Reduced Virulence of *Serpulina hyodysenteriae* Hemolysin-Negative Mutants in Pigs and Their Potential To Protect Pigs against Challenge with a Virulent Strain

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The role of the *Serpulina hyodysenteriae* hemolysin encoded by the *tlyA* gene in the pathogenesis of swine dysentery (SD) was studied. *tlyA* mutants of two *S. hyodysenteriae* strains (B204 and C5) were tested for virulence in pigs. None of the animals developed SD. However, after infection with wild-type strain B204 or C5, the incidence of SD was 100 or 60%, respectively. Thus, the *tlyA*-encoded hemolysin of *S. hyodysenteriae* is an important virulence factor in SD. The potential of these mutants to protect pigs against challenge with a virulent *S. hyodysenteriae* strain was also studied. After challenge with wild-type strain B204, 50% of pigs previously inoculated with the B204 *tlyA* mutant were protected, whereas all control pigs contracted SD. None of the pigs previously inoculated with the C5 *tlyA* mutant developed SD upon challenge with wild-type strain B204, whereas 40% of the control pigs developed SD in this experiment. Thus, previous colonization with *S. hyodysenteriae tlyA* mutants in pigs provides partial protection against challenge with a virulent *S. hyodysenteriae* strain.

Swine dysentery (SD) is a mucohemorrhagic diarrheal disease caused by *Serpulina hyodysenteriae*, an anaerobic spirochete (2, 17). *S. hyodysenteriae* differs from the closely related nonpathogenic *Serpulina innocens* by its high level of beta-hemolysic activity on blood agar plates and by its enteropathogenicity in pigs and mice (3, 7).

*S. hyodysenteriae* hemolysin activity is believed to be a major virulence factor (5, 6, 9, 13, 15, 19). Purified *S. hyodysenteriae* hemolysin is cytotoxic for a number of cell types in vitro (5). Swine intestinal loops exposed to purified *S. hyodysenteriae* hemolysin develop microscopic lesions similar to those seen in natural cases of SD (11).

A gene, *tlyA* (previously named *tly*), encoding an *S. hyodysenteriae* hemolysin was recently cloned and sequenced (13). 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* mutant of Dutch *S. hyodysenteriae* field strain C5 was created by homologous recombination (19). Virulence tests with this mutant in mice indicated that the *tlyA*-encoded hemolysin is an important virulence factor but, after inactivation of this gene, residual virulence remained. Other *S. hyodysenteriae* hemolysin genes, such as *tlyB* and *tlyC* (17a), and lipopolysaccharide (14) 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 strains tested, another mutant strain was used. This mutant was produced from a more virulent wild-type strain, B204, which is of a serotype different from that of the first strain tested, C5.

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

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *S. hyodysenteriae* B204 and C5 (a Dutch field strain) have been described elsewhere (12, 18). The construction of the *tlyA* mutant of strain C5 has been described elsewhere (19). The *tlyA* mutant of strain B204 was constructed in the same way (17b). The isolates were stored at −70°C. The medium used to culture *S. hyodysenteriae* consisted of prerduced Trypticase soy broth supplemented with 5 to 7% fetal calf serum. Cultures were incubated at 37°C in a gas mixture of 50% CO₂-50% H₂ for 16 h. All isolates used to challenge pigs had undergone fewer than 25 in vitro passages.

**Animals.** All piglets used were conventional Yorkshire crosses obtained from the Valley Hog Farm in Wilcox, Ariz. In study A (inoculation with strain C5), the pigs were 4 to 5 weeks old, while for the protection study, extra control pigs 8 to 9 weeks old were obtained. The pigs 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 divided according to size and weight, kept in strict isolation, and fed antibiotic-free grower feed throughout the studies. Animals were checked for serum antibodies to *S. hyodysenteriae* and were tested for the presence of the bacterium in rectal swab samples. Animals inoculated with wild-type *S. hyodysenteriae* strains were necropsied after 30 days, whereas animals inoculated with mutant strains were challenged with wild-type strain B204 and necropsied 30 days after the challenge.

**Inoculation.** Each pig was deprived of food for 24 h before inoculation and inoculated intragastrically with 100 ml of a broth culture containing 10⁷ to 10¹⁰ CFU.

**Observation of clinical signs and fecal sampling.** Daily
observations of each pen were made. Pens were assessed for the presence of diarrhea, mucus, blood, and depression or anorexia. Clinical symptoms in individual pigs were assessed during rectal swabbing. Rectal swab samples were obtained three times a week and plated on blood agar plates containing spectinomycin, spiramycin, rifampin, colistin, and vancomycin (ABAP plates) (10). Selection for the thyA mutants was done on ABAP plates supplemented with 150 μg of kanamycin per ml. The plates were incubated anaerobically for 48 to 72 h and checked for the growth of S. hyodysenteriae. In study B, the plates were rechecked after 5 days for the growth of the thyA mutants when it was found that it sometimes takes more than 72 h for the mutants to grow on plates. Confirmation that the growth on plates was that of the thyA mutants was shown by the PCR as described previously (19).

Necropsy. Colonic lesions were scored blind, and 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 for colonization by the S. hyodysenteriae wild-type or mutant strains.

Data analysis. Pigs were scored positive for clinical signs of SD if they had blood in their stools. However, if they had diarrhea and depression but no blood in their stools, they were scored positive if they had colonic lesions typical of SD. Lesions were considered typical of SD if hemorrhage, fibrin, and/or necrosis was observed in the colon.

Statistical analysis. A one-way randomized analysis of variance was run with the CoStat program.

RESULTS

Virulence of the S. hyodysenteriae thyA mutants. (i) Evaluation of clinical signs and colonic lesions. In both studies, pigs inoculated with the wild-type strains were necropsied for the confirmation of SD, and pigs inoculated with the mutant strains were rechallenged with wild-type strain B204.

In study A, pigs inoculated with either wild-type strain C5 (n = 5) or the C5 thyA mutant strain (n = 5) were compared (Table 1). The pigs inoculated with the thyA mutant did not show signs of SD during the study period. Only three of five pigs inoculated with wild-type strain C5 showed depression and diarrhea, at an average onset of 19 days postinfection (p.i.). However, none of these pigs had blood in their stools. All three 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 inoculated pigs had either no colonic lesions or only slight mucosal hyperemia and were considered negative for SD.

In study B, pigs were inoculated with either wild-type strain B204 (n = 6) or the B204 thyA mutant strain (n = 6) (Table 1). The pigs inoculated with the thyA mutant remained healthy until the end of the study. However, all pigs inoculated with wild-type strain B204 showed diarrhea, depression, mucus, and blood in their stools. The onset of the clinical signs varied from day 7 p.i. to day 21 p.i., with an average of 14 days p.i. The duration of the clinical signs varied from 3 to 13 days. Two pigs died of SD after having had symptoms for 3 and 5 days. All pigs inoculated with wild-type strain B204 had severe colonic lesions, e.g., frank hemorrhage, mucus, fibrin, and/or necrosis typical of SD.

(ii) Colonization with S. hyodysenteriae as detected by fecal shedding or the presence of bacteria in the colon. The direct presence of S. hyodysenteriae in the colon could not be assayed for the pigs inoculated with the thyA mutants, as they were not necropsied after 30 days. In the group inoculated with wild-type strain C5 (study A), four pigs shed S. hyodysenteriae, with an average onset of 17 days p.i. Colonization of the colon occurred in only three pigs, of which one had not been shedding. Colonization as detected by fecal shedding and/or the presence of bacteria in the colon is indicated in Table 1. Plates containing rectal swab samples from pigs inoculated with the C5 thyA mutant were checked for growth after 48 to 72 h of incubation and found to be negative.

When inoculated with wild-type strain B204 (study B), all pigs shed S. hyodysenteriae until death or necropsy, with an average onset of 5 days p.i. S. hyodysenteriae was also reisolated from the colon of all pigs. Pigs inoculated with the B204 thyA mutant also shed Serpulina cells. However, the average onset of shedding in this group was 15 days p.i., while the mean duration was 5 days.

Evaluation of protection obtained by previous inoculation with S. hyodysenteriae thyA mutants against challenge with S. hyodysenteriae wild-type B204. (i) Evaluation of clinical signs and colonic lesions. All pigs were necropsied for the confirmation of SD. In study A, five control pigs and five pigs previously inoculated with the S. hyodysenteriae C5 thyA mutant were challenged with wild-type strain B204. Two control pigs responded with clinical signs of SD, e.g., diarrhea and mucus and blood in their stools (Table 2). One of these pigs died of SD. Clinical signs were observed from days 15 and 29 p.i. At necropsy, lesions were observed only in the pigs that had shown clinical signs of SD. None of the pigs previously inoculated with the C5 thyA mutant responded with clinical signs of SD, nor were colonic lesions observed in any of these pigs. Because of the small number of control pigs contracting SD, the values for this study were not significant.

Six control pigs and six pigs previously inoculated with the S. hyodysenteriae B204 thyA mutant were used in study B. When challenged with wild-type strain B204, five of the six control pigs showed depression, diarrhea, and blood in their stools,
whereas one pig showed only diarrhea and depression (Table 2). The average onset of clinical signs in the five pigs was 14 days p.i. Two pigs died of SD 2 and 7 days after the onset of clinical signs, whereas the average duration of signs in the other pigs was 8 days. All control pigs, including the pig that had not demonstrated blood, had SD lesions, 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 the tlyA mutant strain groups.

In the group previously inoculated with the B204 tlyA mutant, three pigs showed diarrhea and depression, and two of these also had blood in their feces (Table 2). The average onset of clinical signs in these three pigs was 9 days p.i. The pig that had symptoms but not blood died at day 9 p.i., and SD was confirmed by necropsy. The duration of signs of SD in the other two pigs was 4 days. Of the two pigs that had blood in their stools, only one had colonic lesions at necropsy, indicating that one pig had already recovered. The lesions in the test pigs were less severe than those in the control pigs. However, three pigs in the former group were still assessed to have developed SD. The incidence of SD lesions in this study was found to be significantly different (P < 0.01) between the parent strain and the tlyA mutant strain groups.

(ii) Colonization with S. hyodysenteriae as detected by fecal shedding or the presence of bacteria in the colon. In study A, three of five control pigs shed strain C5, with an average onset of 7 days p.i. In the group previously inoculated with the S. hyodysenteriae C5 tlyA mutant, three pigs shed the bacterium, with an average onset of 27 days p.i. Colonization with S. hyodysenteriae in the colon was not assessed at necropsy.

In study B, all control pigs shed S. hyodysenteriae, with an average onset of 10 days p.i. 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. Colonization as detected by fecal shedding and/or the presence of bacteria in the colon is indicated in Table 2. In the group previously inoculated with the S. hyodysenteriae B204 tlyA mutant, all the pigs shed the bacterium, with an average onset of 5 days p.i. However, the duration of shedding was much shorter than in the nonvaccinated control group, and all colonic cultures were negative.

**DISCUSSION**

Virulence tests of a tlyA mutant produced by homologous recombination from an S. hyodysenteriae strain indicated that the tlyA-encoded hemolysin is an important virulence factor in the pathogenesis of S. hyodysenteriae in mice (19). Mutant strains were produced in this study by use of 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 lipopolysaccharide, could have been changed because of downstream changes in the gene, this study demonstrated that TlyA hemolysin production in these strains was eliminated and that this elimination 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 (13) demonstrate 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 a polycistronic messenger would not have occurred. Furthermore, PCR of the mutated strains with primers derived from tlyA yielded products of the expected sizes, indicating that it is unlikely that deletions occurred during the crossover event that created the tlyA mutants. These data demonstrate that the hemolysin was eliminated from the mutants; therefore, mutant strains remain 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 mutant demonstrated that the C5 tlyA mutant was less virulent in pigs. However, the C5 wild-type strain did not infect pigs as well as it did mice (19). Only three of five pigs inoculated with this wild-type strain had a very late onset of depression and diarrhea and never had blood in their feces, the clinical sign characteristic of acute SD (Table 1). Only after necropsy could SD be diagnosed in these pigs. The difference in strain infectivity and/or virulence for mice and pigs was also observed by Kinyon et al. (8). Their findings indicated that some strains had lost virulence for pigs after in vitro passaging while remaining virulent for mice.

Since the tly gene is found in all of the S. hyodysenteriae strains so far tested, the choice of strains for recombination has no bearing on study results. The only item which has an influence is that the strain of S. hyodysenteriae used must be susceptible to kanamycin. Therefore, a tlyA mutant of S. hyodysenteriae B204 was tested, too, since the B204 wild-type strain is extremely virulent in pigs (3a). Also, in this case, the mutant strain was less virulent for 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 typical of SD (Table 1). The presence of mild lesions in pigs

**TABLE 2. Protection by previous inoculation with S. hyodysenteriae C5 and B204 tlyA mutants against challenge with wild-type strain B204**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of pigs with clinical signs/ no. tested</th>
<th>Avg onset of clinical signs, days p.i. (no. of pigs)</th>
<th>No. of pigs with SD lesions/no. tested</th>
<th>No. of pigs colonized/no. tested</th>
<th>No. of dead pigs/no. tested (avg days p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B204 wild type</td>
<td>2/5</td>
<td>+22 (2)</td>
<td>2/5</td>
<td>3/5</td>
<td>1/5 (+20)</td>
</tr>
<tr>
<td>C5 tlyA mutant + B204 wild type</td>
<td>0/5</td>
<td></td>
<td>0/5</td>
<td>3/5</td>
<td>0/5</td>
</tr>
<tr>
<td>B204 wild type</td>
<td>6/6a</td>
<td>+14 (6)</td>
<td>6/6</td>
<td>6/6</td>
<td>2/6 (+25)</td>
</tr>
<tr>
<td>B204 tlyA mutant + B204 wild type</td>
<td>3/6a</td>
<td>+9 (3)</td>
<td>2/6</td>
<td>6/6</td>
<td>1/6 (+9)</td>
</tr>
</tbody>
</table>

a Pigs were considered to have signs of SD if they demonstrated depression, diarrhea, and blood in their stools.

b As detected by fecal shedding and/or the presence of bacteria (wild type) in the colon.

c Control group in study A.

d Control group in study B.

e One pig in each group showed depression and diarrhea but no blood in the feces. SD had to be confirmed by necropsy.
inoculated with the B204 thyA 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 was not very likely.

The data on the shedding of the CS thyA mutant strain could not be interpreted, since in study A plates were kept for only 72 h, and it takes this thyA mutant strain approximately 5 days to grow on ABAP medium. However, the data on the shedding of S. hyodysenteriae B204 indicated that, although the duration of shedding of the thyA mutant strain was shorter than that of the wild-type strain, both the S. hyodysenteriae wild-type strain and the thyA mutant strain were able to colonize the porcine gastrointestinal tract (Table 1). Since S. hyodysenteriae may need the activity of a hemolysin as a scavenger of nutrients both in vitro (16) 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 have recovered naturally from SD usually continue to shed S. hyodysenteriae for a long period and are protected against a subsequent challenge with S. hyodysenteriae (1, 3, 4). 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 thyA mutants were demonstrated to be less virulent for pigs but still able to colonize the porcine gastrointestinal tract and therefore to stimulate the host immune system.

The protection studies undertaken with both S. hyodysenteriae CS and B204 thyA mutants showed that partial protection against a challenge with virulent S. hyodysenteriae B204 was indeed induced. The challenge with B204 was done to test for cross-protection between the two different serotypes in study A and to ensure an effective challenge with a more virulent strain of S. hyodysenteriae. In study A, none of the pigs previously inoculated with the CS thyA mutant displayed clinical signs or lesions typical of SD after a challenge with the B204 wild-type strain, whereas two of five control pigs contracted SD (Table 2). The protection values gained from this study were not statistically significant; there seemed to be an indication of cross-protection from a different challenge serotype, indicating a trend which could be studied further. In study B, all control pigs developed clinical signs and lesions typical of SD after a challenge with the B204 wild-type strain, while only three of six pigs previously inoculated with the B204 thyA mutant contracted SD (Table 2). In this study, both the incidence of SD lesions and the incidence of clinical signs were statistically significantly different when the parent strain group and the thyA mutant strain group were compared. A difference in viability for the inocula of the two B204 challenges may explain the difference in the incidence of SD in the two studies.

Interestingly, the onset of both clinical signs and shedding of S. hyodysenteriae occurred much earlier in the pigs previously inoculated with the B204 thyA 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 observed in the control pigs. A possible explanation for these observations is that the pigs which did contract SD after a challenge with the B204 wild-type strain may have had minor lesions caused by other virulence factors introduced by inoculation with the B204 thyA mutant strain. Such lesions may have allowed the wild-type strain to colonize and induce clinical signs faster. However, since the immune systems 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 shorter in duration and less severe than in the control pigs.

These studies indicated that the thyA-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 thyA-encoded hemolysin. Moreover, it was demonstrated that colonization with S. hyodysenteriae thyA mutants provides partial protection from a challenge with a virulent S. hyodysenteriae strain. Since the experimental challenge inoculum of wild-type B204 was extremely high (10⁹ to 10¹⁰ CFU) in this study, more protection may be afforded under natural challenge conditions.

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