Original Article

Comparison of Lipopolysaccharide and Protein Immunogens from Pathogenic *Yersinia enterocolitica* Bio-serotype 1B/O:8 and 2/O:9 using SDS-PAGE*

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Abstract

Objective *Yersinia enterocolitica* is an extracellular pathogen and its related antigens interact with the host immune system. We investigated the difference in immunological characteristics between a highly pathogenic and poorly pathogenic strain of *Y. enterocolitica.*

Methods We used SDS-PAGE and western blotting to characterize lipopolysaccharide (LPS), *Yersinia* outer membrane proteins (Yops), membrane proteins, and whole-cell proteins from poorly pathogenic *Y. enterocolitica* bio-serotype 2/O:9, isolated from China, and highly pathogenic bio-serotype 1B/O:8, isolated from Japan.

Results These two strains of *Y. enterocolitica* had different LPS immune response patterns. Comparison of their Yops also showed differences that could have accounted for their differences in pathogenicity. The membrane and whole-cell proteins of both strains were similar; immunoblottting showed that the 35 kD and perhaps the 10 kD proteins were immunogens in both strains.

Conclusion The major antigens of the two strains eliciting the host immune response were the LPS and membrane proteins, as shown by comparing protein samples with reference and purified preparations.

Key words: Immunogens; Lipopolysaccharides; Outer membrane proteins; Whole-cell proteins; Pathogenic *Yersinia enterocolitica*

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INTRODUCTION

Yersinia enterocolitica is a Gram-negative enteropathogen causing gastroenteritis and severe diarrhea in children. In adults, typical symptoms include terminal ileitis and mesenteric lymphadenitis. The bacterium invades the Peyer's patches through the M cells; this leads to proliferation of polymorphonuclear leukocytes and formation of abscesses infected with extracellular *Yersinia*; finally, the cytoarchitecture of the Peyer's patches is completely destroyed[1-2]. The optimum growth temperature for this bacterium is 25 °C; however, pathogenicity is

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acquired when the temperature rises to 37 °C. There is evidence for differences in growth and metabolism when the organism is cultivated at these two temperatures[3-4].

Lipopolysaccharide (LPS) may be one of the principal pathogenic factors of *Y. enterocolitica*. The typical LPS has three major domains: O antigen, core region, and lipid A[5]. Pathogenic *Yersinia* have a 70 kb virulence plasmid called pYV that codes for the pathogenicity loci[1]. When incubated at 37 °C in the absence of Ca^{2+}, *Y. enterocolitica* produces large amounts of plasmid-encoded proteins called *Yersinia* outer membrane proteins (Yops)[6]. Heesemann et al. has shown that Yops are secreted proteins that suppress and damage the host's immune system[10]. The Yops comprise three groups of components: the injectisome, translocators and effectors[7].

Y. enterocolitica is divided into 6 distinct biotypes and 60 serotypes. The 1B/O:8 strain is common and is a highly pathogenic bio-serotype, whereas bio-serotype 2/O:9 is the prominent type in China. We used these strains here because we considered them to be typical examples. *Y. enterocolitica* is an extracellular pathogen and its related antigens interact with the host immune system. To determine the difference in immunological characteristics between a highly pathogenic strain (bio-serotype 1B/O:8) and a poorly pathogenic strain (bio-serotype 2/O:9), we selected LPS, Yops, membrane proteins and whole-cell proteins as antigens and compared host immune responses to them.

MATERIALS AND METHODS

Bacterial Strains

Y. enterocolitica bio-serotype 1B/O:8 (Ye92010) was provided by Dr. H. Fukushima of the Public Health Institute of Shimane Prefecture, Japan[8], and *Y. enterocolitica* bio-serotype 2/O:9 (Ye8629) and *Brucella abortus* biotype 3 (2006018) were isolated by our laboratory[9] (Table 1). We used the serological cross-reaction with *B. abortus* for comparison purposes.

Table 1. Bacterial Strains Used in This Study

Strain Number	Isolate	Source	Location	Isolation Date	Serotype	Biotype
Ye92010	*Yersinia enterocolitica*	Patient	Aomori Prefecture/Japan	1992	O:8	1b
Ye8629	*Yersinia enterocolitica*	Patient	Zheng zhou city/China	1986	O:9	2
2006018	*Brucella abortus*	Patient	Zhe jiang Province/China	2006	-	3

Antiserum Preparation

Healthy female New Zealand rabbits weighing 2.5-3.0 kg were used. Antigens were prepared from *Y. enterocolitica* strains Ye92010 and Ye8629 cultured for 24 h at 25 °C on Brain Heart Infusion (BHI) agar; *B. abortus* strain 2006018 was cultured for 24 h at 37 °C on Brucella agar. The cultures were inactivated using 1% formaldehyde NaCl and the McFarland (McF) values of the suspensions were adjusted to 1.2 (Ye92010), 1.6 (Ye8629), and 1.2 (2006018). The rabbits were injected through the auricular veins with 1 mL of the suspension, and injected again 3 weeks later as a booster. Sera were collected 14 days after the booster.

LPS Preparation

The Ye92010 and Ye8629 strains were grown on BHI agar for 24 h at 25 and 37 °C, respectively, then harvested by centrifugation (6 000 × g at 4 °C for 10 min), suspended in 24 mL 0.85% NaCl solution and adjusted to McF >7.5, but the numbers of bacteria did not exceed 10^{10}cfu/mL. Strain 2006018 was grown on Brucella agar for 24 h at 37 °C. The culture was harvested in 24 mL 0.85% NaCl solution as above.

The bacterial suspension was heat-inactivated for 40 min at 95 °C. The LPS from these bacteria was extracted using an LPS Extraction Kit (iNtRON Biotechnology, Korea) following the manufacturer's instructions.

Isolation of Yops Secreted by Y. enterocolitica

Using the methods of Heesemann et al.[10] and Michiels et al.[6], Ye92010 and Ye8629 strains were inoculated on BHI agar for 24 h at 25 °C, and then single colonies were inoculated into BHI broth at 25 °C with overnight shaking. The two cultures were diluted 1:20 with fresh BHI broth (Ye92010 OD_{600} =0.09, Ye8629 OD_{600} =0.10) and incubated with shaking at

37 °C for 90 min. The media were supplemented with filter-sterilized 10 mmol/L EGTA, and the incubation was continued for 90 min at 37 °C (Ye92010 OD_{600} =0.97, Ye8629 OD_{600} =0.91). The bacterial cells were removed by centrifugation at 6 000 ×g at 4 °C for 10 min, and the clarified culture supernatants were filter-sterilized (0.22 μm). Proteins were precipitated from the culture supernatant at 4 °C overnight after addition of solid ammonium sulfate (40 g/100 mL supernatant). On the next day, proteins were pelleted by centrifugation at 10 000 ×g at 4 °C for 20 min and dissolved with a solution containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, and 1% DTT.

PCR Test for yadA and virF Genes Located on pYV plasmid

To verify the different pYV plasmid sequence between two strains, we performed a PCR test for *yadA* and *virF* genes located on pYV plasmid. Primers for *yadA* were 5'CTTCAGATACTGGTGTCGCTGT3' and 5'ATGCCTGACTAGAGCGATATCC3'; and *virF* were 5'GGCAGAACAGCAGTCAGACATA3' and 5'GGTGAGC ATAGAGAATACGTCG3'. Amplification was performed in a 20-μL reaction volume. Annealing temperature for *yadA* was 55 °C and 63 °C for *virF*.

Isolation of Bacterial Membrane Protein

Using the methods of Heesemann et al.[10] and Mazza et al.[11], Ye92010 and Ye8629 were cultured as Yops above for 24 h at 25 and 37 °C, respectively. The cultures were diluted 1:100 with fresh BHI broth and incubation was continued with shaking at 25 and 37 °C for 8 h (stationary phase), and cells were collected by centrifugation (6 000 × g at 4 °C for 10 min). The pellets were resuspended in 50 mmol/L Tris–HCl (pH 7.5) and lysed using a SONICS VCX 750 ultrasonic cell disrupter (Sonics, USA) (2 s pulses and 2 s pauses). Unbroken cells were removed by centrifugation at 6 000 × g at 4 °C for 10 min. We added 0.1 mol/L Na_2CO_3 (pH 11) to the supernatants and mixed in an ice bath gently for 1 h, and then centrifuged the suspensions at 100 000 ×g at 4 °C for 1 h. The pellets were resuspended in 50 mmol/L Tris–HCl (pH 7.5) and centrifuged again at 100 000 ×g at 4 °C for 1 h. Finally, the membrane proteins were dissolved in a solution of 5 mol/L urea, 2 mol/L thiourea, 1% SB3-10, 1% ASB-14, 2% CHAPS, and 1% DTT.

Isolation of Whole-cell Protein

Using the methods of Görg et al.[12], we cultured the bacteria as described above for isolation of membrane protein. The bacteria were resuspended in distilled water and sonicated using three bursts with a SONICS VCX 750 (2 s pulses, 2 s pauses). The resulting suspension was incubated with 125 μg/mL RNase A, 10 U/mL DNase Ⅰ (Promega, USA) for 1 h at room temperature. The suspension was dissolved in a solution of 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, and 1% DTT.

Purification of Protein Samples

To eliminate lipids, polysaccharides and other non-protein components, we purified the 3 protein fractions using a protein cleaning kit (Amersham, British) and compared the immunoblotting results of the reference preparations with the purified samples.

SDS-PAGE

Using the method of Mazza et al.[11], SDS-PAGE was performed at a constant voltage of 100 V in a discontinuous system with a 12.5% polyacrylamide separating gel and a 5% polyacrylamide stacking gel. Each sample was mixed with an identical volume of 2× loading buffer, and boiled for 10 min at 100 °C. Molecular weight standards (ProSieve Color Protein Markers) were used to estimate the molecular weights of the separated components.

Silver and Coomassie Blue Staining

A ProteoSilver™ Silver Stain Kit (Sigma–Aldrich, St Louis, MO, USA) was used for silver staining and the method of Fazekas de St. Groth et al.[13] was used for Coomassie blue staining. All of the stained gels were scanned by UMAX PowerLook 2100XL scanner and analyzed with MagicScan software (UMAX Technologies, USA).

Western Blotting

For western blotting, the polyacrylamide gel was prepared as described above. After electrophoresis, the separated proteins were transferred to a PVDF membrane in a transblot cell (Amersham) at 100 V for 1.5 h in an ice bath. The western blotting procedure was based on that described by Li et al.[14]. The membrane was blocked with 5% skimmed milk in PBS at 4 °C overnight, and washed on the next day at room temperature by shaking with two changes of PBS containing 0.05% Tween-20 (PBST). Antiserum for each strain was diluted in PBS containing 5% skimmed milk and 0.05% Tween-20, and incubated with slow shaking at room temperature for 2 h. The Ye92010

antiserum was diluted 1:1 000, the Ye8629 antiserum 1:2 000 and the 2006018 antiserum 1:500. After incubation with the first antibody, membrane was washed at room temperature by shaking with three changes of PBST. Fluorescein-conjugated goat anti-rabbit IgG (IRDye800; Rockland, Gilbertsville, PA, USA) was diluted to 1:10 000 as the second antibody. The PVDF membrane was washed again in PBST by shaking with three changes at room temperature, and scanned with LI-COR Odyssey scanner (LI-COR Biosciences, USA) and analyzed with Odyssey software.

RESULTS

LPS

Silver Staining The SDS-PAGE profiles of LPS had similar visible band distributions for *Y. enterocolitica* bio-serotype 1B/O:8 (Ye92010) and 2/O:9 (Ye8629) incubated at 25 °C (Figure 1A). All the bands were between 12 and 176 kD according to the molecular markers. *B. abortus* biotype 3 (2006018) incubated at 37 °C had a different silver staining pattern; the region between 41 and 65 kD was dispersed and showed no typical band pattern.

When the growth temperature was raised to 37 °C, the band patterns of *Y. enterocolitica* bio-serotype 1B/O:8 and 2/O:9 became indistinct and changed for both strains, especially in the high-molecular-weight region (Figure 1B).

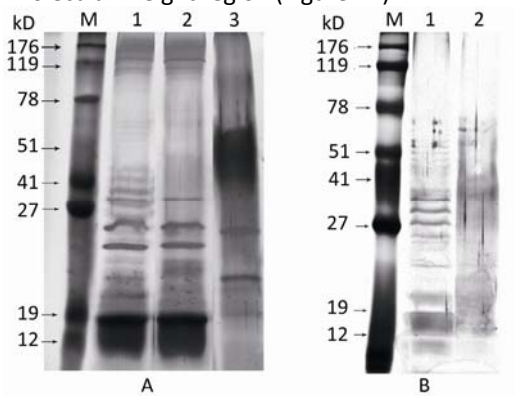

Figure 1. A. Silver staining of LPS from *Y. enterocolitica* bio-serotype 1B/O: 8 (Ye92010) and 2/O: 9 (Ye8629) incubated at 25 °C and *B. abortus* (2006018) at 37 °C. Lane 1, Ye92010 (20 μg); lane 2, Ye8629 (20 μg); lane 3, 2006018 (20 μg). B. Silver staining of LPS profiles of the strains incubated at 37 °C. Lane 1, Ye92010 (20 μg); lane 2, Ye8629 (20 μg).

Western Blotting The bands visible in western blots of LPS at different growth temperatures ranged from 12 to 78 kD. *Y. enterocolitica* bio-serotype 1B/O:8 had typical bands (Figure 2A, lane 1), whereas the bands from *Y. enterocolitica* bio-serotype 2/O: 9 and *B. abortus* biotype 3 were smeared (Figure 2B, lane 2; 2C, lane 3); neither showed obvious bands. *Y. enterocolitica* bio-serotype 1B/O:8 did not cross-react with *B. abortus* biotype 3, however, *Y. enterocolitica* bio-serotype 2/O:9 showed some cross-reactivity in the region 23-45 kD with *B. abortus* biotype 3 (Figure 2B and C).

Western blots of the LPS band distribution of two pathogenic *Y. enterocolitica* strains after incubation at 25 and 37 °C were substantially alike (Figure 2A and D, lane1; 2B and E, lane 2).

All LPSs were tested with preimmune rabbit serum and showed no bands.

Yops

Coomassie Blue Staining and PCR We extracted the Yops of *Y. enterocolitica* at 37 °C in the absence of Ca^{2+}. There were differences between the two strains: bio-serotype 1B/O:8 yielded typical protein patterns[6,15], but only 3 proteins were induced in bio-serotype 2/O:9 (Figure 3A).

PCR of the genes *yadA* and *virF*, located on the pYV plasmid, gave fragments showing that yadA was not in the same position in the two strains, but *virF* was (Figure 3B).

Western Blotting After immunoblotting, we found no bands using purified samples from Ye92010 and Ye8629. *Yops tested* with preimmune rabbit serum showed no bands.

Membrane Proteins

Coomassie Blue Staining The membrane proteins were distributed between 26 and 118 kD and Ye92010 and Ye8629 were similar. The membrane protein band profiles after incubation at different temperatures were almost identical for each strain (Figure 4A).

Western Blotting The immunoblot bands for the purified membrane proteins of *Y. enterocolitica* bio-s erotype 1B/O:8 and 2/O:9 were between 10 and 39 kD. Four bands in both strains reacted with bio-serotype 1B/O:8 (Ye92010) antiserum; their molecular weights were 35, 26, 18, and 10 kD. Two bands reacted with preimmune serum at 26 and 18 kD (Figure 4B). The immunoblot of bio-serotype 2/O:9 (Ye8629) was similar to that of bio-serotype 1B/O:8;

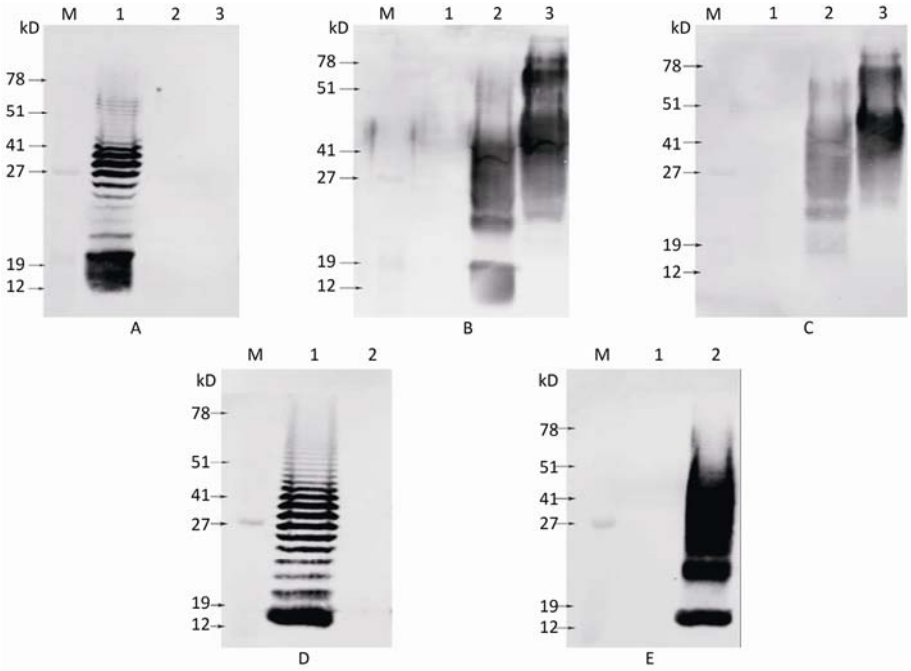

Figure 2. A. Immunoblot of LPS from *Y. enterocolitica* bio-serotype 1B/O:8 (Ye92010) and 2/O:9 (Ye8629) incubated at 25 °C and *B. abortus* (2006018) at 37 °C, using anti-Ye92010 serum. Lane 1, Ye92010 (7 μg); lane 2, Ye8629 (7 μg); lane 3, 2006018 (7 μg). B. Immunoblot of LPS using anti-Ye8629 serum. Lane 1, Ye92010 (7 μg); lane 2, Ye8629 (7 μg); lane 3, 2006018 (7 μg). C. Immunoblot of LPS using anti-*B. abortus* 2006018 serum. Lane 1, Ye92010 (7 μg); lane 2, Ye8629 (7μg); lane 3, 2006018 (7 μg). D. Immunoblot of LPS from *Y. enterocolitica* bio-serotype 1B/O:8 (Ye92010) and 2/O:9 (Ye8629) incubated at 37 °C using anti-Ye92010 serum. Lane 1, Ye92010 (7 μg); lane 2, Ye8629 (7 μg). E. Immunoblot of LPS incubated at 37 °C using anti-Ye8629 serum. Lane 1, Ye92010 (7 μg); lane 2, Ye8629 (7 μg).

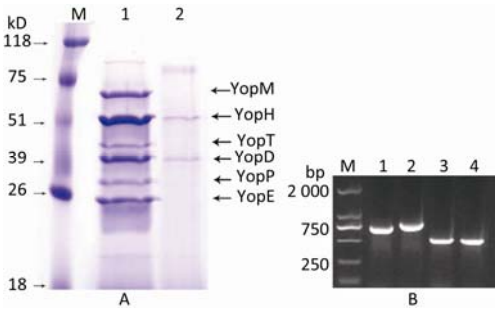

Figure 3. A. Coomassie blue staining for the Yops of *Y. enterocolitica* bio-serotype 1B/O:8 (Ye92010) and 2/O:9 (Ye8629). Lane 1, Ye92010 (40 μg); lane 2, Ye8629 (40 μg). B. PCR results of plasmid pYV *yadA* and *virF* in the two strains. Lane 1, *yadA*-Ye92010; lane 2, *yadA*-Ye8629; lane 3, *virF*-Ye92010; lane 4, *virF*-Ye8629.

we observed 3 bands after reaction with bio-serotype 2/O:9 (Ye8629) antiserum, with molecular weights 35, 18, and 10 kD. Two bands, again with molecular weights of 26 and 18 kD, were present after immunoblotting with preimmune serum (Figure 4C). In both strains, the purified membrane proteins after incubation at 25 and 37 °C were almost the same and the bands appeared to be identical.

We compared the immunoblots with purified membrane proteins using the method of Heesemann et al.[10]. The blots showed many bands between 39 and 118 kD, and between 18 and 26 kD (Figure 4D), and the patterns were similar to the LPS immunoblots for each strain. We believe that the bands were LPS contained in the protein samples prepared using the reference method[10]. The immunogens of the membrane proteins for both strains were revealed after reaction with the purified samples.

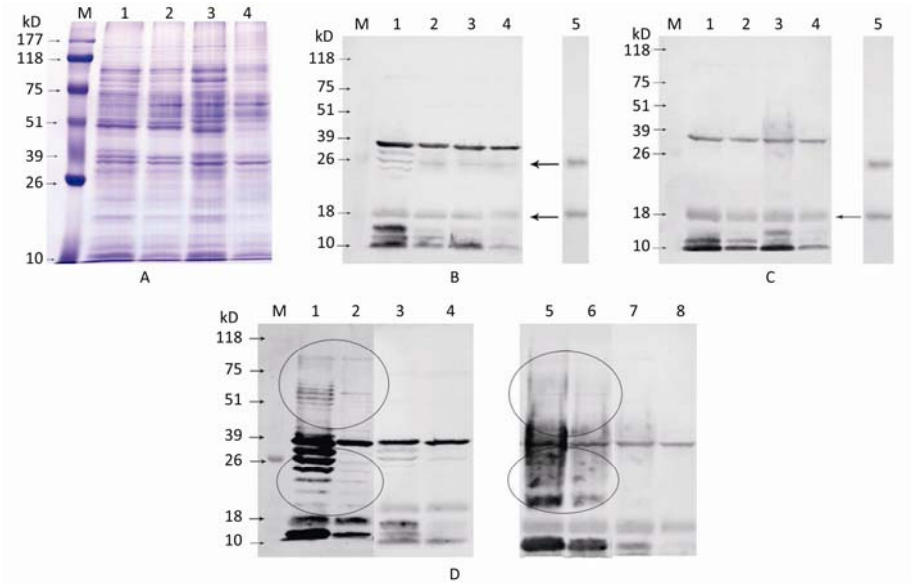

Figure 4. A. Coomassie blue staining of *Y. enterocolitica* bio-serotype 1B/O:8 (Ye92010) and 2/O:9 (Ye8629). Lane 1, Ye92010 at 25 °C (40 μg); lane 2, Ye92010 at 37 °C (40 μg); lane 3, Ye8629 at 25 °C (40 μg); lane 4, Ye8629 at 37 °C (40 μg). B. Immunoblots using anti-Ye92010 serum. Lane 1, Ye92010 at 25 °C (20 μg); lane 2, Ye92010 at 37 °C (20 μg); lane 3, Ye8629 at 25 °C (20 μg); lane 4, Ye8629 at 37 °C (20 μg); lane 5, preimmune serum. C. Immunoblots using anti-Ye8629 serum. Lane 1, Ye92010 at 25 °C (20 μg); lane 2, Ye92010 at 37 °C (20 μg); lane 3, Ye8629 at 25 °C (20 μg); lane 4, Ye8629 at 37 °C (20 μg); lane 5, preimmune serum. D. Comparison immunoblots using unpurified and purified protein samples with each antiserum (left: immunoblot using anti-Ye92010 serum; and right: immunoblot using anti-Ye8629 serum). Lane 1, Ye92010 at 25 °C unpurified (20 μg); lane 2, Ye92010 at 37 °C unpurified (20 μg); lane 3, Ye92010 at 25 °C purified (20 μg); lane 4, Ye92010 at 37 °C purified (20 μg); lane 5, Ye8629 at 25 °C unpurified (20 μg); lane 6, Ye8629 at 37 °C unpurified (20 μg); lane 7, Ye8629 at 25 °C purified (20 μg); and lane 8, Ye8629 at 37 °C purified (20 μg). The cycles of Figure 4D stand for different immunoblot results for the membrane proteins of the two strains incubated at different temperatures.

Whole-cell Proteins

Coomassie Blue Staining In Ye92010 and Ye8629, the whole-cell proteins were between 19 and 176 kD, and the band patterns were similar. They were almost identical after incubation at different temperatures (Figure 5A).

Western Blotting The immunoblot bands of the purified whole-cell proteins from *Y. enterocolitica* bio-serotype 1B/O:8 and 2/O:9 were between 10 and 118 kD. Five bands in both strains reacted with bio-serotype 1B/O:8 (Ye92010) antiserum; the molecular weights were 63, 35, 26, 18, and 10 kD. Two bands at 26 and 18 kD reacted with preimmune serum (Figure 5B). The immunoblots for bio-serotype 2/O:9 (Ye8629) were similar to those for bio-serotype 1B/O: 8; again, there were 5 bands after reaction with bio-serotype 2/O:9 (Ye8629) antiserum at 97, 63, 35, 18, and 10 kD. Again, 2 bands at 26 and 18 kD reacted with preimmune serum (Figure 5C). The purified whole-cell proteins from each strain yielded similar band patterns. They were almost identical after incubation at different temperatures.

We performed the same procedure as for the membrane proteins and compared the immunoblots of purified whole-cell proteins to those using the method of Görg et al [12] . There were many bands between 39 and 51 kD and between 18 and 26 kD (Figure 5D). As previously, we supposed that these bands were LPS contained in the protein samples prepared using the reference method.

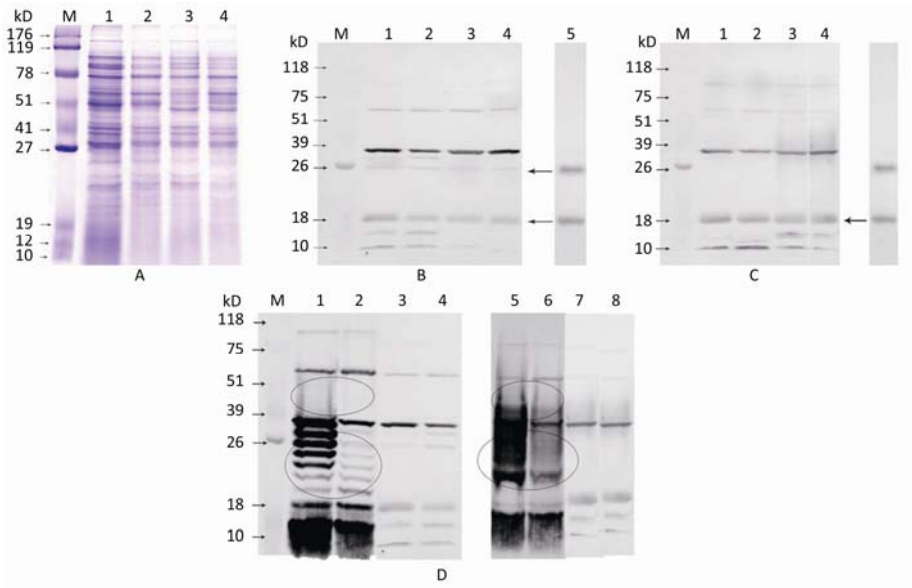

Figure 5. A. Coomassie blue staining of Y. enterocolitica bio-serotype 1B/O:8 (Ye92010), 2/O:9 (Ye8629). Lane 1, Ye92010 at 25 °C (40 μg); lane 2, Ye92010 at 37 °C (40 μg); lane 3, Ye8629 at 25 °C (40 μg); lane 4, Ye8629 at 37 °C (40 μg). B. Immunoblot using anti-Ye92010 serum. Lane 1, Ye92010 at 25 °C (20 μg); lane 2, Ye92010 at 37 °C (20 μg); lane 3, Ye8629 at 25 °C (20 μg); lane 4, Ye8629 at 37 °C (20 μg); lane 5, preimmune serum. C. Immunoblot using anti-Ye8629 serum. Lane 1, Ye92010 at 25 °C (20 μg); lane 2, Ye92010 at 37 °C (20 μg); lane 3, Ye8629 at 25 °C (20 μg); lane 4, Ye8629 at 37 °C (20 μg); lane 5, preimmune sera. D. Comparison of immunoblots with unpurified and purified protein samples using each antiserum (left: immunoblot using anti-Ye92010 serum; right: immunoblot using anti-Ye8629 serum). Lane 1, Ye92010 at 25 °C unpurified (20 μg); lane 2, Ye92010 at 37 °C unpurified (20 μg); lane 3, Ye92010 at 25 °C purified (20 μg); lane 4, Ye92010 at 37 °C purified (20 μg); lane 5, Ye8629 at 25 °C unpurified (20 μg); lane 6, Ye8629 at 37 °C unpurified (20 μg); lane 7, Ye8629 at 25 °C purified (20 μg); lane 8, Ye8629 at 37 °C purified (20 μg).The cycles of Figure 5D stand for different immunoblot results for the whole-cell proteins of the two strains incubated at different temperatures.

DISCUSSION

LPS is the major immunogenic component of the Gram-negative enterobacterium *Y. enterocolitica*. Recent studies have shown the central role of serotype O:8 and O:5 LPS in the development of reactive arthritis[16], whereas other *Yersinia* virulence factors depend on the presence of the O antigen for function and/or expression[17-18]. The O antigen polysaccharide of *Y. enterocolitica* serotype O:8 is a branched pentasaccharide repeat containing *N*-acetylgalactosamine, L-fucose, D-galactose, D-mannose and 6-deoxy-D-gulose[19-20]. LPS has branched repeating pentasaccharide units, therefore, we observed the distinct repeating bands shown in Figures 1 and 2. The O antigen chain of serotype O:9 is a linear polymer of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units terminated at the reducing end by core residues[21]. Therefore, the bands of its LPS appear diffuse on immunoblots. The O polysaccharide of *B. abortus* is covalently linked to a core oligosaccharide composed of 10–100 units of a non-branching linear polymer of 1,2-linked 4,6-dideoxy-4-formamido-D-mannose units[22-23]; therefore, the banding pattern shows dispersion after silver staining and immunoblotting. The serological cross-reaction of *Y. enterocolitica* serotype O: 9 with LPS from the *Brucella* species may be due to the presence of N-acylated 4-amino-4,6-dideoxy-α-D-mannopyranosyl residues in their respective O-antigenic chains[24]; the cross-reacting region was from 23 to 45 kD (Figure 2B and C). We found no serum cross-reaction between *Y. enterocolitica* bio-serotype 1B/O:8 and *B. abortus*.

Y. enterocolitica cultures incubated at 25 and 37 °C differ in several characteristics. Acker et al. have found a growth-temperature-dependent difference in the thickness of the outer membrane[4]. Kawaoka et al. have shown that *Y. enterocolitica* serotype O:3

contains relatively more of the R-core fraction and fewer O antigens in the LPS at 37 than 25 °C[25]. We found that the O antigen band profiles in the immunoblots of both *Y. enterocolitica* strains were similar at different temperatures. Several studies[26-29] have shown that the LPS of Gram-negative bacteria, especially the enteric bacteria, are divided into 3 fractions after SDS-PAGE. We found these band patterns and distribution in bio-serotypes 1B/O:8 and 2/O:9 to be substantially the same compared to the molecular markers. Furthermore, in serum agglutination tests for each strain incubated at different temperatures, the agglutination titer was 1:2 560 for bio-serotype 1B/O:8 at both temperatures and 1:5 120 for bio-serotype 2/O:9. We therefore believe the changes in the O antigens during temperature shifts of *Y. enterocolitica* bio-serotypes 1B/O:8 and 2/O:9 to be slight.

Y. enterocolitica bio-serotype 1B/O:8 induces typical Yops when incubated at 37 °C in the absence of Ca^{2+}[6,10], whereas bio-serotype 2/O:9 has few such proteins (Figure 4A). We speculate that the two strains differ in their pYV plasmids, and the pathogenicity difference might be attributable to the differing Yops and type III secretion systems, the core of the pathogenic mechanism for *Y. enterocolitica*[1]. Our PCR observations suggest variations in the pYV plasmids of *Y. enterocolitica* bio-serotype 1B/O:8 and 2/O:9.

Furthermore, we found that the immunoblots for the Yops were not consistent with the Coomassie blue staining pattern. This suggests that the Yops do not participate in the host immune response. The Yop effectors inhibit signaling cascades and block the ability of the cell to respond to infection. Six effectors have been identified and 4 of these inhibit cytoskeleton dynamics (YopH, YopE, YopT, and YopO/YpkA). They contribute to the strong resistance of pathogenic *Yersinia* to phagocytosis by macrophages[1]. Yop effectors also promote the intracellular survival of *Yersinia* by counteracting the normal proinflammatory response of cells to infection[7]. Naumann et al. could not detect Yops in the ileal lumen or in infected Peyer's patches[30]. Hanski et al. have discovered that only small numbers of bacteria are phagocytosed if no released Yops are detectable. This is similar to the finding of Rosqvist et al. that *Yersinia pestis* grown intracellularly in HeLa cells does not express plasmid-coded proteins[31]. Therefore, when antigen-presenting cells present the Yops of *Y. enterocolitica*, the effect is to inhibit the immune response, because Yop effectors counteract immune cell activity.

The band profiles of membrane proteins after incubation at different temperatures were similar between *Y. enterocolitica* bio-serotypes 1B/O:8 and 2/O:9 with both Coomassie blue staining and immunoblotting. We speculate that the 35 and 10 kD proteins are the immunogens for the 2 strains, and the 26 and 18 kD proteins may be cross-reacting antigens that react non-specifically with natural antibodies in rabbits or with antibodies against other bacterial infections (Figure 4B and C). There were no data on the immune responses in naturally infected hosts, because it was the first time that we performed this experiment.

Comparing the immunoblotting results of membrane proteins extracted by the 2 methods, it seems that the membrane proteins and LPS located on the bacterial surface may be the major antigens provoking immune responses against *Y. enterocolitica* bio-serotypes 1B/O:8 and 2/O:9.

The whole-cell proteins of *Y. enterocolitica* bio-serotype 1B/O:8 and 2/O:9 incubated at 25 and 37 °C were similar and the 2 strains gave identical bands. Comparing the immunoblots for whole-cell proteins from the 2 strains suggests that the constituents of the bacterial surface are the predominant antigens, because the immunoblotting band profiles for the membrane and whole-cell proteins of each strain were identical.

Comparing the LPS, Yops, membrane protein and whole-cell protein band patterns, we concluded that the LPS and membrane proteins were the important antigens for *Y. enterocolitica* bio-serotypes 1B/O:8 and 2/O:9. The membrane and whole-cell proteins were conserved in these two *Y. enterocolitica* strains. The biggest differences between the 2 strains were in the Yops and in the composition and structure of the LPS O antigens.

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