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Lipooligosaccharides from *Treponema hyodysenteriae* and *Treponema innocens*

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Lipooligosaccharides from *Treponema hyodysenteriae* serotypes 1 through 7, attenuated *T. hyodysenteriae* serotypes 1 and 2, and five strains of *T. innocens* were extracted with hot phenol water. The extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation and analyzed by lipopolysaccharide selective silver staining and Western blot (immunoblot) immunodetection. Silver staining revealed the presence of two bands that ranged between 18,000 and 24,000 daltons and that were serotype specific for *T. hyodysenteriae*. Attenuation of pathogenic strains resulted in the loss of the higher-molecular-weight band. Four of five *T. innocens* strains also lacked this particular band. *T. innocens* 421 had six bands between 17,000 and 26,900 daltons. Western blots with hyperimmune rabbit sera and convalescent-phase swine sera revealed antigenic variation among serotypes of *T. hyodysenteriae* and attenuated serotypes of *T. hyodysenteriae*. Convalescent-phase swine sera failed to recognize lipopolysaccharides from *T. innocens*. Differences in results obtained by lipopolysaccharide selective silver staining versus immunoblotting of the lipopolysaccharide preparations probably indicate that these two methods identify separate characteristics of the same molecule.

Treponema hyodysenteriae, the etiological agent of swine dysentery, produces mucohemorrhagic diarrhea in affected pigs (6). Unlike members of the *Enterobacteriaceae* capable of inducing diarrhea, *T. hyodysenteriae* produces no detectable exotoxin, and during the clinical course of disease, invasion of colonic epithelial cells does not occur (22, 23). A prerequisite for pathology is the attachment of *T. hyodysenteriae* to epithelial cells of the colon (unpublished data). Following colonization by *T. hyodysenteriae*, mice demonstrate lesions within their ceca (9). However, C3H/HeJ mice, which are nonresponsive to lipopolysaccharide (LPS) toxicity, do not develop lesions, despite the attachment of *T. hyodysenteriae* (16). In common with other gram-negative organisms, *T. hyodysenteriae* possesses an extractable outer membrane with LPS-like properties. The effects of purified LPS from *Enterobacteriaceae* on a variety of mammalian systems are diverse and well established. Grossly, rabbit pyrogenicity and murine mortality can be demonstrated (3, 4). On a cellular level, LPS can act as a B-cell mitogen and exhibit anticomplementary activity (5, 17). Nuessen et al. have shown that purified outer cell surface extracts from *T. hyodysenteriae* are chemotactic for murine macrophages and, in conjunction with actinomycin D, a potentiating agent for LPS, are toxic for FeJ mice, normal responders to LPS, but harmless to HeJ mice, genetic nonresponders to LPS (15).

Being surface-associated structures, LPS molecules interact with the immune system of the host (20). Convalescent animals produce serotype-specific antibodies to the LPS-like structure of *T. hyodysenteriae*. This fact has been exploited in Ouchterlony double-immunodiffusion identification of strains differing in serospecificities (1). Of clinical importance, this has led to the development of accurate, serologically based enzyme-linked immunosorbent assay screening of swine herds affected by swine dysentery (10). It was our intent to determine if differences exist in the lipopolysaccharide (LOS) extracted from different serotypes of *T. hyodysenteriae*, how it changes with attenuation of the pathogens, and how it compares with the LOS from nonpathogenic strains of *T. innocens*.

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MATERIALS AND METHODS

Cell culture and media. *T. hyodysenteriae* B234, B204, B169, A-1, 8044, 6933, and Ack300/8 (serotypes 1, 2, 3, 4, 5, 6, and 7, respectively), attenuated *T. hyodysenteriae* T-22 (serotype 1) and B204 (serotype 2, 131 passages in culture), and *T. innocens* B256, 421, Dys676, Taylor, and 1555a were grown in sealed flasks containing 500 ml of oxygen-reduced Trypticase soy broth (BBL Microbiology Systems) supplemented with 4% fetal bovine serum (GIBCO Laboratories) on a shaker platform for 48 h at 37°C in an atmosphere of 50% H_2 and 50% CO_2.

Cell harvesting and LOS extraction. Cultures of *T. hyodysenteriae* and *T. innocens* were centrifuged at 7,000 × *g* for 30 min at 4°C. The supernatants were discarded, and the pellets were stored at −70°C until extracted. Pellets from 500-ml cultures were suspended in 10 ml of 68°C dH_2O, combined with 10 ml of 68°C 88% aqueous phenol (Mallinckrodt, Inc.), and extracted by the method of Westphal et al. (21). Extracted LOS crystals were suspended in 2 ml of dH_2O and assayed for total carbohydrate content by reaction with L-cysteine hydrochloride and sulfuric acid (2). The Lowry protein assay was also done and revealed no detectable levels of protein contamination in the LOS extracts (11).

Preparation of samples for electrophoresis. Aqueous suspensions of LOS containing 2.6 μg of total carbohydrate for silver staining or 26 μg of total carbohydrate for Western blots (immunoblots) were solubilized and denatured by heating for 4 min at 95°C in 40% dH_2O–20% glycerol (Mallinckrodt)–20% sodium dodecyl sulfate (SDS; 10% [wt/vol]; Sigma Chemical Co.)–5% 2-mercaptoethanol (Fisher Scientific Co.)–2.5% bromophenol blue (0.05% [wt/vol]; Fisher) in a final volume of 30 μl.

Polyacrylamide gel electrophoresis (PAGE) was performed in a continuous buffer system containing 0.1% SDS with a linear gradient of 10 to 20% acrylamide (Bio-Rad Laboratories) cross-linked with 0.8% *N,N'*-methylene-bisacrylamide (Bio-Rad Laboratories). Slabs were electropho-

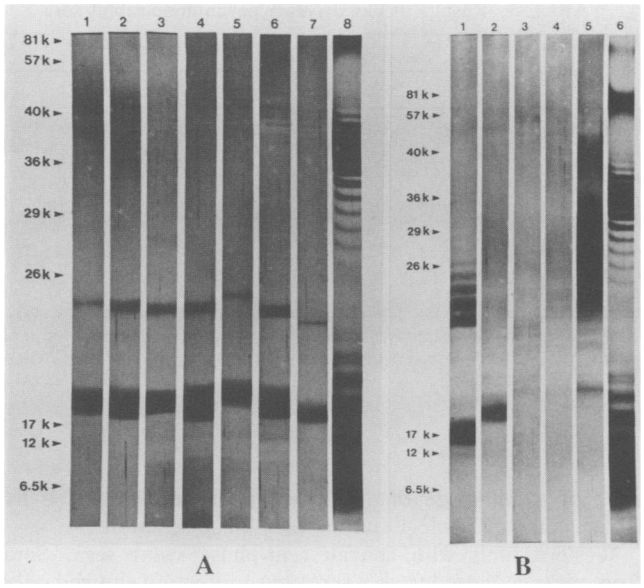

FIG. 1. (A) Visualization of *T. hyodysenteriae* LOS by carbohydrate-enhanced silver staining of SDS-PAGE slabs. Lanes 1 to 7 show serotypes 1 to 7, respectively. Lane 8 shows LPS extracted from *E. coli*. (B) Visualization of *T. innocens* LOS by the same staining method as in panel A. Lanes: 1, strain 421; 2, strain B256; 3, strain Dys676; 4, strain Taylor; 5, strain 1555a; 6, LPS from *E. coli*.

resed at a constant current of 35 mA per slab at 12°C. Proteins included for calibration of molecular masses were 110, 87, 57, 40, 36, 26, 17, 12, and 6.5 kilodaltons (kDa) (courteously supplied by the Center for Separation Science, University of Arizona).

Staining of SDS-PAGE slabs. Slabs were fixed with 10% acetic acid in 50% ethanol overnight. Visualization was accomplished with the LPS silver stain of Tsai and Frasch based upon oxidation of organic groups by periodic acid (19).

Immunoblotting of SDS-PAGE slabs. Immunological analysis of LOSs was performed by the Western blot method of Towbin et al. (18). Electrophoresed samples were transferred to Immobilon paper (Millipore Corp.) at a constant voltage of 20 V for 12 h and at a constant voltage of 30 V for 3 h in a buffer system containing 0.19 M glycine (Sigma) and 0.025 M Trizma base (Sigma) in 20% methanol.

Detection of antigens. Sera containing the primary antibodies were obtained from New Zealand White rabbits by separately hyperimmunizing them with serotype-specific Formalin-treated whole-cell extracts of *T. hyodysenteriae*. Convalescent-phase swine sera were obtained from animals recovered from either a serotype 1 or a serotype 2 swine dysentery infection. These sera contained antibodies to antigens elicited in the host by *T. hyodysenteriae* during the course of clinical disease in contrast to whole-cell immunization. Sera from unexposed rabbits and pigs were used as negative controls. Hyperimmune rabbit sera were diluted 1:400, while convalescent-phase swine sera were diluted 1:200. Binding of the primary antibodies to antigens was visualized with biotin-labeled anti-rabbit or anti-swine immunoglobulin G (1:400; Kirkegaard & Perry Laboratories, Inc.) reacted with peroxidase-labeled strepavidin (1:400; Kirkegaard & Perry) and developed by mixing equal parts of H_2O_2 solution (Kirkegaard & Perry) and 4-chloro-1-naphthol (Kirkegaard & Perry).

RESULTS

SDS-PAGE silver stains. Electrophoresis of the *T. hyodysenteriae* LOS demonstrated separation differences unique to each serotype. All of the pathogenic strains exhibited two characteristic bands that ranged between 18 and 24 kDa. Notably missing was repetitive banding, or the stepladder effect, normally seen in electrophoresed LPS from smooth strains of *Escherichia coli* (Fig. 1A). *T. innocens* strains (Fig. 1B), with the exception of strain 421, lacked the distinct higher-molecular-weight band of the pathogenic strains. *T. innocens* 421 contained bands at approximately 26.9, 25.7, 24.5, 22.9, 21.9, and 17 kDa, producing a partial stepladder effect. A comparison of pathogenic serotypes 1 and 2 with attenuated serotypes 1 and 2 (Fig. 2) revealed the disappearance of the higher-molecular-weight band characteristic of the pathogens. In addition, attenuated serotype 2 developed three new bands at 20.4, 25.1, and 30.9 kDa.

Western blots with hyperimmune rabbit antisera. Homologous rabbit antisera to *T. hyodysenteriae* serotype 1 reacted strongly with the transblotted serotype 1 LOS but also cross-reacted, to variable degrees, with all of the *T. hyodysenteriae* LOS extracts (Fig. 3A). Rabbit antisera to *T. hyodysenteriae* serotype 2 demonstrated a strong homologous reaction but exhibited cross-reactivity with serotypes 6 and 7 (Fig. 3B). Rabbit antisera to serotype 6 reacted with homologous LOS but also faintly cross-reacted with serotype 2 LOS (Fig. 4B). Rabbit antisera against serotype 3, 4, 5, or 7 showed only a homologous reaction (Fig. 3C and D and 4A and C). The banding pattern produced by serotypes 3, 4, 5, and 7 was unique for each pair of a homologous antiserum-LOS reaction. Rabbit antisera revealed the pres-

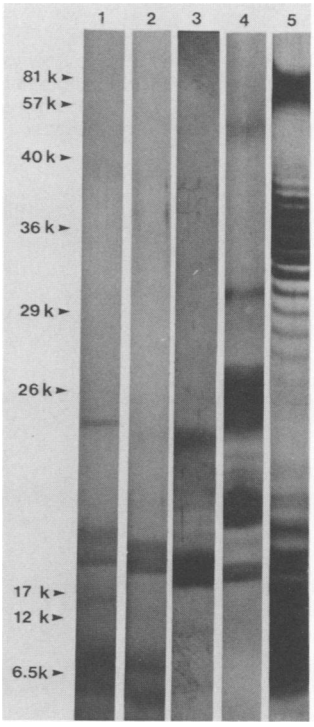

FIG. 2. Comparison of LOSs from pathogenic *T. hyodysenteriae* serotypes 1 and 2 (lanes 1 and 3, respectively) and attenuated *T. hyodysenteriae* serotypes 1 and 2 (lanes 2 and 4, respectively). Lane 5 shows LPS from *E. coli*.

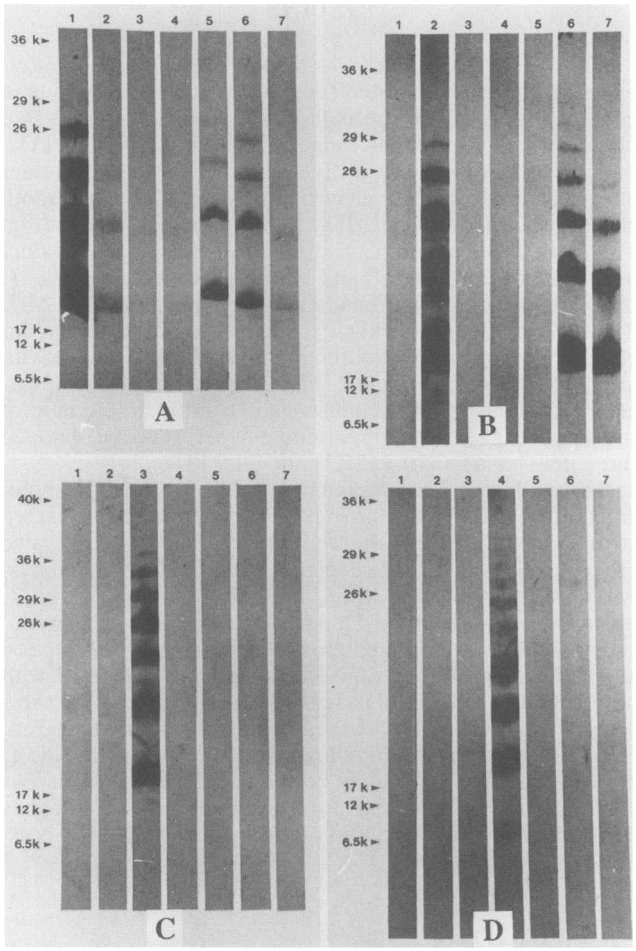

FIG. 3. Immunoblots of LOSs from *T. hyodysenteriae* serotypes 1 to 7 (lanes 1 to 7, respectively) with homologous rabbit antisera to serotype 1 LOS (A), serotype 2 LOS (B), serotype 3 LOS (C), and serotype 4 LOS (D).

TABLE 1. Results of blots with hyperimmune rabbit sera

Serum	Reaction with LOS of serotype[a]:						
	1	2	3	4	5	6	7
Anti-1	3+	+	+	+	2+	2+	+
Anti-2	–	3+	–	–	–	2+	2+
Anti-3	–	–	3+	–	–	–	–
Anti-4	–	–	–	3+	–	–	–
Anti-5	–	–	–	–	3+	–	–
Anti-6	–	+	–	–	–	3+	–
Anti-7	–	–	–	–	–	–	2+
Normal rabbit	–	–	–	–	–	–	–

[a] Data represent the intensity of reactions of hyperimmune rabbit sera against transblotted *T. hyodysenteriae* serotype 1 through 7 LOSs subjected to SDS-PAGE separation.

ence of a multitude of antigenic bands not visualized by silver staining. Table 1 summarizes these reactions.

Western blots with convalescent-phase swine sera. Sera from pigs recovered from a serotype 1 infection showed only a homologous reaction with the LOSs (Fig. 5A). Convalescent-phase sera from a serotype 2 infection reacted strongly with homologous LOS but also cross-reacted with serotype 6 and 7 LOSs (Fig. 5B). Sera from convalescent-phase swine yielded banding differences in Western blots when compared with hyperimmune rabbit antisera. Hyperimmune rabbit antisera induced by formalinized whole cells of serotype 1 reacted against antigens at 26 and 22.9 kDa. Convalescent-phase swine sera from a serotype 1 infection did not recognize these two bands. Convalescent-phase swine sera from serotype 2 infections reacted with an antigen at 27.5 kDa in addition to the antigens recognized by serotype 2 hyperimmune rabbit antisera. Table 2 summarizes Western blot analyses of convalescent-phase swine sera reacted against the *T. hyodysenteriae* and *T. innocens* LOS extracts.

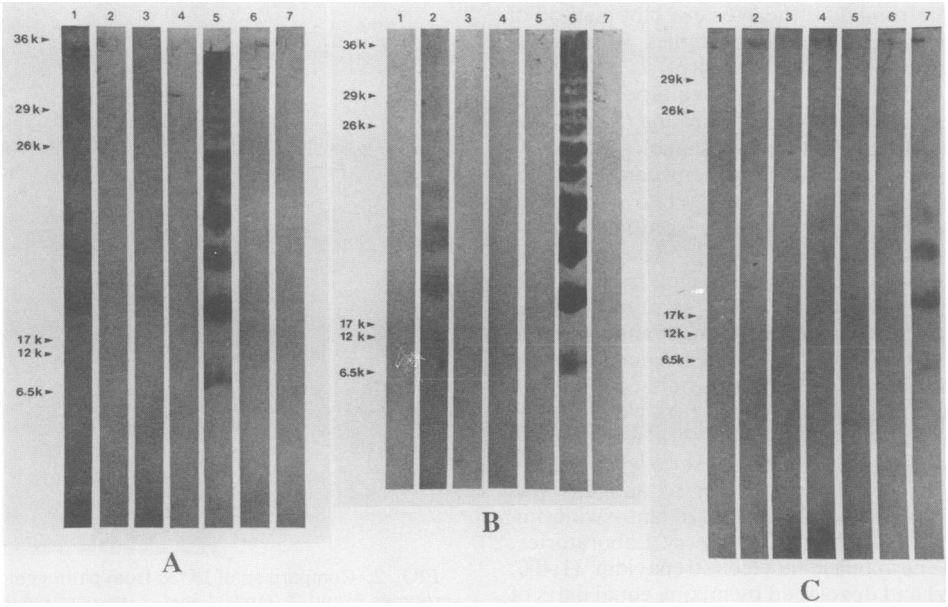

FIG. 4. Immunoblots of LOSs from *T. hyodysenteriae* serotypes 1 to 7 (lanes 1 to 7, respectively) with homologous rabbit antisera to serotype 5 LOS (A), serotype 6 LOS (B), and serotype 7 LOS (C).

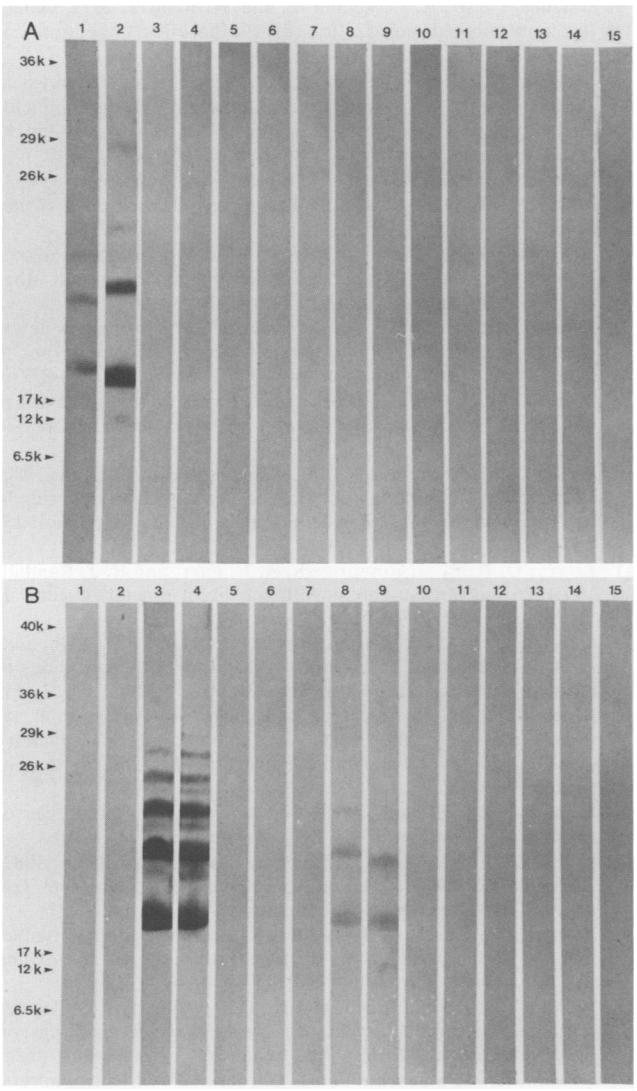

FIG. 5. Immunoblots of LOSs from *T. hyodysenteriae* pathogenic serotypes 1 to 7, attenuated pathogenic serotypes 1 and 2, and *T. innocens* reacted with convalescent-phase swine sera from a serotype 1 infection (A) or a serotype 2 infection (B). Lanes: 1, serotype 1 LOS; 2, attenuated serotype 1 LOS; 3, serotype 2 LOS; 4, attenuated serotype 2; 5, serotype 3 LOS; 6, serotype 4 LOS; 7, serotype 5 LOS; 8, serotype 6 LOS; 9, serotype 7 LOS; 10 to 14, *T. innocens* LOS from strains 421 (lane 10), B256 (lane 11), Dys676 (lane 12), Taylor (lane 13), and 1555a (lane 14); 15, normal swine sera reacted against serotype 1 LOS (A) and serotype 2 LOS (B).

DISCUSSION

SDS-PAGE of hot phenol water extracts of *T. hyodysenteriae* outer membranes demonstrated qualitative differences among all seven known serotypes. A comparison with LPS from *E. coli* showed that the *T. hyodysenteriae* extracts possess only low-molecular-weight banding patterns. This implies the presence of LOS instead of the heavier LPS. LPSs commonly consist of a lipid molecule, a core sugar, and a carbohydrate or O-specific chain (12). If the lipid moiety and the core carbohydrate linkage of the LOSs of *T. hyodysenteriae* are conserved among serotypes, then the observed molecular weight differences should be due to variations in the O-specific chains. The carbohydrate backbones of LPSs are immunogenic (20). Variations in the O-specific chains among *T. hyodysenteriae* serotypes may be the basis for serospecificity. LOSs extracted from *T. hyodysenteriae* are used as the primary antibody in ELISA serological confirmation for the presence of this organism in swine herds (10). For identification of the serotype of the causative agent in field cases of swine dysentery, LOS is extracted from the isolated organism and reacted with known antisera in Ouchterlony double-immunodiffusion tests (1).

Western blot analysis demonstrated complete specificity of some of the homologous antisera against serotype-specific LOSs, while in several cases, cross-reactivity occurred. This result is not inconsistent, since cross-reactivity has been demonstrated between distantly related organisms such as *Staphylococcus aureus* and *Streptococcus faecalis* (7). The appearance of cross-reactivity between *T. hyodysenteriae* serotypes probably results from common carbohydrate sequences that form portions of the LOS O-specific chains. Despite the occurrence of cross-reactivity, homologous antisera react more vigorously to the complementary serotype-specific LOSs. Cross-reactivity can be eliminated by absorption of homologous sera against whole cells of the cross-reacting strains of *T. hyodysenteriae* (14). Immunodetection by Western blots increases the number of bands that can be visualized in the LOS extracts, as compared with silver-stained SDS-PAGE gels. Similar visualization differences between Western blots and silver staining have also been shown for LOSs from *Neisseria gonorrhoeae* (13). This result implies that the extracts contain substances which are unable to complex with ammoniacal silver but which do react with antibodies. The possibility exists that ammoniacal silver and antibodies react to different aspects of the same molecule. Ammoniacal silver is thought to react with aldehyde and prealdehyde groups of periodate-oxidized LPS (8). Conversely, immunoblotting with antisera reveals specific epitopes that form the antigenic character of a molecule. Although only low-molecular-weight species are involved,

TABLE 2. Results of blots with convalescent-phase swine sera

Serum	Reaction with LOS from[a]:													
	T. hyodysenteriae serotype:									*T. innocens*				
	1	1 (strain T-22)	2	2 (attenuated)	3	4	5	6	7	421	B256	Dys676	Taylor	1555a
Serotype 1 convalescent phase	2+	3+	–	–	–	–	–	–	–	–	–	–	–	–
Serotype 2 convalescent phase	–	–	3+	3+	–	–	–	2+	2+	–	–	–	–	–
Normal swine	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Data represent the intensity of reactions of convalescent-phase swine sera against transblotted *T. hyodysenteriae* serotype 1 through 7 and attenuated serotype 1 and 2 LOSs and *T. innocens* LOSs subjected to SDS-PAGE separation.

the banding pattern seen in Western blots reveals a profile that partially mimics the stepladder effect produced by LPSs separated on silver-stained SDS-PAGE gels.

The larger of the two bands, characteristic of LOS from the pathogenic species of *T. hyodysenteriae*, disappears when this same species becomes attenuated, as demonstrated on silver-stained SDS-PAGE gels. However, the presence of this band in Western blots implies that a structural change which prevents the LOS from complexing with silver ions has taken place. The attenuated serotype 1 isolate used in this study is a naturally occurring organism that is unable to produce characteristic lesions in swine. However, the attenuated serotype 2 isolate that was used was artificially produced by in vitro passages. An SDS-PAGE comparison of pathogenic serotype 2 with attenuated serotype 2 shows the attenuated strain losing one band while gaining three new bands. Western blot analysis of the same two strains shows virtually identical banding patterns between them. It appears that laboratory attenuation may produce subtle changes which affect the ability of the LOS components to complex with silver. Silver staining shows the presence of a new band at approximately 30.9 kDa in attenuated serotype 2 LOS. Serotype 2 convalescent-phase swine sera do not recognize this particular band, possibly indicating that this component is absent in pathogenic serotype 2 LOS.

Despite the possibility of structural changes in the higher-molecular weight bands of attenuated *T. hyodysenteriae*, the ability of the LOS to react specifically in Ouchterlony double-immunodiffusion tests remains unaffected. This result may indicate that the lower of the two bands, visualized by silver staining, is a major determinant responsible for contributing to serospecificity, since it appears to be conserved. Demonstrable differences exist among the LOSs of *T. hyodysenteriae*, owing to strain variation. While strains of *T. hyodysenteriae* vary in their degree of pathogenicity, their pathogenic mechanisms are poorly understood. Changes that occur in the LOSs of *T. hyodysenteriae* during attenuation, in conjunction with comparisons to nonpathogenic *T. innocens* strains, may further an understanding of the events that take place within the host.

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LITERATURE CITED

1. **Baum, D. H., and L. A. Joens.** 1979. Serotypes of beta-hemolytic *Treponema hyodysenteriae*. Infect. Immun. **25**:792–796.
2. **Chaplin, M. F., and J. F. Kennedy.** 1986. Carbohydrate analysis: a practical approach, p. 1. IRL Press, Oxford.
3. **Elin, R., A. L. Sanderg, and D. L. Rosenstreich.** 1976. Comparisons of the pyrogenicity, Limulus activity, mitogenicity and complement reactivity of several bacterial endotoxins and related compounds. J. Immunol. **117**:1238–1242.
4. **Galanos, C., M. A. Freudenberg, and W. Reutter.** 1979. Galactosamine induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA **76**:5939–5943.
5. **Galanos, C., and O. Luderitz.** 1976. The role of the physical state of lipopolysaccharides in the interaction with complement. High molecular weight as prerequisite for the expression of anticomplementary activity. Eur. J. Biochem. **65**:403–408.
6. **Harris, D. L., and R. D. Glock.** 1981. Swine dysentery, p. 432–444. *In* H. W. Dunne and A.D. Leham (ed.), Diseases of swine. Iowa State University Press, Ames.
7. **Heidelberger, M.** 1984. Cross-reactions of polysaccharides of staphylococci and streptococci in antipneumococcal and other antisera. Mol. Immunol. **21**:1011–1013.
8. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
9. **Joens, L. A., and R. D. Glock.** 1979. Experimental infection in mice with *Treponema hyodysenteriae*. Infect. Immun. **25**:757–760.
10. **Joens, L. A., N. A. Nord, J. M. Kinyon, and I. T. Egan.** 1982. Enzyme-linked immunosorbent assay for detection of antibody to *Treponema hyodysenteriae* antigens. J. Clin. Microbiol. **15**: 249–252.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **Luderitz, O., M. A. Freudenburg, C. Galanos, V. Lehmann, E. T. Rietschel, and D. H. Shaw.** 1982. Lipopolysaccharides of gram negative bacteria. Curr. Top. Membr. Transp. **17**:79–151.
13. **Mandrell, R., H. Schneider, M. Apicella, W. Zollinger, P. A. Rice, and J. M. Griffiss.** 1986. Antigenic and physical diversity of *Neisseria gonorrhoeae* lipooligosaccharides. Infect. Immun. **54**:63–69.
14. **Mapother, M. E., and L. A. Joens.** 1985. New serotypes of *Treponema hyodysenteriae*. J. Clin. Microbiol. **22**:161–164.
15. **Nuessen, M. E., J. R. Birmingham, and L. A. Joens.** 1982. Biological activity of a lipopolysaccharide extracted from *Treponema hyodysenteriae*. Infect. Immun. **37**:138–142.
16. **Nuessen, M. E., L. A. Joens, and R. D. Glock.** 1983. Involvement of lipopolysaccharide in the pathogenicity of *Treponema hyodysenteriae*. J. Immunol. **131**:997–999.
17. **Raetz, C. H. R., S. Purcell, and K. Takayama.** 1983. Molecular requirements for B-lymphocyte activation by *Escherichia coli* lipopolysaccharide. Proc. Natl. Acad. Sci. USA **80**:4624–4628.
18. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
19. **Tsai, C.-M., and L. F. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115–119.
20. **Westphal, O., K. Jann, and K. Himmelspach.** 1983. Chemistry and immunochemistry of bacterial lipopolysaccharides as cell wall antigens and endotoxins. Prog. Allergy **33**:9–39.
21. **Westphal, O., O. Luderitz, and F. Bister.** 1952. Über die Extraktion von Bakterien mit Phenol-Wasser. Z. Naturforsch. Teil B **7**:148–155.
22. **Wilcock, B. D., and H. J. Olander.** 1979. Studies on the pathogenesis of swine dysentery. I. Characterizations of the lesions in colons and colonic segments inoculated with pure cultures containing *Treponema hyodysenteriae*. Vet. Pathol. **16**: 450–465.
23. **Wilcock, B. D., and H. J. Olander.** 1979. Studies on the pathogenesis of swine dysentery. II. Search for a cytotoxin in spirochetal broth cultures and colon content. Vet. Pathol. **16**: 567–573.