INFECTION AND IMMUNITY, May 1988, p. 1070–1075
0019-9567/88/051070-06$02.00/0

Vol. 56, No. 5

Identification of the Major Antigens of *Treponema hyodysenteriae* and Comparison with Those of *Treponema innocens*

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Received 5 November 1987/Accepted 7 February 1988

Eleven strains of *Treponema hyodysenteriae* isolated from pigs with swine dysentery were examined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. *T. hyodysenteriae* strains formed a homogeneous group with respect to sodium dodecyl sulfate-soluble proteins. However, immunoblotting with antiserum from rabbits immunized with *T. hyodysenteriae* CN8368 revealed heterogeneity among the lipopolysaccharide complexes of different strains. Polypeptides of molecular weights between 30,000 and 36,000 were the predominant *T. hyodysenteriae* polypeptides detected by porcine immune serum. In contrast, *Treponema innocens* did not form a homogeneous group with respect to sodium dodecyl sulfate-soluble proteins. Adsorption studies and immunoblotting identified polypeptide antigens present on cells of *T. hyodysenteriae* which were not detected on cells of *T. innocens*. These unique antigens may play a role in the virulence of *T. hyodysenteriae*.

Treponema hyodysenteriae is the etiological agent of swine dysentery (SD) (4, 8, 23). The disease occurs post-weaning in pigs between 5 and 16 weeks of age and is most common in fattening units where pigs are kept in large groups. It may also occur in breeding herds and affect sows and suckling pigs. After infection with *T. hyodysenteriae*, swine frequently develop mucohemorrhagic diarrhea, which is characterized postmortem by extensive necrosis of the mucosal epithelium of the colon and cecum. This results in dehydration, emaciation, and rapid weight loss, followed in severe cases by death (1, 20). Another group of spirochetes has been isolated from healthy swine and those with post-weaning scours. These spirochetes are morphologically similar to *T. hyodysenteriae* and cross-react serologically with *T. hyodysenteriae* (6). However, they do not cause the clinical symptoms or the lesions typical of SD in swine challenged experimentally (15). This group of commensal spirochetes was proposed as a new species, called *Treponema innocens*, by Kinyon and Harris (14).

Although *T. hyodysenteriae* is recognized as the primary cause of SD, very little is known about the mechanism of virulence involved. However, several factors have been identified which may be associated with the virulence of *T. hyodysenteriae*. In common with other gram-negative bacteria, *T. hyodysenteriae* possesses a hot phenol-water-extractable antigen that has been referred to as a lipopolysaccharide (LPS) (2). This LPS has been shown to be biologically active (18) and has been implicated in the pathogenicity of *T. hyodysenteriae* (19). The LPS has also been used for serological classification of the organism (2, 17). There is some evidence to suggest that protection against infection with *T. hyodysenteriae* is serotype specific (13). *T. hyodysenteriae* grown in vitro produces a hemolysin which causes complete hemolysis on blood agar plates. Most strains exhibiting this hemolytic pattern are virulent in swine (15). In contrast, *T. innocens* strains do not exhibit complete hemolysis and are avirulent in swine (15). There is also evidence that the hemolysin of *T. hyodysenteriae* has cytotoxic activity (K. A. Kent and R. M. Lemcke, Proc. 8th Int. Pig. Vet. Conf. 1984, p. 185). To date, there is no evidence that *T. hyodysenteriae* produces an enterotoxin. Other antigens not yet identified may also be involved in virulence and protection.

A recent study using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting has identified a number of *T. hyodysenteriae* antigens, some of which are shared with *T. innocens* (12). The object of the present study was to examine and compare a larger number of isolates of *T. hyodysenteriae* and *T. innocens* by using biochemical and immunochemical techniques to identify and characterize antigens that may be involved in protection or that may be associated with the virulence of *T. hyodysenteriae*.

MATERIALS AND METHODS

Bacterial strains. *T. hyodysenteriae* CN8368 (Wellcome Culture Collection) was originally isolated from a pig with experimentally induced SD. Isolates B78, B204, and B169 were obtained from the National Animal Disease Center, Ames, Iowa. Strains S73 and S75 were donated by R. J. Lysons, Institute for Research on Animal Diseases, Compton, England. Strains 2805, MB, I19, and I13 were isolated by the authors from outbreaks of naturally occurring SD in the United Kingdom. Isolate S2 was provided by D. Hunter, Veterinary Investigation Centre, Leeds, England. All isolates of *T. hyodysenteriae* used in this study exhibited complete hemolysis on sheep blood agar plates. The serotypes of these isolates are unknown, with the exception of B78, B204, and B169, which are representatives of serotypes I, II, and III, respectively. All strains of *T. innocens* were isolated from healthy pigs in the United Kingdom at Wellcome Research Laboratories and exhibited incomplete hemolysis on sheep blood agar plates.

Cultivation. Cultivation on solid medium used blood agar base no. 2 (Oxoid Ltd., Hampshire, England) containing defibrinated sheep blood (GIBCO, Middlesex, England) to a

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final concentration of 5% (vol/vol). Plates were incubated in an atmosphere of 95% H_2 and 5% CO_2 at 37°C for 48 h. For liquid cultivation, Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 0.1% (wt/vol) sodium pyruvate (BDH, Poole, England) and heat-inactivated (56°C for 30 min) porcine serum to a final concentration of 5% (vol/vol) was used. Cultures were grown in a shaking incubator (orbital, 200 rpm) at 37°C for 20 h in an atmosphere of 88% N_2, 7% H_2, and 5% CO_2.

SDS-PAGE. Samples for electrophoresis were prepared by removing cells from a blood agar plate, washing them twice in 0.01 M phosphate-buffered saline (pH 7.2), and solubilizing 10^{10} cells in 0.5 ml of 0.0625 M Tris hydrochloride buffer (pH 6.8) containing 3% (wt/vol) SDS, 5% (vol/vol) mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% (wt/vol) bromophenol blue. Samples were boiled for 5 min before 50-μl samples were loaded onto gels containing 12.5% polyacrylamide. For cell envelope preparations, 50 μg of protein was loaded into each well. Samples were separated by using the method of Laemmli (16). Gels were stained with either 0.25% (wt/vol) PAGE blue 83 (BDH) or silver nitrate by the method of Hitchcock and Brown (5) to visualize LPSs. Molecular weights were estimated by using SDS markers (Sigma Chemical Co., St. Louis, Mo.).

Immunoblotting. Polypeptides separated in gels were transferred electrophoretically onto nitrocellulose membranes (pore size, 0.4 μm; Schleicher & Schuell, Dassel, Federal Republic of Germany) according to the method of Towbin et al. (24) at 110 mA for 18 h by using a Bio-Rad Transblot cell (Bio-Rad, Hertfordshire, England). The blotting buffer contained 20% (vol/vol) methanol, 20 mM Tris hydrochloride (pH 8.3), and 150 mM glycine. Nitrocellulose sheets were subsequently blocked in 10 mM Tris hydrochloride (pH 7.4) containing 0.85% (wt/vol) sodium chloride (Tris-saline) and 5% (wt/vol) bovine serum albumin fraction V (Sigma) for 3 h at room temperature. The nitrocellulose membranes were then incubated in antisera diluted 1:100 in Tris-saline for 2 h at room temperature, followed by washes for 15 min in Tris-saline, 15 min in Tris-saline containing 0.01% (vol/vol) Nonidet P-40 (BDH), and 15 min in Tris-saline. Membranes were then incubated for 2 h in a solution containing 0.33% (vol/vol) protein A conjugated to horseradish peroxidase (stock, 0.5 mg suspended in 2 ml of Tris-saline; Sigma), and the three washes were repeated as before. The immunoblots were developed in a solution containing 10 ml of 0.6% (wt/vol) 4-chloro-1-naphthol (Sigma) freshly prepared in methanol, 90 ml of Tris-saline, and 0.05 ml of hydrogen peroxide. Bands developed within 0.5 min, and the reaction was stopped by immersion in distilled water.

Antisera. Antiserum to *T. hyodysenteriae* CN8368 was raised in New Zealand White rabbits by using whole cells washed in 0.85% (wt/vol) saline, adjusted to 10^8 cells per ml (counting chamber from Gallenkamp, Leicestershire, England), and preserved with 0.01% (wt/vol) thimerosal. Rabbits were inoculated intravenously with 0.5 ml on day 1, 1 ml on day 5, 1.5 ml on day 8, and 2 ml on days 12, 15, 20, 24, 29, 33, and 38. Rabbits were exsanguinated on day 52, and the pooled serum was reduced to the immunoglobulin G fraction by using the rivanol precipitation method of Walker et al. (25). The final protein concentration of the fraction was 16 mg/ml, determined by the method of Redinbough and Campbell (22).

Each of five pigs at 3 to 4 weeks of age was vaccinated with 1 ml of formolized *T. hyodysenteriae* CN8368 in oil adjuvant (3). This vaccine was administered intramuscularly on a single occasion. The challenge was done 4 weeks postvaccination with virulent *T. hyodysenteriae* CN8368. Blood was collected immediately before challenge, and sera were stored at −20°C until required. Serum from one representative animal was used in this study.

Cell envelope preparation. Cell envelopes were prepared by the method of Owen et al. (21). Washed cells (20 ml) adjusted to 10^{10} cells per ml (Gallenkemp counting chamber) were suspended in 20 ml of 10 mM Tris hydrochloride (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (Sigma) and DNase and RNase (Sigma), adjusted to a final concentration of 0.05 mg/ml. A small sample was retained for comparison. Cells were disrupted at 10,000 lb/in^2 by using a prechilled pressure cell (Stanstead; Fluid Power Ltd., Stanstead, Essex, England) at 4°C. Intact cells were removed by centrifuging them twice at 7,000 × *g* for 20 min at 4°C. Cell envelopes were harvested by centrifugation at 48,000 × *g* for 1 h at 4°C. The envelopes were then washed four times in 10 mM Tris hydrochloride (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride and were finally suspended in 20 ml of washing buffer. The protein concentration was estimated to be 5.4 mg/ml by using the method of Redinbough and Campbell (22).

Adsorption of antisera. *T. innocens* (strains BL and SM) were grown in liquid medium for 18 h at 37°C. Aliquots (0.2 ml) were spread on sheep blood agar plates and incubated for 72 h. Cells were harvested from plates and washed twice in phosphate-buffered saline before they were divided into three aliquots. Each strain was used to separately adsorb 1.5 ml of the immunoglobulin G fraction of antiserum (raised against *T. hyodysenteriae* CN8368 in rabbits) three times for 1 h at 37°C on a rotator. A sample from each adsorption step was retained for comparison.

RESULTS

SDS-soluble protein and immunoblot profiles of *T. hyodysenteriae*. SDS-soluble proteins from 11 strains of *T. hyodysenteriae* isolated from pigs with SD in the United Kingdom and United States were analyzed by SDS-PAGE (Fig. 1). Each of the strains possessed a similar polypeptide profile, including representatives of serotypes I, II, and III (lanes A to C). Immunoblotting with an immunoglobulin G enriched fraction of hyperimmune rabbit serum raised against whole *T. hyodysenteriae* CN8368 cells revealed a similar immunoblot profile for each strain with respect to polypeptides (Fig. 2), indicating that each strain of *T. hyodysenteriae* possesses similar major polypeptide antigens. Differences between strains were demonstrated by the reaction of antibody with antigen in the 14,000- to 24,000-molecular-weight region. This antigen is the LPS complex of *T. hyodysenteriae* (unpublished results). The LPSs of *T. hyodysenteriae* strains B78, B204, and B169 (representatives of serotypes I, II, and III) and S73 and S75 did not react with the hyperimmune rabbit serum raised against *T. hyodysenteriae*, demonstrating immunological heterogeneity in the LPSs of these strains. Silver staining of the polyacrylamide gels revealed that these strains did possess LPS complexes (data not shown).

Figure 3 shows the SDS-soluble proteins from 11 strains of *T. hyodysenteriae*, reacted with antiserum from a pig taken 4 weeks postvaccination with an experimental *T. hyodysenteriae* CN8368 vaccine. The vaccine protected the pig against a severe experimental challenge with *T. hyodysenteriae* CN8368 (data not shown). This antiserum contains antibodies to a number of polypeptides, but it reacted

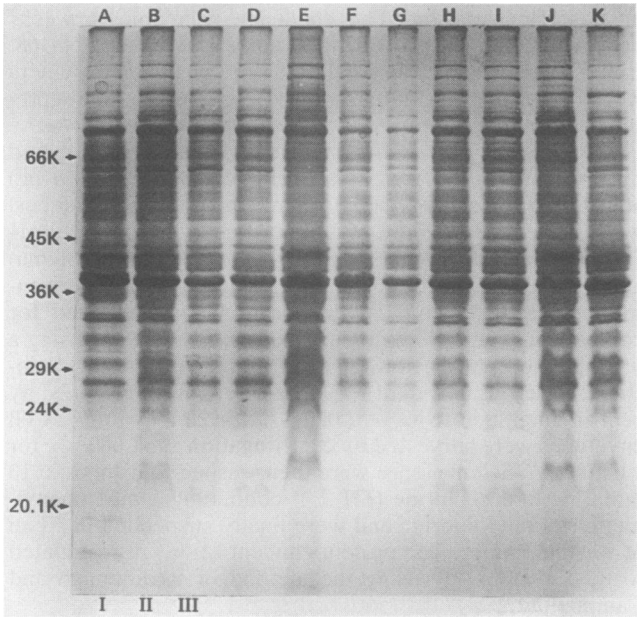

FIG. 1. Comparison of SDS-solubilized *T. hyodysenteriae* strains separated by SDS-PAGE and stained with Coomassie blue to visualize protein. Lanes: A, B78; B, B204; C, B169; D, S73; E, S75; F, 2805; G, MB; H, I19; I, I13; J, S2; K, CN8368. Molecular weight markers are indicated on the left. I, II, and III at the bottom of the gel indicate that these strains are representative of three serotypes of *T. hyodysenteriae*.

predominantly with antigens in the 29,000- to 45,000-molecular-weight region. Each of the different *T. hyodysenteriae* isolates possessed conserved antigens in the 30,000- to 36,000-molecular-weight region. The immune pig serum exhibited a much weaker reaction with LPS than hyperimmune

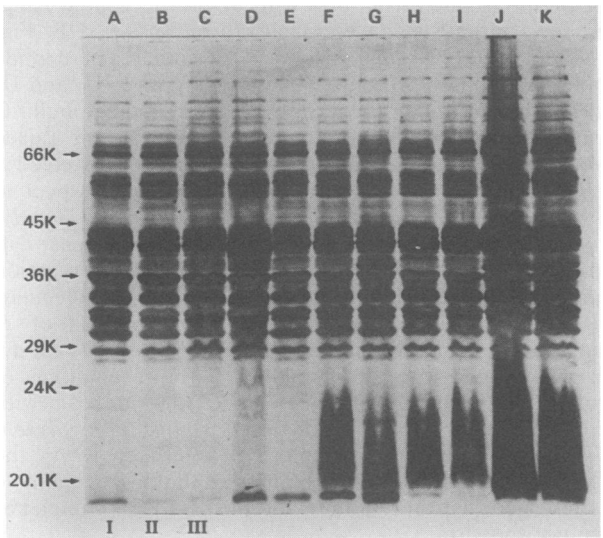

FIG. 2. Identification of antigens from *T. hyodysenteriae* strains by using immunoblotting. The antiserum is an immunoglobulin G fraction of serum raised against *T. hyodysenteriae* CN8368 in rabbits. Lanes: A, B78; B, B204; C, B169; D, S73; E, S75; F, 2805; G, MB; H, I19; I, I13; J, S2; K, CN8368. Molecular weight markers are indicated on the left. I, II, and III at the bottom of the gel indicate that these strains are representative of three serotypes of *T. hyodysenteriae*.

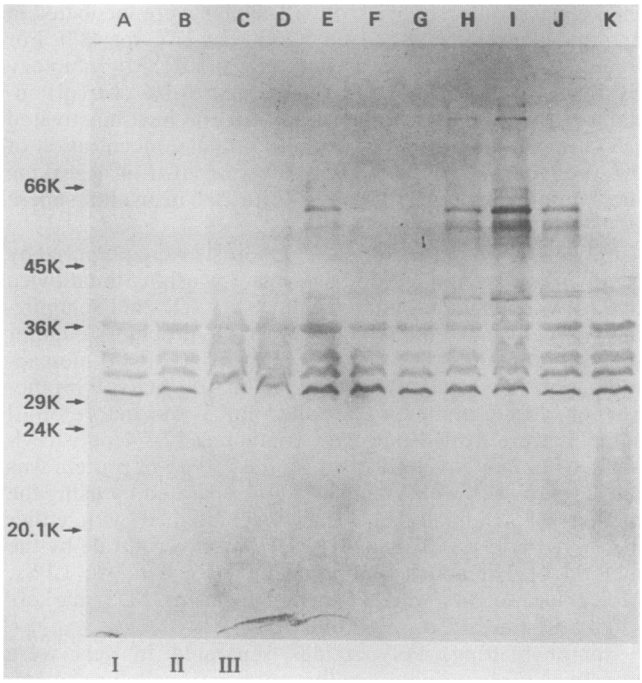

FIG. 3. Identification of antigens of *T. hyodysenteriae* with serum from a vaccinated pig resistant to experimental challenge. Lanes: A, B78; B, B204; C, B169; D, S73; E, S75; F, 2805; G, MB; H, I19; I, I13; J, S2; K, CN8368. Molecular weight markers are indicated on the left. I, II, and III at the bottom of the gel indicate that these strains are representative of three serotypes of *T. hyodysenteriae*.

rabbit serum did after immunoblotting. Normal swine serum from the same pig did not recognize antigens of *T. hyodysenteriae* (data not shown).

Cell fractionation of *T. hyodysenteriae*. Cells of *T. hyodysenteriae* CN8368 were fractionated to isolate cell envelopes. Figure 4 shows the SDS-soluble proteins of unfractionated cells (lane A), the soluble cell fraction (lane B), and cell envelopes (lane C), visualized by Coomassie blue staining. The cell envelope fraction was enriched for polypeptides in the 24,000- to 45,000-molecular-weight region. Immunoblotting revealed that the conserved antigens of molecular weights between 30,000 and 36,000 were predominantly associated with the cell envelope (lane D). A number of additional polypeptides in the cell envelope preparation were recognized weakly by the immune pig serum. Presumably, these polypeptides were present in higher concentrations in the cell envelope preparation than in whole cells.

SDS-soluble protein profile of *T. innocens*. SDS-soluble proteins from eight strains of *T. innocens* were analyzed by SDS-PAGE (Fig. 5) and stained with Coomassie blue. Unlike *T. hyodysenteriae* isolates, *T. innocens* strains did not present a conserved polypeptide profile. In addition, marked differences were apparent between *T. innocens* and the virulent strain of *T. hyodysenteriae* CN8368.

Adsorption of *T. hyodysenteriae* antisera with *T. innocens*. Immunoglobulin G-enriched fractions of antiserum raised in rabbits against *T. hyodysenteriae* CN8368 were adsorbed independently with two strains of *T. innocens*, either BL or SM. Figure 6 shows the immunoblot profiles of *T. hyodysenteriae* CN8368 (lane A) and *T. innocens* (lanes B and C) after reaction with the adsorbed and unadsorbed antisera. The immunoblots with unadsorbed antiserum demonstrated that

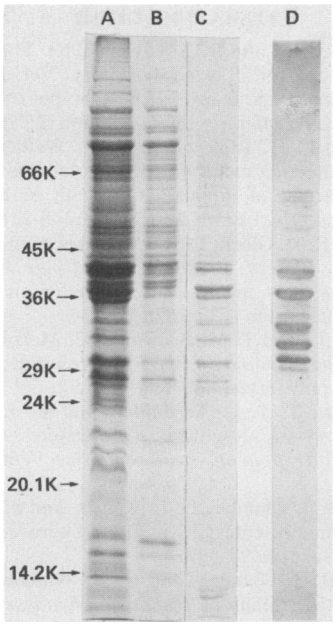

FIG. 4. Cell fractionation of *T. hyodysenteriae* and analysis by SDS-PAGE and immunoblotting. Lanes: A, unfractionated cells; B, soluble protein from supernatant after fractionation; C, cell envelope; D, immunoblot of cell envelope with serum from a vaccinated pig resistant to experimental challenge.

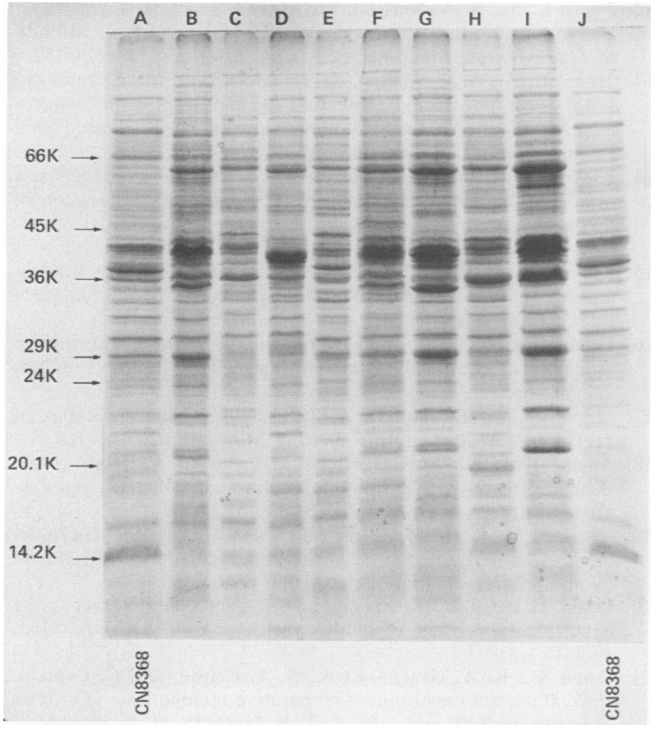

FIG. 5. SDS-soluble proteins from *T. innocens* strains compared with those from *T. hyodysenteriae* CN8368. The protein was separated by SDS-PAGE and stained with Coomassie blue. Lanes: A, CN8368; B, 3625; C, BL; D, 3030; E, PN4; F, 3636; G, 3629; H, SM; I, 916; J, CN8368. Molecular weight markers are indicated on the left.

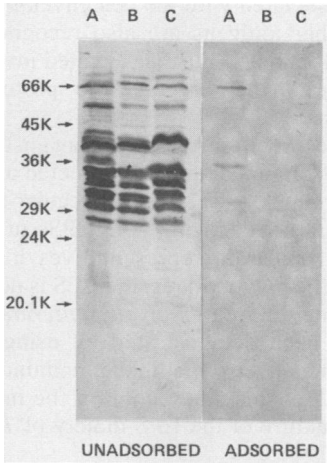

FIG. 6. Reaction of adsorbed antiserum with SDS-soluble proteins of *T. hyodysenteriae* and *T. innocens* after separation by SDS-PAGE and immunoblotting. Lanes: A, CN8368; B, SM; C, BL. Molecular weight markers are indicated on the left.

antibody to *T. hyodysenteriae* cross-reacts with many of the polypeptide antigens possessed by *T. innocens*. After sequential adsorption with whole *T. innocens* BL cells, cross-reactive antibodies were removed, leaving three major polypeptides of *T. hyodysenteriae* that *T. innocens* did not possess. The approximate molecular weights of these polypeptides are 68,000, 36,000, and 31,000. A similar pattern was seen when *T. innocens* SM was used for adsorption.

DISCUSSION

T. hyodysenteriae is a fastidious anaerobic pathogen which has only recently been cultivated in vitro in sufficient quantities to carry out biochemical analysis of cellular structures. This study reports the preliminary characterization of some *T. hyodysenteriae* antigens and cell envelope components and the vaccine-induced humoral immune responses to several antigens in swine after vaccination. *T. hyodysenteriae* strains isolated from several geographical locations in the United States and United Kingdom displayed a conserved polypeptide profile when whole cells were analyzed by SDS-PAGE, irrespective of serotype. This conservation of polypeptides is in contrast to the profiles seen with independent isolates of commensal *T. innocens* spirochetes, which showed heterogeneous polypeptides. Thus, *T. hyodysenteriae* may represent a relatively conserved or related group of spirochetes. This observation is in line with other characteristics of *T. hyodysenteriae*, including its virulence for swine and ability to produce complete hemolysis on blood agar plates. This conservation of polypeptides was also confirmed by immunoblotting experiments with rabbit or swine antibodies raised against whole *T. hyodysenteriae* cells and may prove to be extremely useful in identifying *T. hyodysenteriae* strains in spirochete field isolates.

SDS-PAGE and immunoblotting demonstrated that the majority of strongly immunogenic polypeptides of *T. hyodysenteriae* have molecular weights below 70,000. Of particular interest are several polypeptides of molecular weights between 30,000 and 36,000. These are the predominant polypeptide antigens recognized by serum from pigs vaccinated with an experimental *T. hyodysenteriae* vaccine and are conserved among different *T. hyodysenteriae* isolates. Cell fractionation and immunoblotting also demonstrated

that they are associated with the cell envelope. However, it is clear that other antigens are also recognized by serum from vaccinated animals. Further detailed investigations are required to clarify the targets for a protective immune response against SD.

Hyperimmune rabbit serum raised against whole cell *T. hyodysenteriae* contained antibody directed against the LPS antigen in the 14,000- to 24,000-molecular-weight region. The antibody did not recognize the LPSs of all the strains examined, in particular the representatives of serotypes I, II, and III. Unlike the polypeptides, the LPS is not antigenically conserved among different *T. hyodysenteriae* isolates. This has previously been demonstrated by using gel precipitin tests (2, 17) and an enzyme-linked immunosorbent assay (12). The results of this study confirm the immunologically heterogeneous nature of the LPS moiety of *T. hyodysenteriae*.

In previous studies, it has been shown that pigs that have recovered from SD are no longer susceptible to challenge with *T. hyodysenteriae* (10, 13, 20). The antibody response of swine to infection with *T. hyodysenteriae* is both humoral and mucosal (10; L. A. Joens, D. W. Deyoung, R. D. Glock, M. E. Mapother, J. D. Cramer, and H. E. Wilcox III, Proc. 8th Int. Pig Vet. Conf., 1984, p. 187), but its role in protection is unknown and the antigens to which it is directed have not been clearly identified. However, it has been demonstrated that swine colonic loops can be protected against *T. hyodysenteriae* challenge by passive transfer of serum from convalescent swine (9). These observations suggest that antibody has a role in protection against SD. Antibody in serum from convalescent pigs is known to be directed in part against the LPS of the organism. This has been demonstrated by using swine ligated loops from convalescent pigs that have been challenged with virulent *T. hyodysenteriae*. Protection was only demonstrated in those loops challenged with the homologous serotype, suggesting that protection against *T. hyodysenteriae* infection is serotype specific (13). In the present study, serum from a vaccinated pig that was subsequently protected against experimental challenge recognized several polypeptide antigens that were common to each strain of *T. hyodysenteriae* examined. The serum was taken before challenge, and, therefore, antibody raised against *T. hyodysenteriae* was a consequence of vaccination alone. The serum reacted weakly with the LPS moiety of *T. hyodysenteriae*, although the LPS content of the vaccine was monitored and found to be satisfactory (data not shown). These results suggest that antibody to the cross-reactive polypeptides may also play a role in protection against SD. A recent study by Joens and Marquez (11) also identified several antigens, including polypeptides that were recognized by serum and colonic washings from recovered pigs.

Immunoblotting indicated there are a number of cross-reactive antigens shared by *T. hyodysenteriae* and *T. innocens*. This is in agreement with observations made by other researchers (6, 7, 11). By adsorbing antisera against *T. hyodysenteriae* with *T. innocens* cells, we were able to identify three major polypeptide antigens apparently possessed by *T. hyodysenteriae* but not by *T. innocens*. These polypeptides could possibly play a role in virulence. We are using immune electron microscopy to see whether these antigens are exposed at or close to the surface of *T. hyodysenteriae*.

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