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Partial Characterization of *Streptococcus suis* Type 2 Hemolysin†

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***Streptococcus suis* type 2 was evaluated for hemolysin production. Supernatants of *S. suis* type 2 grown in Todd-Hewitt broth were assayed for hemolytic activity by a photometric assay. Twenty-two additional serotypes of *S. suis* (1, 3 to 22, and 1/2) were evaluated for hemolysin production; nine of them (1/2, 1, 4, 5, 14, 15, 17, 19, and 20) were positive. The effects of temperature, atmosphere, centrifugation, sonication, chemicals, bovine serum albumin, fetal calf serum, and enzymes on *S. suis* type 2 hemolysin activity were studied. Maximum hemolysis occurred after incubation in RPMI 1640 medium at 40°C in 6% CO_2 and after growth in Todd-Hewitt broth at 37°C under anaerobic conditions. Hemolytic activity was absent after the addition of fetal calf serum and decreased after the addition of trypsin or amylase. However, treatment of erythrocytes with amylase or trypsin prior to incubation with supernatant also resulted in a decrease in hemolytic activity. The addition of bovine serum albumin caused increased hemolytic activity. Dipyridyl and EDTA had negligible effects on hemolysis. Hemolytic *S. suis* type 2 culture supernatant injected intraperitoneally failed to cause death in BALB/c mice. Data from our study indicate that *S. suis* type 2 hemolysin is a secreted or loosely cell bound, thermolabile molecule whose activity is growth condition dependent.**

Streptococcus suis type 2 is a frequent cause of arthritis, meningitis, pneumonia, and septicemia in swine and, less commonly, is associated with endocarditis, encephalitis, polyserositis, abortions, and abscesses (1, 4, 5). Of the 30 capsular serotypes of *S. suis*, type 2 is associated most often with disease in swine (1, 4). In humans, *S. suis* type 2 causes meningitis or septicemia followed by permanent deafness and also has caused death in pork meat handlers (1, 22). The economic impact of *S. suis* on the swine industry is substantial (5) and has been the basis for extensive research efforts on the prevention and control of *S. suis* infections. Because the use of antibiotics in feed and drinking water has not successfully controlled the disease (5) and vaccination against the disease also has been largely unsuccessful (12), more effective measures of prevention and control are needed.

Little is known about the virulence factors and protective antigens of *S. suis* type 2. Two virulence markers have been described: a cell wall-associated protein (muramidase-released protein) and an extracellular protein (extracellular factor) (26). A recent study suggested that the gene coding for the pathogenic extracellular protein factor may have evolved from a similar, but larger, gene and that one recombination event could cause a weakly pathogenic *S. suis* strain to become pathogenic (24). Sialic acid in the polysaccharide capsule was hypothesized to promote the invasiveness of *S. suis* type 2 (7, 15). Additionally, surface fimbriae have been described on *S. suis*, but their function is unknown (15).

Hemolysin activity is associated with the virulence and pathogenicity of several gram-positive and gram-negative bacteria (6, 10, 16, 20, 25). The significance of the *S. suis* hemolysin as a virulence factor or protective antigen is not known. The purpose of the study described here was partial characterization of the *S. suis* type 2 hemolysin as a preliminary step toward investigation of its virulence or protective properties.

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MATERIALS AND METHODS

Bacterial strains. Twenty-three serotypes (1 to 22 and 1/2) of *S. suis* recovered from pigs in Europe and North America were screened for hemolytic activity (Table 1). Stock cultures were maintained on glass beads in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, Mich.) at −70°C.

Preparation of hemolysin. Two methods were used to grow *S. suis* for evaluation of hemolysin production. In the first method, single-colony isolates grown on tryptic soy agar (TSA) plates (Difco) containing 5% sheep blood (24 h at 37°C in 6% CO_2) were transferred to THB containing 0.6% yeast extract (Difco). Broth cultures were incubated at 40°C to an A_{540} of 0.9 and were then diluted 1:10 in THB. This suspension was then incubated aerobically for an additional 16 h at 40°C.

In the second method, single-colony isolates were grown on sheep blood agar plates as described above, subcultured onto TSA plates, and incubated in 6% CO_2 for 16 h at 37°C. Colonies were harvested from TSA plates with cold RPMI 1640 medium (RPMI; GIBCO Laboratories, Grand Island, N.Y.) and were transferred to an ice-chilled flask. The RPMI suspension was incubated aerobically for 1 h at 40°C.

Cultures were centrifuged at 12,000 × *g* for 10 min (4°C), and the supernatant was filtered through a 0.45-μm-pore-size nylon filter (Micron Separations Inc., Westboro, Mass.). Supernatants were chilled on ice, and all hemolysin assays were conducted immediately following collection of the chilled supernatants. The remaining supernatant was stored at −70°C unless stated otherwise.

Kinetics of hemolysin production. In order to determine the conditions necessary for maximum hemolysin production, the kinetics of hemolysin production over a 48-h period were investigated. THB was inoculated with *S. suis* type 2 as described above, and the mixture was incubated aerobically for 48 h at 40°C. Ten-milliliter aliquots of *S. suis* culture were collected before incubation (0 h) and at 6, 12, 18, 24, 30, 36,

and 48 h for colony counts and determination of hemolysin activity.

Hemolysin assay. Culture supernatants were added in triplicate to round-bottom, 96-well titer plates in the following amounts: 2, 5, 10, 15, 20, 30, 50, 70, and 100 μl. None was added to control wells. The volume of each well was standardized to 100 μl with uninoculated RPMI or THB.

Sheep erythrocytes were washed twice with 0.9% NaCl and were resuspended in citrate-phosphate-glucose buffer (osmolarity, 280 to 300) to a hematocrit of 10% for the hemolysin assay. One hundred microliters of stock sheep erythrocytes, diluted to a hematocrit of 1% with 10 mM Tris buffer in 0.9% NaCl, was added to each well. Plates were incubated at 37°C (6% CO_2) for 2 h. Unlysed erythrocytes were removed by centrifugation at 100 × *g* for 10 min. The resultant supernatant was transferred to a flat-bottom, 96-well titer plate for spectrophotometric analysis at A_{410}. Nonhemolytic controls consisted of 100 μl of Tris buffer or growth medium (RPMI or THB) and 100 μl of 1% sheep erythrocytes. Maximum levels of hemolysis were determined by lysing sheep erythrocytes with 100 μl of water. One hemolytic unit (HU) was defined as the greatest dilution of supernatant that resulted in 50% hemolysis of 1 ml of 1% sheep erythrocytes.

Effects of incubation temperatures, incubation times, and oxygen tension on hemolysin activity. The temperature at which maximal hemolytic activity of *S. suis* type 2 was induced was investigated. Organisms were incubated at 4, 24, 37, 40, and 43°C in RPMI and at 24, 37, 40, and 43°C in THB. The effect of incubation time on hemolytic activity was determined. *S. suis* type 2 colonies suspended in RPMI were incubated aerobically for 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 h at 40°C. Hemolysin assays were conducted as described above.

To evaluate the effect of oxygen on hemolytic activity, *S. suis* type 2 cultures (in RPMI or THB) were divided into three fractions and the fractions were incubated at 37°C aerobically, anaerobically, or aerobically with 6% CO_2, respectively.

Effects of sonication and centrifugation of whole-cell cultures. The effect of centrifugation on the hemolytic activity of the *S. suis* type 2 supernatant was determined by varying the centrifugation time and speed as follows: 1,000 × *g* for 10 min, 1,000 × *g* for 30 min, or 12,000 × *g* for 10 min. To determine the effect of sonication on the hemolytic activity of the *S. suis* type 2 supernatant and to aid in the determination of the cellular location of the hemolysin, the following suspensions were assayed for hemolytic activity: supernatant from nonsonicated cells, supernatant from sonicated cells, supernatant from RPMI-washed pellets, and supernatant from RPMI-washed and sonicated pellets. Sonication of *S. suis* was performed for 10 min at 80% of maximum output in a Vibra cell sonicator (Sonics and Materials Inc., Danbury, Conn.).

Effects of medium supplements and chemicals on hemolysin production or activity. Various medium supplements and chemicals were added to the cell-free culture supernatant prior to assay and were evaluated for their effects on hemolysin activity. These included 1, 5, and 7% bovine serum albumin (BSA; Fisher Biotech, Fair Lawn, N.Y.) in RPMI, 5% fetal calf serum (FCS; Flow Laboratories, McLean, Va.) in RPMI or THB, 6.0% EDTA (Fisher) in THB, and 0.2 M dipyridyl (Sigma) in THB. All media were inoculated and incubated as described above. The hemolysin activities of the culture supernatants were determined immediately following collection (0 h); BSA-supplemented samples, however, were also evaluated after 12, 24, 48, and 96 h at 24°C. The hemolysin activities of unsupplemented culture supernatants were determined for comparison with the activities of each of the supplemented supernatants.

TABLE 1. Serotype designation, origin, sources, and hemolytic activity of *S. suis*

Serotype	Origin	Source[a]	HU
1/2	North America	M. M. Chengappa	625
1	North America	M. M. Chengappa	666
2	North America	M. M. Chengappa	666
3	North America	M. M. Chengappa	0
4	North America	M. M. Chengappa	83
5	North America	M. M. Chengappa	17
6	North America	M. M. Chengappa	0
7	North America	M. M. Chengappa	0
8	North America	M. M. Chengappa	0
9	North America	M. M. Chengappa	0
10	Denmark	J. Henrichsen	0
11	Denmark	J. Henrichsen	0
12	Denmark	J. Henrichsen	0
13	Denmark	J. Henrichsen	0
14	Denmark	J. Henrichsen	100
15	Denmark	J. Henrichsen	111
16	Denmark	J. Henrichsen	0
17	Denmark	J. Henrichsen	20
18	Denmark	J. Henrichsen	0
19	Denmark	J. Henrichsen	50
20	Denmark	J. Henrichsen	20
21	North America	M. M. Chengappa	0
22	North America	M. M. Chengappa	0

[a] M. M. Chengappa, College of Veterinary Medicine, Kansas State University, Manhattan; J. Henrichsen, State Serum Institute, Copenhagen, Denmark.

Effect of enzymes on hemolysin activity. *S. suis* type 2 cultures were harvested from TSA plates with RPMI containing 0.03% (wt/vol) trypsin or 0.06% (wt/vol) amylase and were incubated aerobically at 40°C for 1 h. Following incubation, hemolysin activity was measured as described above. To determine the effects of enzymes on erythrocytes, sheep erythrocytes were treated with 0.03% trypsin or 0.06% amylase for 2 h, washed three times with 0.9% NaCl, and used in the hemolysin assay.

Effect of temperature on stability of hemolytic activity. The effects of ambient temperatures (−70, 4, and 24°C) on the hemolytic activities of the supernatants were evaluated. Hemolysin assays on supernatants incubated at 4 and 24°C were conducted at 3, 6, 8, and 24 h; assays on supernatants stored at −70°C were conducted at 1, 2, and 3 weeks and compared with the results of the assays done before incubation and storage.

Mouse inoculation. The pathogenicity of the *S. suis* type 2 supernatant was evaluated. Groups of five BALB/c mice each were inoculated intraperitoneally with 1.5 ml of *S. suis* type 2 supernatant (999 HU), *S. suis* type 2 culture (999 HU), or RPMI (0 HU). Mice were observed for 1 week postinoculation for clinical signs of disease and death.

RESULTS

Hemolytic activities of *S. suis* types 1 to 22 and 1/2. *S. suis* serotypes 1 to 22 and 1/2 caused alpha-hemolysis on sheep blood agar plates. All 23 serotypes were screened for their hemolytic activities, and 10 serotypes (1/2, 1, 2, 4, 5, 14, 15, 17, 19, and 20) showed hemolytic activity. The HUs varied among serotypes; serotypes 1/2, 1, and 2 had the greatest activity (Table 1).

Bacterial growth and hemolysin production. The relationship between bacterial growth in THB and hemolysin activity is shown in Fig. 1. Because *S. suis* type 2 cells from a logarithmic-phase culture were used for inoculation, the lag phase was absent. Maximal hemolysin activity was obtained from cell-free

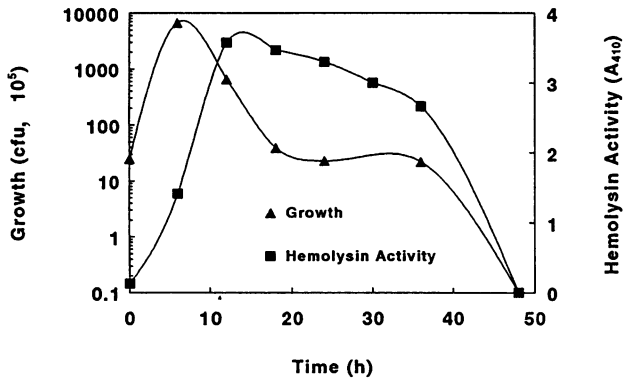

FIG. 1. Hemolysin activity (A_{410}) in relation to the growth of *S. suis* type 2 in THB.

supernatants between 12 and 18 h. No hemolytic activity was observed at 48 h.

Incubation temperature, incubation times, and atmospheric conditions. Maximum hemolytic activity (666 HUs) in THB was obtained from cultures incubated anaerobically at 37°C. Under aerobic conditions, maximum hemolytic activity was obtained from *S. suis* cultures in RPMI at 40°C. Cultures incubated at 37 and 43°C had slightly less activity, while cultures incubated at 4, 24, and 30°C showed substantially decreased hemolytic activity (Table 2). In THB, maximum hemolytic activity was obtained from cultures incubated at 37°C, and the activities from the cultures incubated at 30, 40, and 43°C were slightly lower. Both THB and RPMI cultures, incubated at 37°C, had greater hemolytic activity when they were grown in 6% CO_2 than when they were grown under aerobic conditions without CO_2. Cultures suspended in RPMI and incubated aerobically had hemolytic activity that peaked at 1 h. A longer incubation time caused a sharp decline in activity.

Sonication and centrifugation studies. With regard to whole-cell cultures, an increase in the speed of centrifugation (from 1,000 to 12,000 × *g* for 10 min) or an increase in the time of centrifugation (from 10 to 30 min at 1,000 × *g*) resulted in increased hemolysin activity, from 512 to 666 HUs and from 512 to 538 HUs, respectively. However, no hemolysin activity was detected in supernatants from sonicated cultures or from washed and sonicated cultures.

Effect of medium supplements and chemicals. The addition of dipyridyl or EDTA to THB cell-free culture supernatant did not change the activity of hemolysin, whereas hemolysin activity could not be detected after the addition of 5% FCS. Adding 5% FCS after the addition of erythrocytes had no effect on hemolysin activity. However, if 5% FCS was added to THB before inoculation, an increase in the number of *S. suis* type 2 CFU was noted.

TABLE 2. Hemolysin production at different incubation temperatures under aerobic conditions

Incubation (°C)	HU	
	THB	RPMI
4	0	143
24	100	91
30	80	22
37	125	222
40	111	666
43	0	333

TABLE 3. Effect of BSA on the stability of hemolysin activity at room temperature (24°C)

% BSA	HU at the following incubation times[a]:			
	0 h	12 h	24 h	48 h
0	144	0	0	0
1	100	83	12.5	0
5	666	83	25	14
7	666	83	0	0

[a] No hemolysin activity was detected at 96 h.

The addition of 5% BSA to RPMI cell-free culture supernatant resulted in higher hemolysin activity at 0 h and increased stability through 48 h when compared with the hemolysin activity in supernatants without BSA or containing 1% BSA (Table 3). Similar activity was detected from supernatants containing 7% BSA at 0 and 12 h, but no activity was detected at 24 h. Hemolysin activity was not detected in any culture supernatants at 96 h.

Effects of enzymatic treatments. Supernatants containing amylase or trypsin had no hemolytic activity. When erythrocytes were pretreated with amylase or trypsin and were then used to assay hemolysin-containing supernatants, no lysis of amylase-treated erythrocytes occurred, and decreased lysis occurred with trypsin-treated erythrocytes.

Stability of hemolysin at −70, 4, and 24°C. Hemolytic activity was maintained at a higher level at 4°C than at 24°C for 24 h. After 24 h, activity dropped from 500 to 83 HUs at 4°C, while activity dropped from 500 to 20 HUs at 24°C.

Mouse lethality. No clinical signs were apparent after intraperitoneal injection of *S. suis* type 2 supernatant containing hemolysin to mice. Intraperitoneal injections of whole-cell preparations of *S. suis* type 2 from the same culture caused death within 5 days in all mice.

DISCUSSION

In the study described here, hemolysin activity was detected in 10 of 23 *S. suis* serotypes. Of these 10, serotypes 1/2, 1, and 2 had similar hemolytic activities under identical growth and assay conditions. Maximum hemolytic activity was noticed in RPMI incubated at 40°C in 6% CO_2 and in THB incubated anaerobically at 37°C. Whether or not modification of the hemolysin assay or growth conditions could result in the detection of hemolytic activity from the 12 serotypes that did not hemolyse sheep erythrocytes needs further study.

Various laboratory media have been used to obtain hemolysin in the culture supernatant for a number of different organisms (3, 6, 17, 27). Generally, defined media are recommended for the production and characterization of bacterial hemolysins. Because *S. suis* does not grow well in unsupplemented RPMI, cells were grown on TSA plates for 16 h before being transferred to RPMI for a final 1 h of incubation.

The nonproportional relationship between *S. suis* type 2 numbers and hemolysin activity in THB was greatest during the death phase, when hemolysin was released from dying cells. Similar results were reported for *Helicobacter pylori* hemolysin (27). Conversely, *Streptococcus pyogenes*, *Escherichia coli*, and *Proteus penneri* produced the greatest amount of hemolysin during the exponential phase of growth (3, 9, 23). *S. suis* type 2 hemolysin appears to be heat labile, because hemolytic activity in a noncooled supernatant decreased rapidly and was

not detectable after 24 h at 24°C. Heat-labile hemolysins are well documented for *Pseudomonas aeruginosa* (21) *Clostridium botulinum* (10), *Aeromonas hydrophila* (11), *Actinobacillus pleuropneumoniae* (17), *Pseudomonas pseudomallei* (2), and *E. coli* (3).

S. suis type 2 hemolysin is most likely secreted or loosely cell bound rather than tightly cell bound or intracellularly located, because hemolysin activity was not detected in washed, sonicated, or washed and sonicated cells. Sonication or heat resulting from sonication may break down any hemolysin that may be present. Alternatively, hemolysin may be continually released by *S. suis*, but it may be rapidly inactivated unless it is associated with carrier molecules such as starch or proteins, as is the case for certain streptococci (19). Hemolysin may exist as a loosely bound molecule, because increased centrifugation speed or time resulted in greater hemolysin activity. Hughes (13) showed that centrifugation of cells can stimulate the release of metabolites and that centrifugation of virus-infected cultures can increase cytopathic effects.

BSA aids in the stabilization of hemolysins of streptococci and *Helicobacter pylori* (19, 27), as was the case for *S. suis* type 2. Although 7% BSA seemed to stabilize the hemolysin activity early, the loss of activity at later sampling times may have been a result of blocking of hemolysin-binding sites because of the presence of undissolved BSA. Although BSA stabilized hemolysin activity in RPMI, it was not used in all experiments because it could interfere with hemolysin characterization. No hemolysin activity was detected when FCS was added to the *S. suis* supernatant prior to the addition of erythrocytes. This was unexpected, because serum stabilizes the hemolysins of the group B streptococci, *H. pylori*, and *S. pyogenes* (9, 19, 27). Supernatants to which amylase or trypsin was added had no hemolytic activity. However, sheep erythrocytes incubated with amylase or trypsin were less susceptible to lysis in the hemolysin assays, suggesting that the erythrocyte receptor for *S. suis* hemolysin is a glycoprotein. Protease treatment of cytolysin-erythrocyte intermediates inhibited subsequent hemolysis (18). The *S. suis* hemolysin, like many other bacterial hemolysins (8, 10), is probably a protein. The lack of a change in hemolysin activity after the addition of dipyridyl or EDTA to cell-free supernatants suggests that neither calcium nor iron is necessary for hemolysin activity.

Mice inoculated intraperitoneally with virulent strains of *S. suis* type 2 died within 2 to 7 days. However, supernatant containing hemolysin activity, injected intraperitoneally, failed to kill mice. Whether or not concentrated hemolysin would be lethal remains to be elucidated.

Previous studies have demonstrated that hemolysins can be important virulence and antigenic factors (16, 25). Listeriolysin O, a hemolytic exotoxin related to streptolysin O, is a 58-kDa protein and a major determinant of the virulence of *Listeria monocytogenes* (20). Pigs immunized with the purified 104-kDa hemolysin from *A. pleuropneumoniae* serotype 1 were protected against challenge (6). In addition, pigs immunized with *A. pleuropneumoniae* serotype 5 survived a challenge with the wild-type strain, whereas those immunized with a nonhemolytic mutant from the parent strain were not protected (14). Recently, the proteins of the 110-kDa extracellular protein factor, which was shown to be important in the pathogenicity and virulence of *S. suis* type 2, have been identified (26).

The precise role of *S. suis* hemolysin in disease can be defined more clearly by additional studies with purified hemolysin to determine its toxic, virulence, and immunogenic properties. Demonstration and characterization of *S. suis* type 2 hemolysin or a hemolysin-like substance have not been reported previously. The intent of the present study was to determine the factors that would enhance hemolysin activity and enable its purification and further characterization.

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