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Monoclonal Antibodies to Lipopolysaccharide Antigens of *Salmonella enterica* serotype Typhimurium DT104

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Salmonella enterica subsp. *enterica* serotype Typhimurium is one of the major causative agents of human gastroenteritis. Here we raised a panel of 45 monoclonal antibodies (MAbs) against ser. Typhimurium DT104 by immunizing mice with formalin-killed bacteria and demonstrated that all the MAbs recognized the bacterial lipopolysaccharide (LPS) antigen. These MAbs were specific for group O:4 *Salmonella* with very little or no cross-reactivity with other closely related bacteria and were able to bind to the cell surface of live bacterial cells, making them potential candidates for capture and concentration of the pathogen in food and water samples. Epitope characterization revealed that the O:5 antigen present in the LPS of some serogroup 4 *Salmonella* is the critical factor for the binding of these MAbs to LPS. This study has provided some insights into the structure of the *Salmonella* LPS and its influence on the antigenicity of LPS.

Introduction

Acute foodborne gastrointestinal infections are commonly caused by the gram-negative, rod-shaped bacterium *Salmonella enterica* subsp. *enterica* serotype Typhimurium.[1] Although most individuals experience acute gastroenteritis, *Salmonella* infection in infants, the elderly, or immunocompromised patients can become systemic and result in death.[2] In otherwise healthy patients, *Salmonella* infection can lead to additional complications such as chronic acute reactive arthritis.[3]

Salmonella enterica subsp. *enterica* is classified into over 1500 serotypes based on antigenic differences in lipopolysaccharide (LPS) (O) and flagellar (H) antigens. The H-antigenic determinants are carried in the flagellin protein, the main structural component of flagella. Through phase variation, *Salmonella* can express antigenically different flagella.[4] Lipopolysaccharide is a major component of the outer surface of gram-negative bacteria, composed of a hydrophobic lipid A, which anchors LPS to the membrane, a core oligosaccharide region, and an O-polysaccharide polymer (O-chain) composed of oligosaccharide-repeating units. While the lipid A and the LPS-core region are relatively conserved among gram-negative organisms, there is a substantial difference in the composition of the O-chain-repeating units, which leads to a large antigenic diversity in O-antigens.

Monoclonal antibodies (MAbs) have been raised against the surface antigens of *Salmonella*, several of which were generated in an attempt to seek a MAb that would be specific for a certain serotype. The MAb 23D4 directed against the *Salmonella* H antigen appeared to be highly specific for ser. Typhimurium but did cross-react with monophasic [4,5,12:i:-] *Salmonella.*[5] Other attempts to produce MAbs specific for the H antigen of ser. Typhimurium have been less successful, showing the ability of these MAbs to differentiate between the two phases (H:i, H:1,2), but cross-react with other serotypes.[4] An interesting immunization approach using the outer membrane protein (OMP) extract from ser. Typhimurium led to the creation of 10 MAbs against a 38 kDa OMP, which were specific to serogroup 4 *Salmonella.*[6]

Monoclonal antibodies have also been raised against the structural components of *Salmonella* LPS. Antibodies against the core region of LPS tend to react with a broad range of *Salmonella* and other enterobacteriaceae. The T6 MAb directed against a highly conserved N-acetylglucosamine and glucose portion of the Ra-type core reacts with a broad range of *Salmonella*, although it does not react with several *Salmonella* isolates of subsp. *arizonae*, lacking the Ra-type core.[7,8] The MAb M105 had slightly more success in recognizing a larger portion of *Salmonella* isolates by binding to both the Ra-type and less effectively to the Rb1 and Rb2-type core,[9] although it does not react with all *Salmonella* isolates.[10]

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Attempts have been made to raise MAbs against the O-chain of LPS that may be able to recognize specific serogroups or even serotypes. *Salmonella* ser. Typhimurium belongs to the O:4 (formerly B) serogroup. The LPS of group 4 organisms contains the O:12 antigen, which is comprised of a repeating unit of mannose, rhamnose, and galactose in the O-chain, although this sugar arrangement is common to a number of serogroups. The defining factor of serogroup 4 LPS is the O:4 antigen, which is abequose α-1, 3 linked to the mannose. The stereochemistry of this abequose residue is the sole determinant of the immunodominant epitope within the serogroup 4 LPS.[11] Acetylation of the 2-hydroxyl group of the abequose residue defines the O:5 antigen, which is not expressed by all Typhimurium strains. As the attachment of this acetyl group changes the stereochemistry of the abequose residue, it is predictable that the presence or absence of the O:5 antigen is critical in defining immunogenic serogroup 4 epitopes.[12] Jaradat and Zawistowski reported that MAbs 4A8, 5B2, and 5A5 all react with O-acetylated variants of serogroup 4 *Salmonella* while failing to react with the non-acetylated counterparts.[13] Slauch and colleagues developed seven MAbs against ser. Typhimurium LPS, five of which recognized acetylated LPS exclusively while two recognized non-acetylated LPS exclusively.[12] Luk and Lindberg reported a number of MAbs specific for various O-groups, including MABO-8 and MABO-10 directed against the serogroup 4 LPS.[14] In contrast to MABO-10 that detects LPS in both the presence and absence of the O:5 antigen, MABO-8, which has a higher affinity for the LPS, only reacted with LPS containing O:5 antigen.[14] The same specificity has also been demonstrated with polyclonal antibodies.[15] The mechanism behind this was suggested in a detailed analysis of the SE115-4 binding site. Through crystallographic studies, antibody-antigen interaction as defined by hydrogen bonding was shown to be dominated by the abequose residue, despite the antibody binding site being complementary to a trisaccharide-sized epitope.[11] A number of MAbs recognizing surface antigens of *Salmonella* have been produced, although none is specific enough to be used in diagnostics or serotyping of certain *Salmonella* serotypes.

A study comparing the ability of participating laboratories to correctly serotype *Salmonella* found significant differences,[16] which suggests that better antisera are required to get clear results in serological assays. The current serotyping antisera are polyclonal, created through immunization with *Salmonella* whole cells. Taking into account the similarities in cell surfaces between gram-negative organisms such antisera may contain many cross-reacting antibodies. Since LPS is known to carry distinct diagnostic markers, which aid in the ability to define serotypes, creation of new MAbs against LPS may increase the accuracy of serological assays. In addition, since this study indicates that LPS plays a critical role in the immune response to *Salmonella* infection, the creation of MAbs against LPS may be useful for mapping potential virulence epitopes.

Given the fact that *Salmonella* ser. Typhimurium is an important agent of foodborne gastrointestinal infections, a MAb specific for this serotype would be very useful in diagnostics. This study was undertaken to create and characterize antibodies directed against surface antigens of *Salmonella* ser. Typhimurium, which may be useful in diagnostics as well as the future study of *Salmonella*.

Materials and Methods

Chemicals and reagents

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (H + L) was bought from Zymed Laboratories (San Francisco, CA). Protein standards (pre-stained), nitrocellulose membranes, and HRP conjugate substrate kit were obtained from Bio-Rad (Mississauga, Canada). Phenol extracted LPS from *Salmonella* ser. Typhimurium ATCC 7823 was obtained from Sigma (Oakville, Canada). All other chemicals were of analytical grade and commercially available.

Bacterial culture

The bacteria species used in this study (Table 1) were cultured at 37°C on Luria-Bertani (LB) agar or in LB broth, with the exception of *Listeria* spp., which was cultured on either brain heart infusion (BHI) agar or in BHI broth, for 16−18 h. Cell concentrations were estimated using OD_{600} $1 = 1.6\times10^9$ cells/mL for *Salmonella* species,[17] OD_{600} $1 = 1\times10^9$ cells/mL *E. coli*, and OD_{620} $0.61 = 1\times10^9$ cells/mL for *Listeria*.[18]

Mouse immunization

A *Salmonella* ser. Typhimurium DT104 strain SA03-1907 was used as an immunogen. Bacterial cells were harvested from a 200 mL overnight culture by centrifugation at 10000 *g* for 10 min at 4°C, washed three times with PBS, and killed by resuspending in 30 mL of 0.3% (v/v) formalin solution in PBS for 24 h at room temperature. Killed cells were collected by centrifugation at 10000 *g* for 10 min at 4°C, washed twice with PBS, and then resuspended in PBS to obtain a concentration of 1×10^9 cells/mL. Formalin-killed cells were stored at −20°C until use. Three ND4 and three BALB/c mice, after collecting pre-immune sera, were immunized intraperitoneally with 1×10^8 cells in 0.5 mL of PBS at days 0 and 8. Two intravenous injections with 1×10^8 cells in 0.1 mL of PBS were given at days 29 and 38. On day 42, three ND4 and two BALB/c mice were sacrificed for fusion with SP2/0-Ag14 myeloma cells as previously described.[18] Animal immunization experiments were approved by the Animal Care Committee at the Ottawa Laboratory Fallowfield (Canadian Food Inspection Agency) and carried out under the guidelines of the Canadian Council on Animal Care.

Screening and isotyping of murine monoclonal antibodies

Culture supernatants from hybridoma cells were tested for *Salmonella*-reactive monoclonal antibodies using the indirect ELISA procedure as previously described.[18] Formalin-killed *Salmonella* ser. Typhimurium DT104 cells were used as the coating antigen at a concentration of 1×10^9 cells/mL in a volume of 100 μL/well. Hybridoma cell lines secreting reactive MAbs were identified by using an OD_{414} cut-off of 0.3 under the conditions used. The subclasses of immunoglobulins (Ig) secreted by hybridoma cell lines were determined as previously described.[18]

Table 1. Bacteria Used in Study

Bacteria	*Strain or isolate*	*O-Antigenic formula*
Salmonella ser. Typhimurium DT104	SA03-1907	1,4,[5],12
Salmonella ser. Typhimurium P10	Unflagellated mutant.	1,4,[5],12
Salmonella ser. Typhimurium Passage 1	ATCC 14028	1,4,[5],12
Salmonella ser. Typhimurium DT108	02D1634 2-4	1,4,[5],12
Salmonella ser. Typhimurium Str. 324	ADRI 5040	1,4,[5],12
Salmonella ser. Typhimurium variant Copenhagen DT104 1	00X344-C5	1,4,12
Salmonella ser. Typhimurium variant Copenhagen DT104 2	01D5997 25-3	1,4,12
Salmonella ser. Typhimurium 14028 *ofaA127*:: TN *10d*- Km	MT120	1,4,12
S. enterica subsp. *arizonae*	ADRI 15	48
Salmonella ser. Abortus equi	ADRI 37	4,12
Salmonella ser. Heidelberg	01D3969-14	1,4,[5],12
Salmonella ser. Heidelberg Type 8	O1D5997 31-7	1,4,[5],12
Salmonella ser. Heidelberg	OLC#502	1,4,[5],12
Salmonella ser. Agona	ADRI 36	1,4,[5],12
Salmonella ser. Chester	OLC#320	1,4,[5],12
Salmonella ser. California		4,12
Salmonella ser. Thompson		6,7,[14]
Salmonella ser. Enteritidis		1,9,12
Salmonella ser. Paratyphi A	OLF#1836	1,2,12
L. monocytogenes ser. 4b	LI0521	
L. grayi	HPB29	
E. coli O157:H7	ATCC 43889	157

*Cross-reactivity of anti-*S. *Typhimurium DT104 MAbs with other microorganisms*

The MAbs, selected based on reactivity with *Salmonella* ser. Typhimurium DT104, were assessed for cross-reactivity with bacterial species (Table 1) by indirect ELISA using respective formalin-killed bacteria ($\sim 1.0 \times 10^8$ cells/well) as described.[18]

Immunofluorescence microscopy

The ability of the MAbs to bind to the surface antigens of live *Salmonella* ser. Typhimurium was assessed using immunofluoresence microscopy.[18]

Preparation of Salmonella LPS

LPS was prepared from *Salmonella* ser. Typhimurium DT104 through a procedure modified from Johnson and colleagues.[19] Briefly, lyophilized bacteria were suspended in water to a concentration of 5% (w/v). The aqueous suspension was heated to 70°C in a water bath for 10 min. An equal volume of preheated 90% (v/v) phenol was added to the bacterial suspension. This was maintained at 70°C while being stirred vigorously for 15 min. The solution was quickly cooled to 10°C. The emulsion was centrifuged at 10,000 *g* for 30 min. The upper aqueous layer was removed and retained. The remaining phenol phase was suspended in one volume of water and extracted again. The aqueous layers from each extraction were combined and dialysed overnight against water. The dialyzed sample was lyophilized, dissolved in PBS, and then centrifuged at 27,000 *g* for 30 min to remove insoluble material. The supernatant was treated with RNase (1 μg/mL) and DNase (1 μg/mL) in the presence of 10 mM $MgCl_2$ at 37°C for 1 h and ultracentrifuged at 100,000 *g* for 10 h. The final pellet was dissolved in water and lyophilized.

*Immunoblot analysis of Salmonella LPS using anti-*S. *Typhimurium DT104 MAbs*

Binding of anti-*S*. Typhimurium DT104 MAbs to *Salmonella* LPS was analyzed by immunoblotting, as described previously.[20] Transfer of the LPS to nitrocellulose essentially took place as previously described[21] using a Bio-Rad wet-electrotransfer apparataus (Hercules, CA). The purified LPS (Sigma) antigens were dissolved in SDS-PAGE sample buffer at 2.3 mg/mL, heated at 100°C for 10 min and loaded into wells of 12% SDS-PAGE gels (20 μL per well). The separated LPS bands were probed with monoclonal antibody-containing hybridoma cell culture fluids at a dilution of 1:20 in PBST containing 5% BSA.

SDS-PAGE analysis of purified Salmonella LPS

Analysis of purified *Salmonella* LPS by SDS-PAGE followed by silver staining were performed using the procedure modified from Tsai and Frasch.[22]

Deacetylation of purified LPS

Hot phenol purified LPS was subjected to treatment with either 4 M acetate buffer (pH 4.6), 4 M sodium hydroxide (pH 9.6), or PBS (pH 7.0). Each LPS solution was either heated at 100°C for 30 min or held at room temperature for 30 min. Carbonate buffer (60 mM $NaHCO_3$-Na_2CO_3, pH 9.6) was used to dilute LPS to 10 μg/mL. This LPS (100 μL) was added to each well of an ELISA plate[18] and used to assess the ability of the MAbs to react with chemically modified LPS.

Results

Identification of hybridoma clones secreting MAbs to Salmonella ser. Typhimurium

ELISA screening of culture supernatants of hybridomas generated by fusion of myeloma cell line SP2/0-Ag14 with

Table 2. Summary of ELISA Data on Cross-reactions of MAbs with *Salmonella* serotypes

	ser. Typhimurium DT104	ser. Typhimurium Strain 324	ser. Typhimurium Passage 1	ser. Typhimurium P10	ser. Typhimurium DT108	ser. Typhimurium variant Copenhagen 1	ser. Typhimurium variant Copenhagen 2	ser. Chester	ser. Heidelber g 1	ser.Heidelberg Type 8	ser. Heidelberg 2	ser. Abortus equi	ser. Paratyphi A	ser. California	ser. Enteritidis	ser. Thompson	ser. Agona	subsp. Arizonae	*E. coli* O157:H7	*L. monocytogenes*	*L. grayi*
M3037	■	■	■	■	■	░	░	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3038	■	■	■	■	▓	□	□	■	■	□	□	□	▓	□	□	□	□	□	□	□	□
M3039	■	■	■	■	■	░	░	■	■	░	░	░	░	□	□	□	□	□	□	□	□
M3040	■	■	■	■	■	░	░	■	■	□	░	░	░	□	□	□	□	□	□	□	□
M3041	■	■	■	■	■	░	░	■	■	□	░	░	■	□	░	□	□	□	□	□	□
M3042	■	■	■	■	■	░	░	■	■	□	░	░	░	□	□	□	□	□	□	□	□
M3043	■	■	■	■	▓	░	░	■	■	□	░	░	░	□	□	□	□	□	□	□	□
M3044	■	■	■	■	■	░	□	■	■	□	░	░	░	□	░	□	□	□	□	□	□
M3045	■	■	■	■	■	░	░	■	■	□	░	░	■	□	□	□	□	□	□	□	□
M3046	■	■	■	■	■	□	□	■	■	□	░	□	░	□	□	□	□	□	□	□	□
M3047	■	■	■	■	■	▓	░	■	■	░	░	░	□	□	□	□	░	□	□	□	□
M3048	■	■	■	■	■	░	░	■	■	░	░	░	░	□	□	□	□	□	□	□	□
M3049	■	■	■	■	■	░	░	■	■	□	░	░	▓	□	□	□	□	□	□	□	□
M3050	■	■	■	■	■	□	□	■	■	□	□	░	░	□	□	□	□	□	□	□	□
M3051	■	■	■	■	■	░	□	■	■	□	░	░	░	□	□	□	□	□	□	□	□
M3052	■	■	■	■	■	░	░	■	■	░	░	░	■	□	□	□	□	□	□	□	□
M3053	■	■	■	■	■	░	░	■	■	░	░	□	░	□	□	□	□	□	□	□	□
M3054	■	■	■	■	■	░	□	■	■	□	□	░	░	□	□	□	□	□	□	□	□
M3055	■	■	■	■	▓	□	□	■	■	░	░	□	░	□	░	░	░	□	□	□	□
M3056	■	■	■	■	■	▓	▓	■	■	□	░	░	■	□	░	□	░	□	□	□	□
M3057	■	■	■	■	■	░	░	■	■	░	░	░	░	□	░	□	░	□	□	□	□
M3058	■	■	■	■	■	░	░	■	■	░	░	░	□	□	□	□	░	□	□	□	□
M3059	■	■	■	■	▓	□	□	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3060	■	■	■	■	▓	□	□	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3061	■	■	▓	▓	▓	□	□	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3062	■	■	■	■	■	░	□	■	■	□	▓	░	░	□	□	□	□	□	□	□	□
M3063	■	■	■	■	■	░	□	■	■	□	░	░	░	□	□	□	□	□	□	□	□
M3064	■	■	■	■	▓	□	□	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3065	■	■	■	■	■	□	□	■	■	□	□	□	■	□	□	□	□	□	□	□	□
M3066	■	■	■	■	■	▓	▓	■	■	░	░	░	■	□	□	□	░	□	□	□	□
M3067	■	■	■	■	▓	░	□	■	■	□	░	░	▓	□	□	□	□	□	□	□	□
M3068	■	■	■	■	■	░	░	■	■	□	░	░	□	□	□	□	□	□	□	□	□
M3069	■	■	■	■	▓	□	□	■	■	□	░	□	░	□	□	□	□	□	□	□	□
M3070	■	■	■	■	■	□	□	■	■	□	□	□	□	□	□	□	□	□	□	□	□
M3071	■	■	▓	■	▓	□	□	■	■	□	□	░	░	□	□	□	□	□	□	□	□
M3072	■	■	■	■	■	□	□	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3073	■	■	■	■	■	□	□	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3074	■	■	■	■	▓	□	░	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3075	■	■	■	■	▓	□	□	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3076	■	■	■	■	■	░	░	■	■	□	░	□	□	□	□	□	□	□	□	□	□
M3077	■	■	■	■	■	□	□	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3078	■	■	■	■	■	□	□	■	■	□	░	□	░	□	□	□	□	□	□	□	□
M3079	■	■	■	■	▓	□	□	■	■	□	□	□	░	□	□	□	□	□	□	□	□
M3080	■	■	■	■	▓	□	□	■	■	□	░	□	░	□	□	□	□	□	□	□	□
M3081	■	■	■	■	■	░	░	■	■	□	░	░	░	□	□	□	░	□	□	□	□

ELISA optical density (OD) readings were determined at 414 nm: ■ represents an OD_{414} of >1, ▓ represents an OD_{414} of <1 but >0.5, ░ represents an OD_{414} of <0.5 but >0.05, □ represents an OD_{414} of <0.05, which was comparable to the negative control. Negative controls were preformed with an irrelevant MAb M2787 raised against *Listeria monocytogenes*.[17] All ELISA OD values are the average of two determinations.

spleen cells from three Balb/c and two ND4 mice receiving formalin-killed *Salmonella* ser. Typhimurium DT104 identified 45 positive clones. ELISA analysis of MAb-containing culture supernatants revealed that all MAbs showed a reaction with the immunizing bacteria with an OD at 414 nm of greater than 1.0 (Table 2). All the MAbs were able to react with other *Salmonella* ser. Typhimurium isolates tested (Table 2). Antibody isotype analysis demonstrated that of these 45 MAbs, four were IgG1 (M3037, M3044, M3061, and M3068); 22 were IgG2a (M3038, M3040, M3041, M3045, M3046, M3049, M3050, M3051, M3054, M3059, M3060, M3064, M3065, M3069, M3070, M3071, M3072, M3073, M3074, M3077, M3078, and M3080), 13 were IgG2b (M3043, M3047, M3048, M3053, M3055, M3057, M3058, M3061, M3063, M3075, M3076, M3079, and M3081); and six were IgG3 (M3039, M3042, M3052, M3056, M3066, and M3067).

Binding of monoclonal antibodies to the surface of live Salmonella ser. Typhimurium

Immunoflourescent staining of live *Salmonella* ser. Typhimurium DT104 showed that all 45 MAbs were able to bind to the cell surface. The result of immunofluoresence staining was exemplified by binding of M3037 to the surface of *Salmonella* ser. Typhimurium (Fig. 1). No fluorescence staining was observed with an irrelevant MAb M2787 raised against *L. monocytogenes*.[17]

Cross-reactivity of monoclonal antibodies within Salmonella serogroup 4

All 45 MAbs were tested for cross-reactivity by indirect ELISA with a number of non-Typhimurium isolates and three other bacterial species (Table 1) as well as 14 ser. Typhimurium isolates. All 45 MAbs strongly reacted with all 14 ser. Typhimurium isolates (data not shown). Two *Salmonella* ser. Typhimurium variant Copenhagen strains tested here showed a similar reaction pattern with all the monoclonal antibodies (Table 2): no cross-reaction with 19 MAbs (M3038, M3046, M3050, M3055, M3059, M3060, M3061, M3064, M3065, M3069, M3070, M3071, M3072, M3073, M3075, M3077, M3078, M3079, and M3080) with OD_{414} readings comparable to the negative control; strong reaction with three MAbs (M3047, M3056, and M3066) with OD_{414} readings above 0.5; and mild cross reactions, having OD_{414} readings less than 0.5 but greater than the negative control with the remaining MAbs. Of seven other isolates from serogroup 4 (three *Salmonella* ser. Heidelberg isolates, *Salmonella* ser.

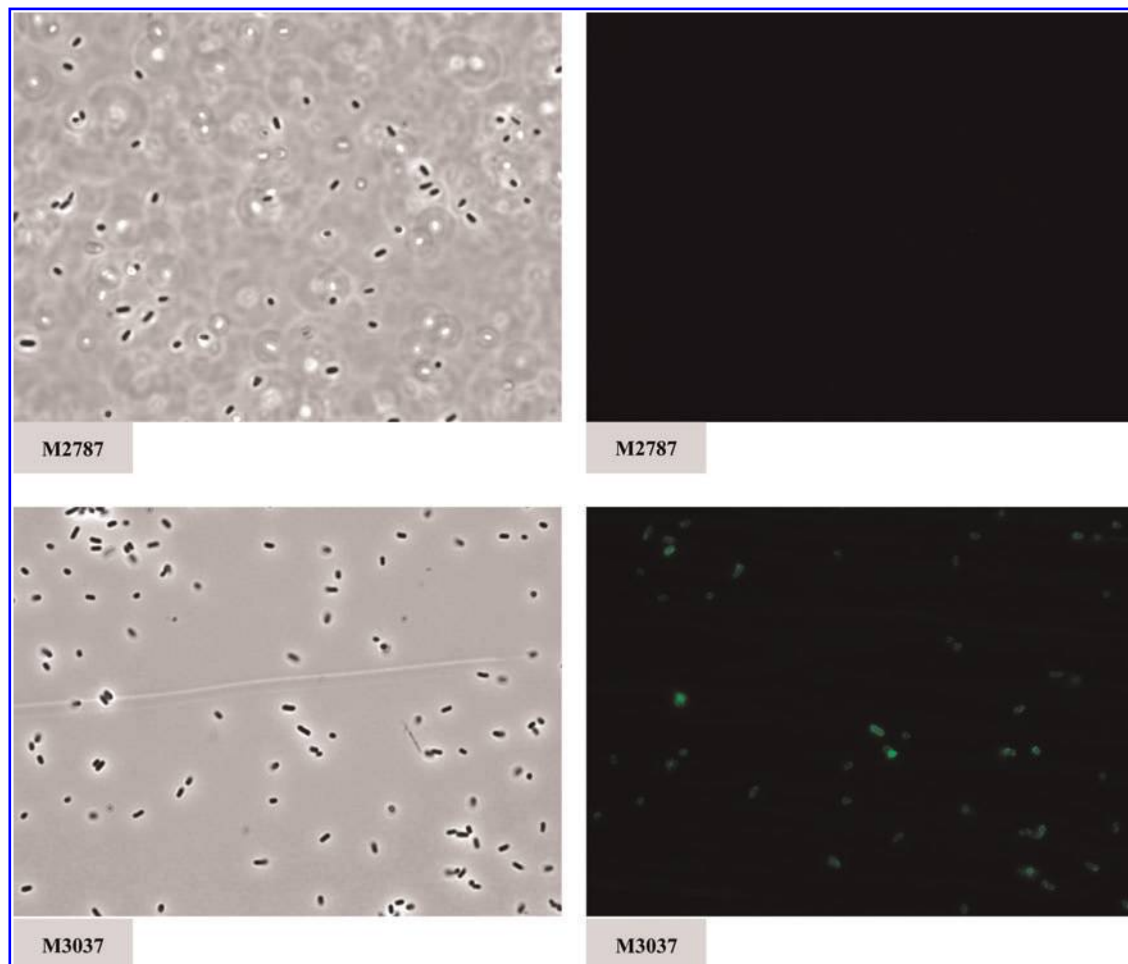

FIG. 1. Immunoflouresence staining of live *Salmonella* ser. Typhimurium DT104 cells with monoclonal antibodies. Bacteria cells were probed with all 45 MAbs followed by reaction with FITC-conjugated goat anti-mouse IgG as described in Materials and Methods. Examples of flouresence images (right) and phase-contrast images (left) of the bacterial cells probed with two MAbs (M2787 and M3037) are shown.

Agona, *Salmonella* ser. California, *Salmonella* ser. Abortus equi and *Salmonella* ser. Chester), most showed negligible cross-reactions with all 45 MAbs. However, one of the three *Salmonella* ser. Heidelberg isolates tested and the *Salmonella* ser. Chester isolate cross-reacted significantly with these MAbs. Significant cross-reactions, having an OD_{414} of greater than 0.5, were observed with *Salmonella* ser. Paratyphi A (a serogroup 1 isolate) and 13 MAbs (M3039, M3040, M3041, M3042, M3044, M3046, M3049, M3052, M3056, M3062, M3065, M3066, and M3067). Several other strains of *Salmonella* serotypes O:6 and O:9 exhibited negligible cross-reactions. All 45 MAbs showed no cross-reaction with the gram-negative bacterium *E. coli* O157:H7 and two gram-positive bacterial species of *Listeria*, *L. monocytogenes* and *L. grayi*.

Molecular nature of antigens recognized by monoclonal antibodies to Salmonella ser. Typhimurium

The ability of all 45 MAbs to bind purified *Salmonella* ser. Typhimurium LPS was demonstrated through immunoblotting. Although some MAbs gave a stronger binding signal than others, all 45 MAbs showed a similar band pattern characteristic of a LPS ladder on the immunoblot, each band differing from the next by one O-chain repeating unit (Fig. 2). The ability of the MAbs to bind equally to both the low molecular weight fragments and the high molecular weight fragments indicates that the antigen is likely the O-chain repeating unit.

Role of acetyl group in LPS in antibody-antigen binding

A highly purified preparation of LPS from *Salmonella* ser. Typhimurium as revealed by SDS-PAGE with silver staining (data not shown) showed strong reactions with all 45 MAbs by indirect ELISA (Fig. 2). This further confirmed that each MAb recognized LPS and also revealed that during the LPS purification process the integrity of the epitopes was not altered. The effect of various temperature and pH conditions on binding of all 45 MAbs to LPS was investigated using an indirect ELISA. No substantial change in the ability of each MAb to bind LPS was observed when LPS was pre-treated with PBS (pH 7.0) or acetate buffer (pH 4.6) at room temperature (Fig. 3a) or at high temperature of 100°C (Fig. 3b). However, LPS pre-treated with alkali conditions at room temperature retained only a moderate ability to interact with M3039, M3047, M3052, M3056, M3058, and M3066 and lost the ability to bind to all other MAbs. Pre-treatment of LPS in alkali conditions at 100°C completely abolished the ability of each MAb to bind LPS (Fig. 3b). Pre-treatment with alkali conditions removes the acetyl group from group B LPS while leaving the remaining structure intact.[23] A similar experiment was also carried out for other group 4 isolates, which appeared to cross-react with the MAbs. The same trend was observed with all other purified LPS from *Salmonella* ser. Heidelberg and *Salmonella* ser. Chester (data not shown). The results indicate that the presence of the acetyl group (O5 antigen) is required for binding of each MAb to LPS.

Role of lipopolysaccharide acetylase in binding of MAbs to Salmonella ser. Typhimurium

Binding of all 45 MAbs to an *oafA* gene inactivation mutant of *Salmonella* ser. Typhimurium was markedly reduced (Fig. 4) compared to *Salmonella* ser. Typhimurium DT104 (Fig. 5). The mutant lost the ability to interact with the majority of the MAbs and retained some binding to M3039, M3047, M3056, and M3058 with OD_{414} readings around 0.5.

FIG. 2. Immunoblot analysis of purified LPS from *Salmonella* ser. Typhimurium DT104. LPS (10 μg) from Sigma was loaded onto each lane of an SDS-PAGE gel, electrophoresed, electrotransferred onto nitrocellulose membranes, and probed with all 45 MAbs, followed by reaction with HRP-conjugated goat anti-mouse IgG. All 45 MAbs, exemplified here by M3037, showed reaction with LPS in a similar band pattern. Each additional band represents the addition or deletion of an O-antigen repeating unit from the band following or preceding it, respectively.

Discussion

In this study, 45 MAbs were generated against formalin-killed *Salmonella* ser. Typhimurium. These MAbs showed a high serological specificity for group 4 *Salmonella*, although they do not detect all group 4 isolates. Immunoblotting with purified LPS showed a distinctive ladder-like pattern under conditions used for carbohydrate antigens, confirming that the epitopes for these MAbs were located on the O-antigen of LPS (Fig. 2). Although the cells used for immunization were formalin killed, immunoflouresence microscopy demonstrated that the MAbs are capable of binding to live bacteria (Fig. 1). This indicates that the epitopes for these MAbs are surface exposed in live cells and were formalin resistant,

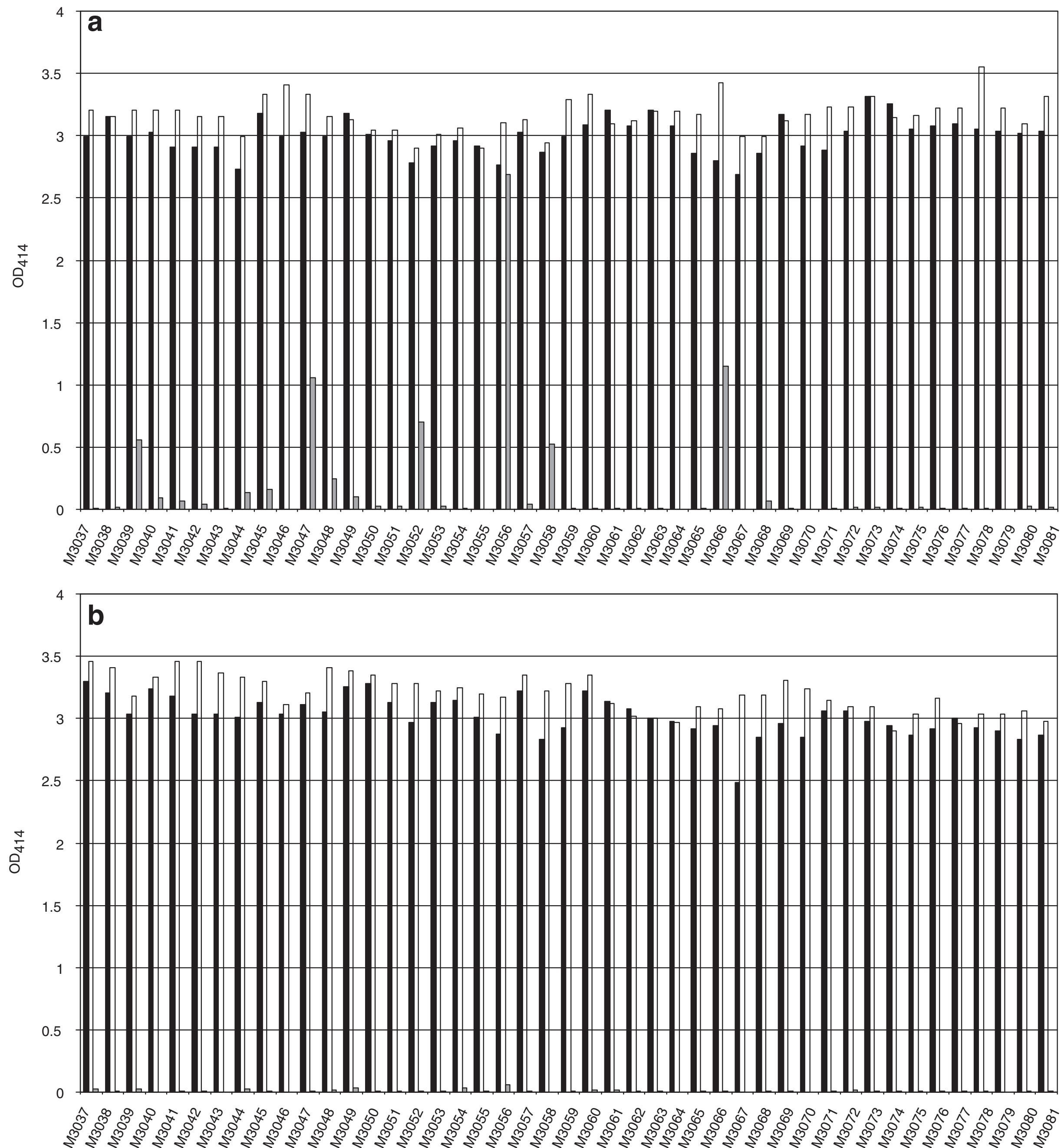

FIG. 3. ELISA analysis of monoclonal antibody interaction with chemically modified purified LPS. The first bar (■) represents LPS treated in acetate buffer at pH 4.6, second bar (□) represents LPS treated in PBS at pH 7.0, and the third bar (■) represents LPS treated in NaOH at pH 9.6 for 30 min at room temperature (**a**) or 100°C (**b**). Each well of the ELISA plate were coated with ~1 μg of pre-treated LPS diluted in 100 μl of carbonate buffer. All ELISA OD values are the average of two determinations.

consistent with the nature of the epitope being the LPS O-antigen. This also suggests that these antibodies may be useful in capturing live *Salmonella* pathogen from food, water, or environmental sources.

Evidence that the presence of the acetyl group, which confers the O:5 serotype, is essential for the binding of all 45 MAbs to the *Salmonella* LPS was shown by assessing the ability of the MAbs to react with *Salmonella* ser. Typhimurium both before and after acetyl-group removal. The acetyl group was removed using two independent methods: first through chemical removal of the acetyl group with heat and alkaline conditions and through creation of a mutant without a functional acetylase, which is required for the synthesis of the O:5 antigen.[12] Although, this study delivers a novel set of 45 MAbs reactive to *Salmonella* ser. Typhimurium LPS, others have made similar findings concerning the significance of the O:5 antigen in serological assays with both MAbs and with polyclonal antisera.[11–13]

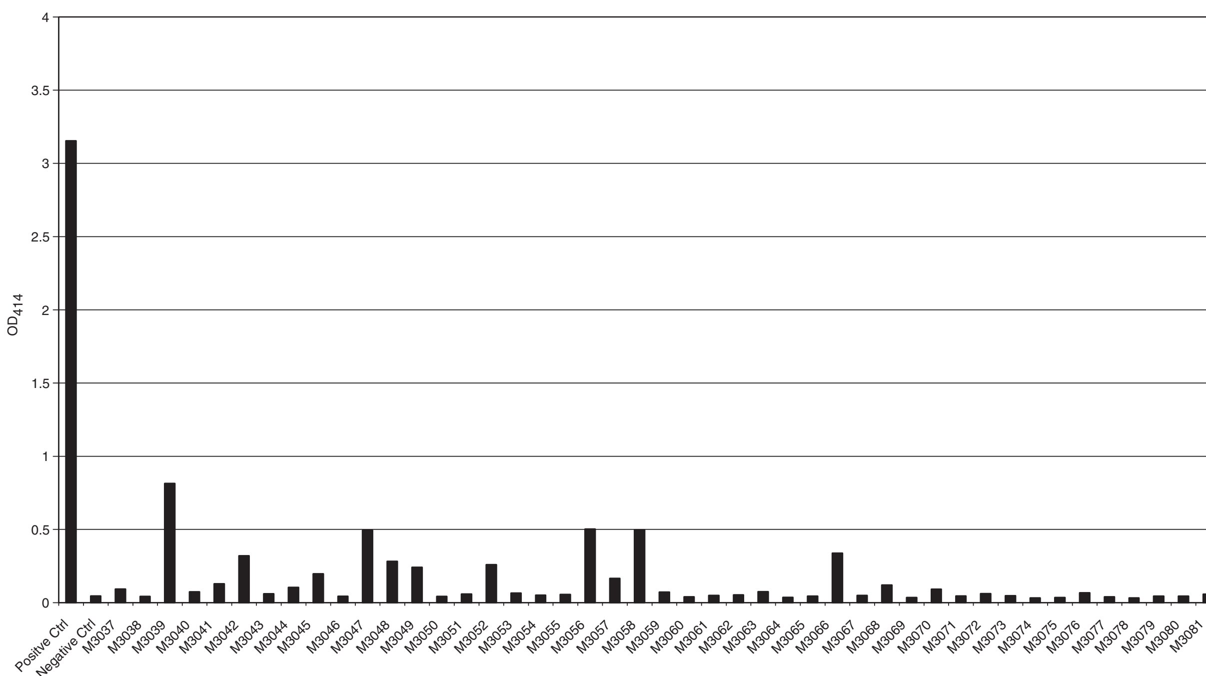

FIG. 4. ELISA analysis of monoclonal antibody interaction with an acetylase negative mutant of *Salmonella* ser. Typhimurium. ELISA plates were coated with formalin-killed cells of *Salmonella* ser. Typhimurium *oafA* 14027 oafA127::TN 10d-Km[15] at a concentration of approximately 1.0×10^8 cells/well. All ELISA OD values are the average of two determinations.

Salmonella ser. Typhimurium is divided into two named serological variants, non-Copenhagen and Copenhagen based on the presence or absence of the O:5 antigen, respectively. The presence of the O:5 antigen requires an LPS acetylase, which is chromosomally encoded, unrelated to phage conversion, and may or may not be present in wild-type *Salmonella* isolates.[12] Other studies have indicated that *Salmonella* ser. Typhimurium variants can differ in the percentage of acetylated abequose residues they possess.[24] It is likely that other group O:4 isolates also vary in the relative abundance of abequose acetylation. Variation in the percentage of acetylated abequose residues, along with their absolute

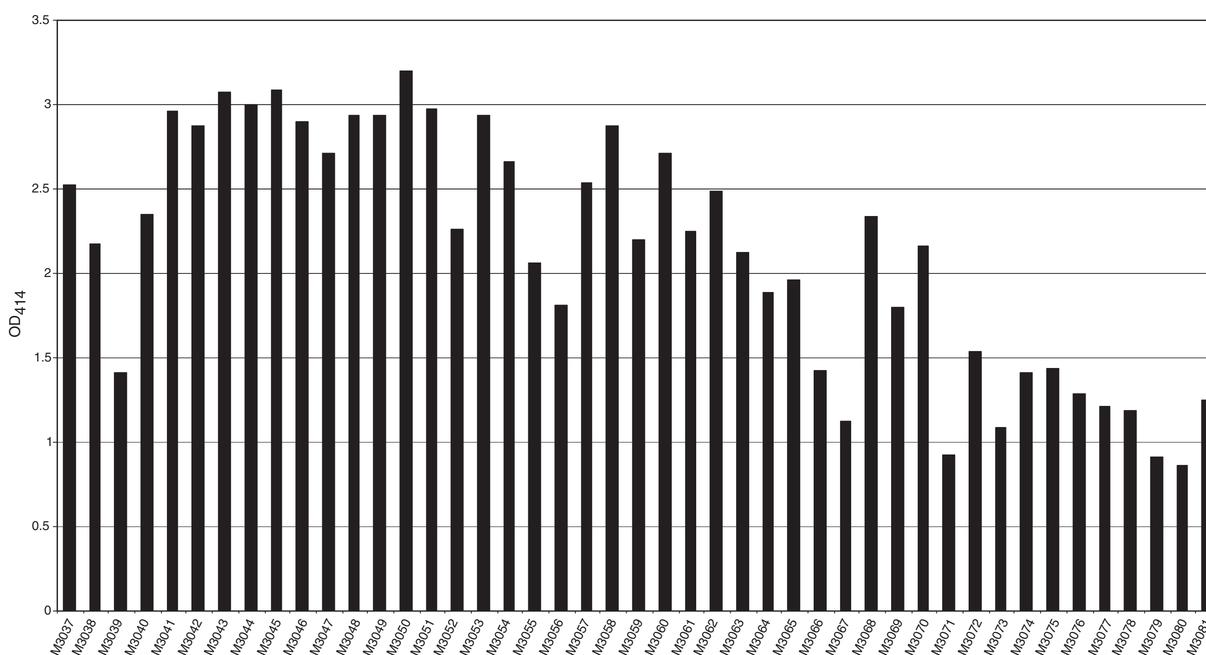

FIG. 5. ELISA analysis of monoclonal antibodies against *Salmonella* ser. Typhimurium DT104. ELISA plates were coated with purified LPS from *Salmonella* ser. Typhimurium DT104 at a concentration of $1\,\mu g/100\,\mu L$. All ELISA OD values are the average of two determinations.

presence or absence is a possible explanation for the variation in reactions between different group O:4 isolates and the panel of 45 MAbs. Evidence for this can be found in the cross-reactivity of the MAbs against *Salmonella* ser. Agona. Although this isolate has the same 1,4,[5],12 serotype as *S.* ser. Typhimurium, it reacted very weakly with most MAbs and not at all with others. This lack of reaction is too pronounced to be explained simply by a difference in the percentage of acetylated residues and is likely the result of the LPS acetylase gene and therefore acetyl group not being present in this particular isolate. This is also the likely cause of the reaction pattern observed for the three *Salmonella* ser. Heidelberg isolates that were examined in this study. One exhibited a strong reaction to the panel of MAbs and the other two did not (Table 2). These observations suggest that other group 4 *Salmonella* such as ser. Heidelberg could be divided into sub-groups based on the presence of the O:5 antigen, similar to *Salmonella* ser. Typhimurium, since they are also serologically distinct.

This study has demonstrated that acetylation of the abequose residue is a critical factor in determining the binding specificity of the MAbs generated here against the O-antigen of LPS. Other studies have shown similar results as antibodies produced against fully acetylated LPS do not recognize deacetylated LPS.[13] There are two proposed models to explain this. The first model suggests that the acetylated abequose residue is the most immunodominant antigen and the majority of the antibodies produced during immunization will be directed against this specific epitope.[25–27] The second and more recent model suggests that the acetylated abequose residue alters the conformation of the entire *Salmonella* O-antigen, thereby changing the spectrum of available conformational epitopes.[15,28] This is reasonable since multiple conformations are known to occur in oligosaccharides due to the fluidity of the glycosidic bonds that connect the sugar residues.[29] Our observations support the second model since minor cross-reactions are observed with non-acetylated but closely related isolates (Table 2). If the actual acetylated abequose residue was the epitope, the reactions would be more binary in contrast to the spectra of cross-reactions that were observed here. It is likely that the spectra of cross-reactions with non-acetylated LPS can be explained by conformations that are slightly less than optimal for antibody binding. This study raises interesting prospects about the humoral immune response to carbohydrate antigens, and more studies are needed to understand what determines the choice antigens for a humoral response.

Another interesting observation produced from this study is the mild cross-reactions with *Salmonella* ser. Abortus equi[4,12] and *Salmonella* ser. Paratyphi A.[1,2,12] The most likely explanation for this fits with the previous explanation in that acetylated abequose alters the immunogenic epitopes present elsewhere on the LPS structure. The epitope that the MAbs are binding to is likely on the main part of the chain, the O:12, which is composed of repeating mannose, rhamnose, and galactose; however, in *S.* ser. Typhimurium, the stereochemistry of this repeating unit is altered by the acetylated abequose residue.[11] This fits with the *Salmonella* ser. Abortus equi and *Salmonella* ser. Paratyphi A data since the O:12 epitope is still present, but not in optimal conformation since it is lacking the acetylated abequose residue, leading to a mild cross-reaction. This observation is interesting and should be examined more fully by testing more isolates that lack the O:12 epitope in addition to *S.* subsp. *arizonae* and *S.* ser. Thompson (Table 2) for cross-reactivity. Some MAbs with similar properties have been produced, such as 8aC10, which reacts optimally with acetylated group 4 LPS but also cross-reacts with LPS containing the O:8 antigen.[30] Interestingly the cross-reactivity of 8aC10 with O:8 containing LPS is observed when using whole cell antigen but is lost when using purified LPS, although it reacts optimally with group 4 LPS regardless of whether whole cell or purified LPS is used. This difference in epitope nature suggests a true cross-reaction where the epitopes are similar but not identical.[30] Our observations of cross-reactions with *Salmonella* ser. Abortus equi and *Salmonella* ser. Paratyphi A could also be explained by the presence of epitopes that are similar but not identical to those in group 4 isolates. An alternate possibility is that the structural changes to the oligosaccharide chain are effected by more than the acetylation status of the abequose residue. Previous work has shown that the conformation of fatty acid chain in glycolipids can regulate the structure of the attached polysaccharide.[29] The structure of the lipid A tail anchoring the oligosaccharide to the cell surface may be playing a role in its structure and therefore affecting the presence and absence of conformational epitopes, in addition to acetylation status, although this would have to be confirmed by future work.

Some MAbs produced in this study, such as M3046, M3059, and M3080, are very specific for group 4 and have very low levels of cross-reactions with isolates in other serogroups. These antibodies may be useful in diagnostics. However, it would be important to develop a second MAb that binds to non-acetylated isolates that could work in synergy with these MAbs so that all group 4 *Salmonella*, even non-acetylated variants, would be detected.

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Author Disclosure Statement

The authors have no financial conflicts to declare.

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