesions in this study was found to be significantly different ($p<0.01$) between the parent strain and the *tlyA*-mutant strain groups.

B. Colonization of *S. hyodysenteriae* as detected by fecal shedding or presence of bacteria in the colon (Table 8).

In study A, 3 out of 5 control pigs were shedding strain C5 during the study period with an average onset of 7 days PI. In the group previously inoculated with the *S. hyodysenteriae* C5 *tlyA*-minus mutant, 3 pigs were shedding with an average onset of 27 days PI. The colonization of *S. hyodysenteriae* in the colon was not assessed at necropsy.

In study B, all control pigs were shedding *S. hyodysenteriae* during the study with an average onset of 10 days PI. All pigs continued to shed until the end of the study or

until death. Colonization of the colon with B204 occurred in each control animal. The colonization as detected by fecal shedding and/or presence in the colon is indicated in Table 8. In the group previously inoculated with the B204 *tlyA*-minus mutant, all the pigs were also shedding, with an average onset of 5 days PI. However, the duration of shedding was much shorter and all colonic cultures were negative.

D. DISCUSSION

Virulence tests of a *tlyA*-minus mutant, produced by homologous recombination, of a *S. hyodysenteriae* strain indicated that the *tlyA* encoded hemolysin is an important virulence factor in the pathogenesis of *S. hyodysenteriae* in mice (101). The production of the mutant strain in this study used the cloned *tlyA* gene and a kanamycin cassette which was inserted into the gene. This construct was then electroporated into a wild-type strain (either C5 or B204) and checked for homologous recombination. Although it is possible that other virulence factors, such as the LPS, could have been changed due to downstream changes in the gene, this study demonstrates that TlyA hemolysin production in this strain is eliminated and that this had a significant effect on the virulence of the organism. Polar effects due to the insertion of the Kanamycin resistance gene are unlikely. The sequence data (78) demonstrates that the *tlyA* encoding gene is not part of a polycistronic messenger that contains genes downstream. In addition, sequence analysis of the inserted Kanamycin resistance gene shows that this gene does not contain a termination signal, therefore, interruption of polycistronic messenger would not have occurred. Furthermore, PCR with primers derived from *tlyA* on the mutated strains gave products of the expected size, indicating that it is unlikely that deletions have occurred during the cross-over even that created the *tlyA*- minus mutants. This data demonstrates that the hemolysin was eliminated in the mutant and, therefore, the mutant strain remains an important tool for the analysis of the hemolysin as a virulence factor in the pathogenesis of SD. Since the pig is the natural host of *S. hyodysenteriae*, the next step was to prove the importance of the

tlyA encoded hemolysin in the pathogenesis of SD.

The differences in clinical signs and lesions caused by the *S. hyodysenteriae* C5 wild-type strain and its *tlyA*-minus mutant, demonstrated that the C5 *tlyA*-minus mutant was less virulent in pigs. However, the C5 wild-type strain did not infect pigs as well as mice (101). Only 3 out of 5 pigs inoculated with this wild-type strain had a very late onset of depression and diarrhea, but never had blood in their feces, the clinical sign characteristic for acute SD (Table 7). Only after necropsy SD could be diagnosed in these pigs. The difference in infectivity and/or virulence in mice and pigs of strains has also been observed by Kinyon et al. (54). Their findings indicated that some strains had lost virulence in pigs after *in vitro* passaging while remaining virulent in mice.

Since the *tly* gene is found in all of the *S. hyodysenteriae* strains tested, the choice of strains for recombination have no bearing. The only thing which has an influence is that the strain of *S. hyodysenteriae* used has to be sensitive to kanamycin. Therefore a *tlyA*-minus mutant of *S. hyodysenteriae* strain B204 was tested too, since the B204 wild-type strain is extremely virulent in pigs (Joens, personal observation). Also in this case the mutant strain was less virulent in pigs than the wild-type strain. All pigs inoculated with the mutant strain remained healthy until the end of the study. However, all pigs inoculated with the wild-type strain showed clinical signs and lesions of SD (Table 7). Mild lesions in the pigs inoculated with the B204 *tlyA*-minus mutant cannot be excluded since these pigs were not necropsied. However, considering the healthy status of these pigs, the presence of colonic lesions, typical of SD, in these pigs is not very likely.

The data on the shedding of the C5 *tlyA*-minus mutant strain could not be interpreted since in study A plates were kept for only 72 hours, while it takes the *tlyA*-minus mutant strain approximately 5 days to grow on the ABAP media. However, the data on the shedding of *S. hyodysenteriae* B204 indicated that, although the duration of shedding of the *tlyA*-minus mutant strain was less than that of the wild-type, both the *S. hyodysenteriae* wild-type and the *tlyA*-minus mutant are able to colonize the porcine gastro-intestinal tract (Table 7). Since *S. hyodysenteriae* may need the activity of a hemolysin as a scavenger of nutrients both *in vitro* (98) and *in vivo*, a strain lacking a hemolysin is probably less able to compete for available nutrients and therefore to survive.

The colonization of the colon by large numbers of *S. hyodysenteriae* could induce immunity, since previous studies demonstrated that pigs which had recovered naturally from SD, usually continue to shed *S. hyodysenteriae* for a long period and are protected against subsequent challenge with *S. hyodysenteriae* (23, 35, 41). Thus a less virulent strain, able to colonize the colon in sufficiently high numbers for an extended period, may induce (partial) protection against a challenge with a virulent *S. hyodysenteriae* strain. In this study the *tlyA*-minus mutants were demonstrated to be less virulent in pigs but still able to colonize the porcine gastro-intestinal tract and therefore stimulate the host immune system.

The protection studies undertaken with both the *S. hyodysenteriae* C5 and B204 *tlyA*-minus mutants showed that indeed partial protection against challenge with the virulent *S. hyodysenteriae* strain B204 was induced. The challenge with B204 was done in

order to test for cross-protection between the two different serotypes in study A and to ensure a good challenge using a more virulent strain of *S. hyodysenteriae*. In study A, none of the pigs previously inoculated with the C5 *tlyA*-minus mutant, displayed clinical signs or lesions of SD after challenge with the B204 wild-type strain, whereas 2 out of 5 control pigs contracted SD (Table 8). The protection values gained from this study were not statistically significant, there seemed to be an indication of cross-protection from the different challenge serotype and indicated a trend which could be studied further. In study B, all control pigs developed clinical signs and lesions of SD after challenge with the B204 wild-type strain, while only 3 out of 6 pigs previously inoculated with the B204 *tlyA*-minus mutant got SD (Table 8). In this study, both the incidence of SD lesions and the incidence of clinical signs were statistically different between the parent strain group and the *tlyA*-minus mutant strain group. A difference in viability between the inocula of the two B204 challenges may explain the differences in the incidence of SD observed between both studies.

Interestingly, the onset of both clinical signs and shedding of *S. hyodysenteriae* was much earlier in the pigs previously inoculated with the B204 *tlyA*-minus mutant than in the control pigs. However the duration of the clinical signs and shedding was much shorter. Moreover, the colonic lesions observed in these pigs were less severe than those of the controls. A possible explanation for these observations could be that the pigs which did get SD after challenge with the B204 wild-type strain, may have had minor lesions caused by other virulence factors introduced by the inoculation with the B204 *tlyA*-minus

mutant strain. This may have allowed the wild-type strain to colonize and induce clinical signs faster. However, since the immune system of these pigs had already been activated, the *S. hyodysenteriae* wild-type strain was neutralized or inactivated more quickly and the clinical signs, shedding and lesions were therefore less in duration and severity than in the control pigs.

These studies indicate that the *tlyA* encoded hemolysin is an important virulence factor in the pathogenesis of SD. While other virulence mechanisms may be involved in the pathogenicity of the organism, currently no other mechanisms have been shown to be as conclusively involved in the pathogenesis of SD as the *S. hyodysenteriae* hemolysin. Moreover, it is demonstrated that colonization with *S. hyodysenteriae tlyA*-minus provides partial protection to challenge with a virulent *S. hyodysenteriae* strain. Since the experimental challenge with the wild-type B204 was extremely high (10^9 to 10^{10} CFU) in this study, protection may be better under natural challenge conditions.

Table 7: Virulence in pigs of *S. hyodysenteriae* C5 and B204 wild-type strains and C5 and B204 *tlyA*-minus mutants.

Group	Incidence of Clin. Signs[a]	Avg. Onset of Clin. Signs (days PI)	Incidence of SD lesions	Incidence of Colonization[b]	Deaths (avg. days PI)
C5 wt	3/5[c]	+19 (3 pigs)	3/5	5/5	3/5 (+19)
C5 (tly-)[d]	0/5	-	ND	ND[e]	0/5
B204 wt	6/6	+14 (6 pigs)	6/6	6/6	2/6 (+12)
B204 (tly-)	0/6	-	ND	6/6	0/6

[a]: Pigs were considered to have signs of SD if they demonstrated depression, diarrhea and blood in their stools.
[b]: The colonization of *S. hyodysenteriae* as detected by fecal shedding and/or presence of bacteria (wild-type or mutant) in the colon is indicated.
[c]: Pigs showed depression and diarrhea, but no blood in feces. SD had to be confirmed by necropsy.
[d]: tly-: *tlyA*-minus mutant.
[e]: The *tlyA*-minus mutant strain could not be re-isolated due to the short incubation period of the plates.
Abbreviations: Clin, clinical; PI, post-infection; SD, swine dysentery; ND, not determined; wt, wild-type.

Table 8: Protection by previous inoculation with *S. hyodysenteriae* C5 and B204 *tlyA*-minus mutants against challenge with the B204 wild-type strain.

Group	Incidence of Clin. Signs[a]	Avg. Onset of Clin. Signs (days PI)	Incidence of SD lesions	Incidence of Colonizatio n[b]	Deaths (avg. days PI)
B204 wt[c]	2/5	+22 (2 pigs)	2/5	3/5	1/5 (+20)
C5(tly-)[d] / B204 wt	0/5	-	0/5	3/5	0/5
B204 wt[e]	6/6 [f]	+14 (6 pigs)	6/6	6/6	2/6 (+25)
B204(tly-) /B204 wt	3/6 [f]	+9 (3 pigs)	2/6	6/6	1/6 (+9)

[a]: Pigs were considered to have signs of SD if they demonstrated depression, diarrhea and blood in their stools.
[b]: The colonization of *S. hyodysenteriae* as detected by fecal shedding and/or presence of bacteria (wild-type) in the colon is indicated.
[c]: Control group in study A.
[d]: tly-: *tlyA*-minus mutant.
[e]: Control group in study B.
[f]: One pig in each group showed depression and diarrhea, but no blood in feces. SD had to be confirmed by necropsy.
Abbreviations: Clin, clinical; PI, post-infection; SD, swine dysentery; ND, not determined; wt, wild-type.

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