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Interaction of human mannose-binding lectin (MBL) with *Yersinia enterocolitica* lipopolysaccharide

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ABSTRACT

The lipopolysaccharide (LPS) is involved in the interaction between Gram-negative pathogenic bacteria and host. Mannose-binding lectin (MBL), complement-activating soluble pattern-recognition receptor targets microbial glycoconjugates, including LPS. We studied its interactions with a set of *Yersinia enterocolitica* O:3 LPS mutants. The wild-type strain LPS consists of lipid A (LA) substituted with an inner core oligosaccharide (IC) which in turn is substituted either with the O-specific polysaccharide (OPS) or the outer core hexasaccharide (OC), and sometimes also with the enterobacterial common antigen (ECA). The LPS mutants produced truncated LPS, missing OPS, OC or both, or, in addition, different IC constituents or ECA. MBL bound to LA-IC, LA-IC-OPS and LA-IC-ECA but not LA-IC-OC structures. Moreover, LA-IC substitution with both OPS and ECA prevented the lectin binding. Sequential truncation of the IC heptoses demonstrated that the MBL targets the IC heptose region. Furthermore, microbial growth temperature influenced MBL binding; binding was stronger to bacteria grown at room temperature (22 °C) than to bacteria grown at 37 °C. In conclusion, our results demonstrate that MBL can interact with *Y. enterocolitica* LPS, however, the *in vivo* significance of that interaction remains to be elucidated.

Introduction

Lipopolysaccharide (LPS), the major surface antigen of Gram-negative bacteria also known as endotoxin, contains three main structural components: the outer membrane integrated lipid A (LA), the core oligosaccharide and, most distal, the O-specific polysaccharide (OPS), the latter consisting of different numbers of repeating units (Raetz and Whitfield, 2002; Alexander and Rietschel, 2001). The core oligosaccharide is generally divided into the LA-linked heptose-containing inner core (IC), and hexose-containing outer core (OC) to which the OPS is linked (Fig. 1A). Rough (R), in contrast to smooth (S), strains lack OPS from their LPS (Ra chemotype). Deep–rough strains (Rb–Re chemotypes) in turn have successive truncations of the core oligosaccharide (Raetz and Whitfield, 2002; Alexander and Rietschel, 2001) as illustrated in Fig. 1B.

Yersinia enterocolitica is a Gram-negative rod being an etiological agent of food- or water-transmitted diseases such as enteritis, enterocolitis, mesenteric lymphadenitis and terminal ileitis (Bottone, 1999). It may also be involved in development of post-infectious sequelae such as reactive arthritis, myocarditis, glomerulonephritis and erythema nodosum (Bottone, 1999; Huovinen et al., 2010). *Y. enterocolitica* is able to grow at a wide range of temperatures (0–42 °C) (Bottone, 1999). The ability of *Y. enterocolitica* to multiply at low temperatures as well as in an iron-rich environment

Abbreviations: ECA, enterobacterial common antigen; IC, inner core oligosaccharide; LA, lipid A; LP, lectin pathway (of complement activation); LPS, lipopolysaccharide; MASP, mannose-binding lectin-associated serine protease; MBL, mannose-binding lectin; OC, outer core oligosaccharide; OPS, O-specific polysaccharide; PAMP, pathogen-associated molecular pattern; RT, room temperature (22 °C).

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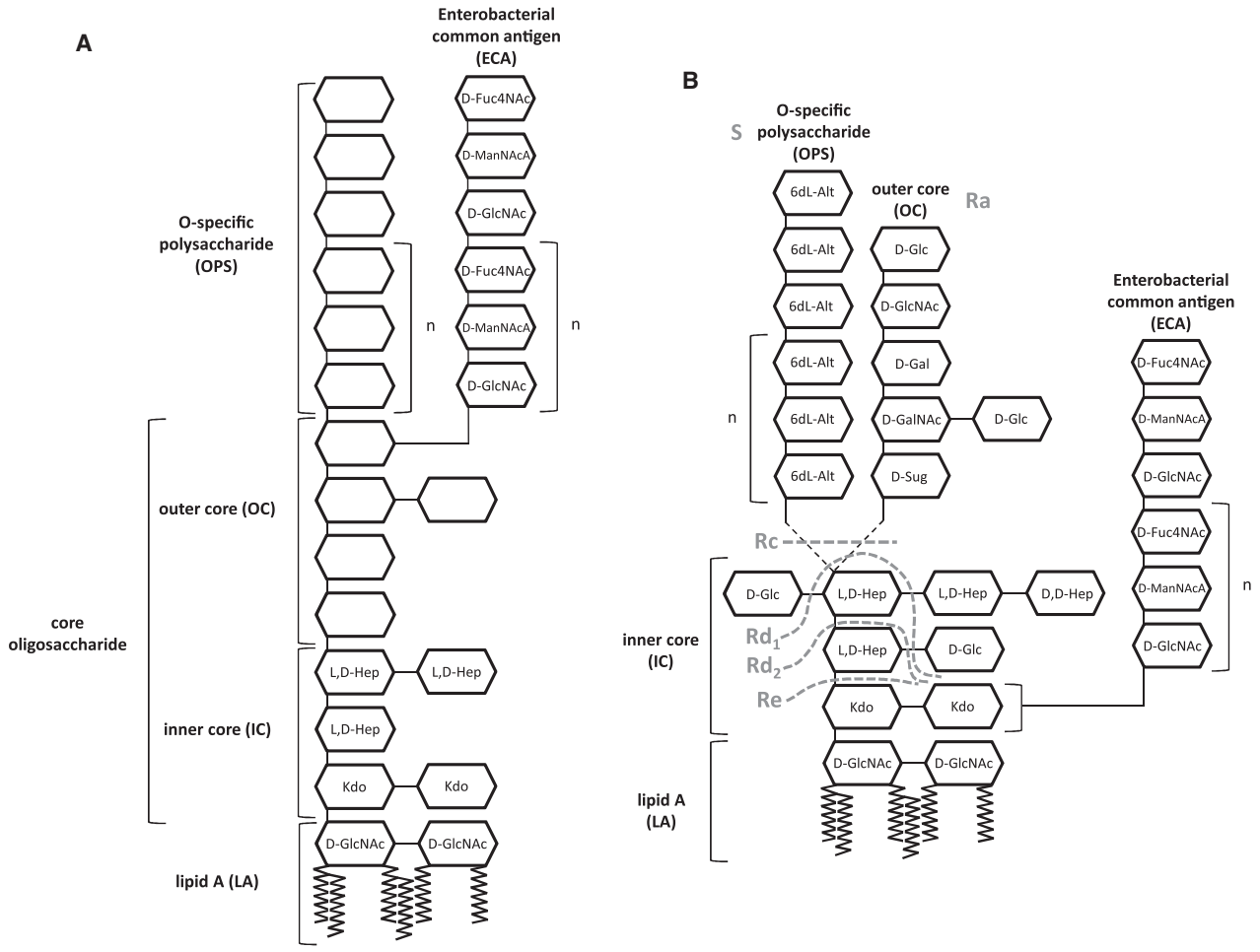

Fig. 1. Schematic structure of LPS. Panel A: LPS typical for majority of *Enterobacteriaceae*. The empty hexagons reflect the sugar residue variability in the OC and OPS structures of different strains. The zigzag lines in LA represent fatty acids. Panel B: Schematic structures of the LPS of the *Y. enterocolitica* serotype O:3 strains used in this work. The chemotypes of the O:3 LPS molecules are indicated by grey letters and the grey dashed lines indicate the point(s) of truncations: S, LA-IC-OPS; Ra, LA-IC-OC; Rc, LA-IC; Rd_1, Rd_2 and Re, LA plus truncated IC. The wild type O:3 bacteria produce a mixture of S- and Ra-type LPS molecules. The sugar residues: 6dL-Alt, 6-deoxy-L-altropyranose; D,D-Hep, D-*glycero*-D-*manno*-heptopyranose; D-Fuc4NAc, *N*-acetyl-D-fucos-4-amine (4-acetamido-4,6-dideoxy-D-galactopyranose); D-GalNAc, *N*-acetyl-D-galactosamine (2-acetamido-2-deoxy-D-galactopyranose); D-Glc, D-glucopyranose; D-GlcNAc, *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucopyranose); D-ManNAcA, *N*-acetyl-D-mannosaminouronic acid (2-acetamido-2-deoxy-D-*manno*-pyranosuronic acid); Kdo, 3-deoxy-D-*manno*-oct-2-ulopyranosonic acid; L,D-Hep, L-*glycero*-D-*manno*-heptopyranose; D-Sug, 2-acetamido-2,6-dideoxy-D-*xylo*-4-ulopyranose.

enhances the risk of life-threatening infections *via* transfusion of contaminated blood or its products and may result in septic shock with overall fatality rate of 54% (Guinet et al., 2011). Moreover, the bacterium withstands freezing very well and may survive for prolonged periods in frozen food (Norberg, 1981). The chromosome- and plasmid-located genes of *Y. enterocolitica* encode for numerous virulence factors enabling the pathogen to invade the host and to evade the immune response. The biosynthesis of various virulence factors is regulated by the bacterial growth temperature. For example, the OPS and Yst enterotoxin are optimally synthesized at room temperature (RT), whereas the virulence factors YadA (*Yersinia* adhesion protein), Ail (attachment-invasion protein) or Yops (*Yersinia* outer proteins) are expressed only at 37 °C (Bottone, 1999; Konkel and Tilly, 2000). The level of OPS synthesis at 37 °C was reported to be seven-fold lower in comparison to RT reflecting the repression of the two promoters controlling the OPS gene cluster at 37 °C (Bengoechea et al., 2002, 2004). Moreover, bacterial growth phase, low pH, iron concentration, oxygen tension and ionic strength modulate OPS expression (reviewed by Skurnik and Bengoechea, 2003).

Y. enterocolitica O:3 (YeO3) is the most frequently isolated causative agent of yersiniosis in Europe (Huovinen et al., 2010; Rastawicki et al., 2009). In contrast to the majority of *Enterobacteriaceae*, (where OPS is linked to the OC, Fig. 1A), the IC of YeO3 LPS was found to be anchoring point either for the OC hexasaccharide or the homopolymeric OPS and/or ECA (Skurnik et al., 1995; Radziejewska-Lebrecht et al., 1998; Pinta et al., 2009, 2012) thereby the wild type bacteria express at the same time S and Ra chemotype LPS molecules (Fig. 1B). As shown in Fig. 1B, the IC of YeO3 is an octasaccharide of two octose, four heptose and two hexose residues (Radziejewska-Lebrecht et al., 1998) while the OC is a hexasaccharide (Pinta et al., 2009). The OPS is a homopolymer of β-(1 → 2)-linked 6-deoxy-L-altropyranose (Hoffman et al., 1980; Gorshkova et al., 1985; Bruneteau and Minka, 2003). The OC, and likely also the OPS, is linked to the second heptose (Hep II) residue of IC (Pinta et al., 2012). Both the OPS and OC substitutions are essential for bacterial virulence (Al-Hendy et al., 1992; Skurnik et al., 1999). The exact linkage of ECA_{LPS} to the LA-IC (Fig. 1B) is not determined yet although it was indirectly shown to be the Kdo region (Noszczynska et al., 2015).

The LPS core region is a target of numerous serum proteins, including the mannose-binding lectin (MBL). MBL was originally described as the murine Ra-reactive factor (RaRF), activating the complement cascade by interaction with *Salmonella* LPS of Ra type but not with the smooth or Re chemotypes (Ihara et al., 1982). Later, MBL has been demonstrated to react with OPS or

Table 1
Description of bacterial strains used in this work.

Strain	Description	LPS chemotype[a]	References
Klebsiella pneumoniae			
KO3	Serotype O:3, OPS is a homopolymer of mannose	S (LA-IC-OC-OPS)	Curvall et al. (1973)
Yersinia enterocolitica			
6471/76	(YeO3) Serotype O:3, patient stool isolate, wild type strain.	S (LA-IC-OPS) and Ra (LA-IC-OC)	Skurnik (1984)
6471/76-c	(YeO3-c) Virulence plasmid cured derivative of 6471/76	S (LA-IC-OPS) and Ra (LA-IC-OC)	Skurnik (1984)
Ye75S	Serotype O:3, wild type strain.	S (LA-IC-OPS) and Ra (LA-IC-OC)	Acker et al. (1981)
YeO3-028	Derivative of 6471/76, ΔyadA::Km-GenBlock, KmR	S (LA-IC-OPS) and Ra (LA-IC-OC)	Biedzka-Sarek et al. (2005)
8081-c	pYV-cured derivative of wild type serotype O:8 strain 8081	S (LA-IC-OPS)	Portnoy et al. (1981)
Ye75R	Spontaneous rough mutant of Ye75S	Rc (LA-IC)	Radziejewska-Lebrecht et al. (1994)
YeO3-c-R1	Spontaneous rough derivative of strain 6471/76-c	Ra (LA-IC-OC)	Al-Hendy et al. (1992)
YeO3-c-Rfb-R7	YeO3-c, Tn*5-phoA* insertion mutant in OC gene cluster, pYV$^-$, Km^R	Rc (LA-IC)	Skurnik et al. (1995, 1999), Radziejewska-Lebrecht et al. (1998)
YeO3-c-trs8-R	YeO3-c Δ(*wzx–wbcKL*), spontaneous rough mutant, pYV$^-$, Km^R	Rc (LA-IC)	Skurnik et al. (1995, 1999), Muszynski et al. (2013)
YeO3-c-trs22-R	YeO3-c Δ(*wbcKL*), spontaneous rough mutant, pYV$^-$	Rc + (LA-IC-4/6OC)[b]	Skurnik et al. (1995, 1999), Muszynski et al. (2013)
YeO3-c-trs24-R	YeO3-c Δ*wbcP*, spontaneous rough mutant, pYV$^-$	Rc (LA-IC)	Skurnik et al. (1995, 1999), Muszynski et al. (2013)
YeO3-c-Ail-R	YeO3-c Δ*ail*, spontaneous rough mutant, pYV$^-$, Km^R	Ra (LA-IC-OC)	Biedzka-Sarek et al. (2005)
YeO3-c-OC	YeO3-c Δ(*wzx–wbcQ*). The whole OC gene cluster deleted	S (LA-IC-OPS) and Rc (LA-IC)	Biedzka-Sarek et al. (2005)
YeO3-c-OCR	YeO3-c Δ(*wzx–wbcQ*). Spontaneous rough mutant	Rc (LA-IC)	Biedzka-Sarek et al. (2005)
YeO3-c-ECA	YeO3-c Δ(*wzzE-wzyE*). ECA gene cluster deleted. Construction as described by Acker et al. (1991)	S (LA-IC-OPS) and Ra (LA-IC-OC)	This work
YeO3-c-R-ECA	YeO3-c-R1 Δ(*wzzE–wzyE*). ECA gene cluster deleted. Construction as described by Acker et al. (1991), pYV, KmR	Ra (LA-IC-OC)	This work
YeO3-c-OCR-ECA	YeO3-c-OCR Δ(*wzzE–wzyE*). ECA gene cluster deleted. pYV$^-$, KmR	Rc (LA-IC)	Rabsztyn et al. (2011)
YeO3-c-OC-ECA	YeO3-c-OC Δ*(wzzE–wzyE).* ECA gene cluster deleted. Construction as described by Acker et al. (1991), pYV$^-$, KmR	S (LA-IC-OPS) and Rc (LA-IC)	This work
YeO3-c-R1-M205	YeO3-c-R1 *hldE*::cat-Mu, clmR	Re (LA-2/8IC)[c]	Noszczynska et al. (2015)
YeO3-c-R1-M196	YeO3-c-R1 *galU*::cat-Mu, clmR	Rd_1 (LA-4/8IC)[c]	Noszczynska et al. (2015)
YeO3-c-R1-M181	YeO3-c-R1 *galU*::cat-Mu, clmR	Rd_1 (LA-4/8IC)[c]	Noszczynska et al. (2015)
YeO3-c-R1-M164	YeO3-c-R1 *waaF*::cat-Mu, clmR	Rd_2 (LA-3/8IC)[c]	Noszczynska et al. (2015)

[a] All the *Y. enterocolitica* strains are ECA-positive, except the ones from which the ECA-gene cluster has been deleted.
[b] The OC hexasaccharide is truncated by two glucose residues.
[c] The IC structures of Rd_1, Rd_2 and Re chemotype LPSs are shown in Fig. 1B. The IC octasaccharide is truncated by 4, 5 or 6 sugar residues, respectively.

core oligosaccharides of some other bacteria (Jiang et al., 1995; Swierzko et al., 2003; Dumestre-Perard et al., 2007). MBL belongs to the collectin family: a group of Ca^{2+}-dependent lectins possessing a collagen-like helical domain. As a pattern-recognition molecule it recognizes pathogen-associated molecular patterns (PAMPs) of variety of microorganisms. It protects the host from infection by lysis of microbial cells involving complement activation *via* the lectin pathway (LP), in which MBL-associated serine proteases (MASPs) take part. Anaphylatoxins released during this process help to limit the infection through their chemotactic activities. In parallel, MBL may enhance phagocytosis by direct opsonization involving MBL receptors on phagocytic cells (reviewed by Cedzynski et al., 2012).

The aim of this study was to investigate the interaction of MBL and *Y. enterocolitica* and to identify the *Yersinia* LPS region targeted by MBL. To this end, wild type strains as well as a set of LPS mutants expressing sequentially truncated LPS molecules were used. Additionally, the influence of growth temperature on MBL binding was tested.

Material and methods

Bacterial strains, growth conditions and LPS isolation

Bacterial strains used in this work are listed in Table 1. Bacteria were grown aerobically at 22 °C (RT) or 37 °C, in LB medium, and in the presence of kanamycin or chloramphenicol, when required. The LPS from smooth *Y. enterocolitica* O:3 and O:8 as well as *Klebsiella pneumoniae* O:3 strains was isolated by hot phenol/water method, according to Westphal and Jann (1965), whereas the LPS of the rough strains was isolated by the hot phenol/water extraction followed by the phenol/chloroform/petroleum ether method (Galanos et al., 1969).

Flow cytometry

Bacterial cultures were suspended from the solid medium into PBS, centrifuged and fixed with 1.5% formaldehyde. After washing with cold PBS cell pellets were suspended in veronal-buffered

saline supplemented with 0.1% gelatine, 0.3 mM $CaCl_2$ and 2 mM $MgCl_2$ (GVB^{2+}) to OD_{600} = 1. Then 1 μg of recombinant human MBL (R&D Systems, USA) was added to a 100 μl aliquot of bacteria and incubated for 1 h at 37 °C. The bound protein was detected with the MBL-specific mAb HYB 131-01 (BioPorto, Denmark) and FITC-labeled anti-mouse Ig secondary antibodies (DAKO, Denmark), with the help of Cytomics FC 500 MPL Beckman-Coulter (USA) flow cytometer. Bacteria were detected using log-forward and log-side scatter dot plot. Gating region was set to exclude debris and larger aggregates of bacteria. A total of 10 000 events were acquired. The MBL binding was considered positive when the fluorescence value exceeded the threshold value that was set to be 2 times the average value of the corresponding negative control (without added MBL).

Western and affinity blotting, and lectin pathway analysis

The formaline-fixed bacterial suspensions were prepared as described above and then incubated for 2 h at 30 °C with normal human serum, pre-diluted 1:10 in GVB^{2+} buffer in a total volume of 100 μl. The serum used as a source of the lectin was obtained from a healthy volunteer with a normal MBL level (2 μg/ml) and wild type *MBL2* genotype (HYPA/LYQA). The samples were then washed with GVB^{2+}, and solubilized in SDS-sample buffer by boiling for 10 min, and aliquots equaling to 100 μl of the original OD_{600} = 1 suspension were separated in 12% SDS–PAGE under reducing conditions. After transfer to PFDV membrane, the bound MBL was detected with mAb HYB 131-01 (BioPorto) as primary and HRP-conjugated anti-mouse Ig antibodies (DAKO) as secondary antibodies, respectively. The bound antibodies were visualized with an ECL substrate (Santa Cruz Biotechnology, USA). For affinity blotting, LPSs were separated using SDS–PAGE and transferred to PVDF membrane. After incubation with human serum, the bound MBL was detected with specific antibodies as described above. To detect lectin pathway activation on the PVDF membrane (Bartlomiejczyk et al., 2014), the serum was diluted 1:10 in the high-ionic strength "MBL-binding buffer" (20 mM Tris, 1 M NaCl, 10 mM $CaCl_2$, pH 7.4, supplemented with 0.1% BSA) to prevent classical pathway activation (Petersen et al., 2001), and, after incubation at 37 °C, the deposited C4c was detected with specific rabbit antibodies and HRP-conjugated goat anti-rabbit Ig (DAKO).

ELISA

Microtitre MaxiSorp U96 plates (NUNC, Denmark) were coated with LPS (5 μg/well). After overnight incubation at 4 °C, plates were washed with calcium-supplemented Tris-buffered saline (TBS-Ca^{2+}) containing 0.05% Tween-20 and blocked with 0.1% BSA in TBS-Ca^{2+} (2 h, 37 °C). Human serum (a source of MBL) was diluted 1:50 in "MBL-binding buffer" supplemented with 0.1% BSA. Plates were incubated overnight at 4 °C. To estimate MBL binding, HYB 131-01 (BioPorto) mAb and HRP-conjugated anti mouse Ig (DAKO) were used as primary and secondary antibodies. As substrate for peroxidase, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulphonic)acid (ABTS, Sigma) was employed. OD_{405} values were measured with the use of a Benchmark Plus microplate spectrophotometer (Bio-Rad, USA).

Statistical analysis

The statistical significance of the differences in the ELISA OD_{405} values obtained from the binding of MBL to wells coated with LPS from different bacteria were compared with the Student's *t* test. The "Statistica" version 10 software (StatSoft Poland) was used for data management and calculations.

Results

Interaction of MBL with whole bacterial cells

A set of LPS mutant strains derived from the *Y. enterocolitica* serotype O:3 strain 6471/76 (YeO3) expressing sequentially truncated LPS molecules were used to map the LPS region responsible for the interaction with MBL. The *K. pneumoniae* O:3 was used as a positive control as its OPS is a homopolymer of D-mannopyranose (D-Man*p*). When analyzed by flow cytometry neither the wild type strain YeO3 nor the spontaneous rough mutant YeO3-c-R1 expressing Ra/S and Ra chemotype LPS, respectively, were able to bind MBL, however, MBL bound efficiently to the Rc chemotype strain YeO3-c-Rfb-R7 (Fig. 2A and B). This was also the case with other Rc chemotype strains (Fig. 2C) suggesting that MBL binds to a structure in the LA-IC part of *Y. enterocolitica* LPS. No significant growth temperature effect was observed (Fig. 2A). Furthermore, we could not detect any differences in MBL binding between parental and virulence plasmid-cured or *ail* (attachment-invasion protein) knock-out strains (data not shown).

The flow cytometry results were confirmed by analyzing MBL binding to bacteria by Western blotting. The bacteria were incubated in 10% human serum, washed thoroughly and the whole cell lysates were subjected to Western blotting to detect MBL. In this experiment, neither YeO3 nor other smooth serotype O:3 or O:8 strains expressing S/Ra chemotype LPS bound MBL (Fig. 3A). This was also the case with the Ra chemotype (LA-IC-OC expressing) YeO3-c-R1 bacteria (Fig. 3B). Interesting results were obtained with the extensive set of YeO3 derivatives that differ in LPS composition and expression of ECA (Fig. 3B–D). While strong MBL binding could be seen to all four Rc chemotype (LA-IC) strains (Fig. 3B), significantly decreased binding was seen to YeO3-c-trs22-R bacteria. This strain, due to deletion of the glucosyltransferase encoding genes *wbcK* and *wbcL* (Skurnik et al., 1999), produces a truncated OC missing both of the glucose residues and only partially substitutes the LA-IC molecules with the truncated OC (Pinta et al., 2009). Thus the bacteria express a mixture of LA-IC and LA-IC-4/6OC molecules, and apparently only the LA-IC binds MBL, explaining the reduced binding. In conclusion, the missing of OC seemed to be a *sine qua non* condition for MBL access to the receptor although some minor (trace) binding could be observed to the Ra chemotype strain YeO3-c-R-ECA (Fig. 3C). Surprisingly, in OC-negative strains the expression of either OPS or ECA did not abolish MBL binding, however, binding was blocked when both OPS and ECA were present simultaneously as in the case of YeO3-c-OC strain (Figs. 2C and 3C).

Finally, the results presented in Fig. 3D suggested that the IC heptoses are the MBL targets. Truncation of the IC from the wild type four heptoses to two (Fig. 1B), as is the case in the *galU* mutant strains YeO3-c-R1-M196 and YeO3-c-R1-M181, did not abolish MBL binding to bacteria (Fig. 3D), however, a further truncation to one heptose in the *waaF* mutant strain YeO3-c-R1-M164 or down to lipid A substituted by the Kdo residues only, as in the *hldE* mutant strainYeO3-c-R1-M205, completely abolished the binding ability of MBL to whole bacteria.

Interaction of MBL with isolated LPS

To confirm the above whole bacterial cell binding data indicating that MBL binds to the IC heptoses we used ELISA to study the MBL binding to LPS isolated from a set of *Yersinia* strains cultivated at 22 °C and 37 °C. Interestingly, in contrast to whole cells, a clear growth temperature dependence could be seen especially with the Rc chemotype LPS such that the LPS isolated from 22 °C grown bacteria bound significantly more MBL than the 37 °C one (Fig. 4A). The effect was not so pronounced with Rd_1 and Rd_2 chemotype LPSs.

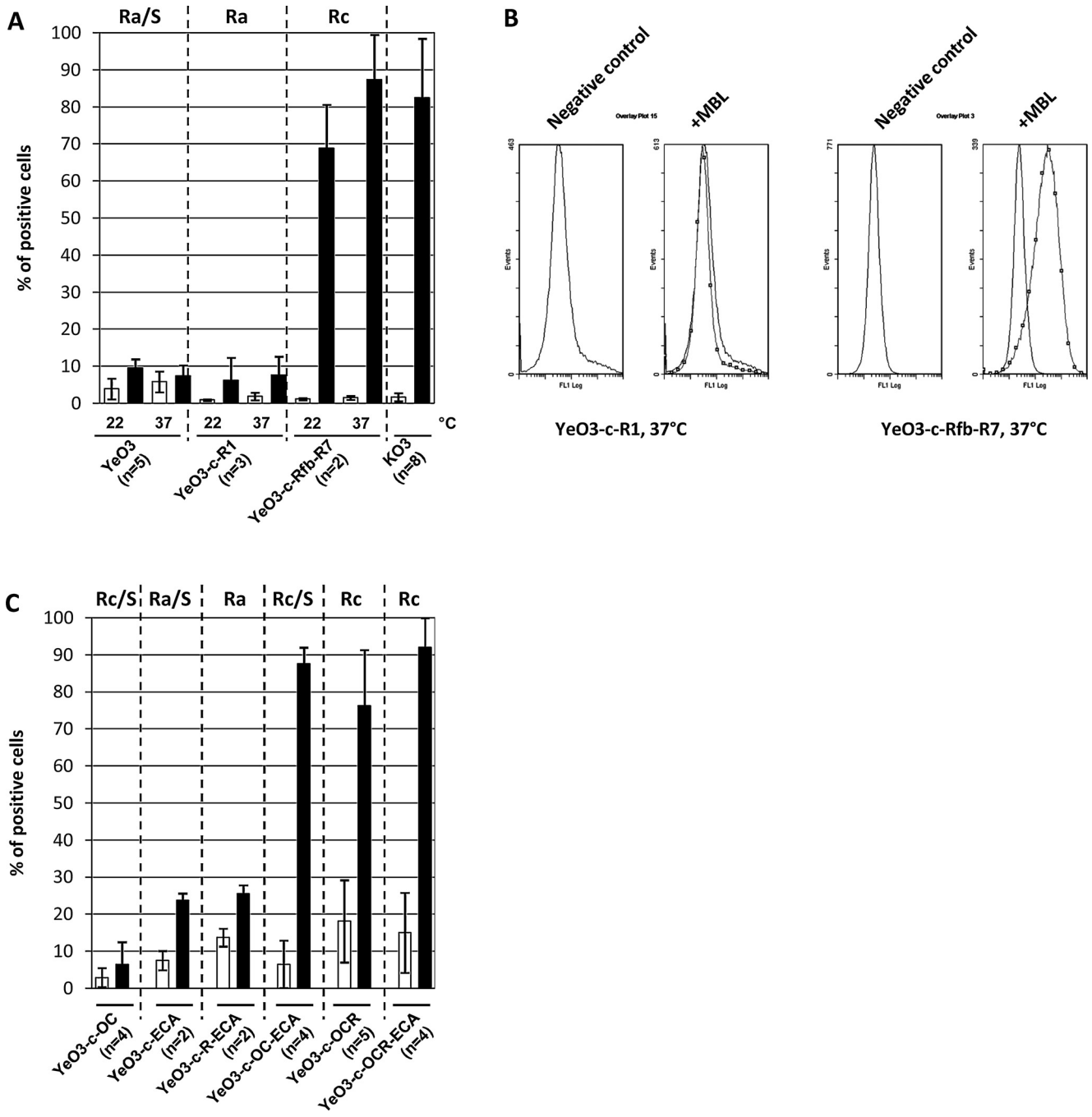

Fig. 2. Analysis of MBL binding to *Y. enterocolitica* by flow cytometry. Bacteria were washed with PBS and fixed with formaldehyde. After washing the bacteria were incubated with recombinant human MBL and the bound MBL was detected with mAb HYB 131-01 and FITC-labeled anti-mouse Ig secondary antibodies. *K. pneumoniae* O:3 (KO3) cells were used as positive control. The results are given as means ± standard deviation. The number of repeats for each strain is indicated in parentheses under the strain designation. The LPS chemotypes of the strains are indicated above the columns. Note that some strains express two different LPS chemotypes at the same time. S, LA-IC-OPS; Ra, LA-IC-OC; Rc, LA-IC. Gating region was set to exclude debris and larger aggregates of bacteria. A total of 10 000 events were acquired. The MBL binding was considered positive when the fluorescence value exceeded the threshold value that was set to be 2 times the average value of the corresponding negative control (without added MBL). Panel A: Binding of recombinant MBL to *Y. enterocolitica* Rc, Ra and Ra/S chemotype strains cultivated at 22 °C and 37 °C. Panel B: Flow cytometry curves for two strains in panel A. Representative data from one of 2 or 3 independent experiments are presented. Panel C: Binding of MBL to *Y. enterocolitica* Rc, Ra, Rc/S and Ra/S chemotype ECA-positive and -negative strains cultivated at 22 °C (black bars). The ECA extension in the strain name indicates ECA-negative mutants. The white bars indicate negative controls (no MBL added).

While MBL did not bind to the Rd_2 chemotype bacteria (YeO3-c-R1-M164) it bound to LPS isolated from them, suggesting that under the conditions used in the ELISA assay a single heptose residue was sufficient for MBL binding. The number of heptoses made a difference as strongest MBL binding took place with the four-heptose Rc chemotype LPS. On the other hand, the Re-, Ra-, and Ra/S chemotype LPSs showed no MBL binding. This was also the case with YeO3-c-trs22-R LPS, carrying the truncated OC, that in contrast to the weak MBL binding in the Western blotting, showed no MBL binding in the ELISA assay (compare Figs. 3B and 4A). Unexpectedly, the binding of MBL to LPS from the Rc chemotype ECA-negative strain YeO3-c-OC-R-ECA was significantly lower than binding to the LPSs of the two Rc chemotype ECA-positive strains, YeO3-c-Rfb-R7 and YeO3-c-trs24-R (Fig. 4B). This could indicate that ECA

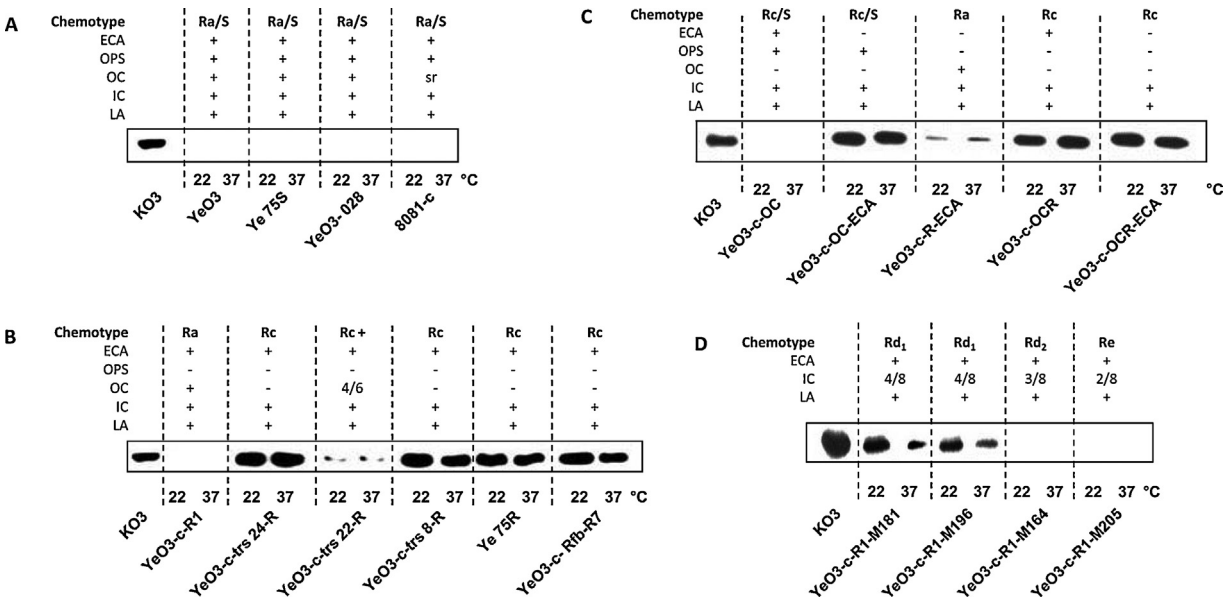

Fig. 3. Analysis of MBL binding to *Y. enterocolitica* bacteria by Western blotting. The bacteria were incubated in 10% human serum, washed thoroughly and equal amounts of the whole cell lysates were subjected to gel-electrophoresis and Western blotting to detect bound MBL. *K. pneumoniae* O:3 (KO3) cells were used as positive control. The negative controls incubated without serum were completely negative (not shown). The LPS and ECA constituents of the tested strains are indicated above the lanes and the strains and growth temperature below the blots. The bound MBL was detected with mAb HYB 131-01 (BioPorto). Panel A, *Y. enterocolitica* serotype O:3 and O:8 strains with wild type LPS and ECA phenotypes. Note that the serotype O:8 strain 8081-c does not express OC, instead it expresses a prominent semi-rough LPS (sr), *i.e.*, LA-IC substituted with a single repeat unit in addition to the LA-IC-OPS (chemotype S) LPS. Panel B, *Y. enterocolitica* O:3 ECA-positive Ra-Rc chemotype strains. Panel C, *Y. enterocolitica* O:3 strains missing OPS and/or OC and/or ECA. The ECA extension in the strain name indicates ECA-negative mutants. Panel D, *Y. enterocolitica* O:3 deep-rough mutants (Rd-Re). 4/8, 3/8, 2/8 indicate number of sugar residues in the IC.

functions as an additional MBL target in the absence of both OC and OPS.

MBL affinity blotting

The MBL binding to purified LPS was further analyzed by affinity blotting. The LPS samples were run in SDS–PAGE and blotted to transfer membrane that was subsequently incubated with human serum. The bound MBL was detected using the MBL-specific mAb (Fig. 5A). In the affinity blotting, in contrast to whole bacteria results, MBL bound to Ra/S and Ra chemotype LPSs but only to the low molecular mass fractions (Fig. 5A). This probably reflects the fact that bacteria produce a heterogenous population of LPS molecules including those with incomplete substitution of the inner core with OC or OPS. No MBL binding took place with Re chemotype LPS.

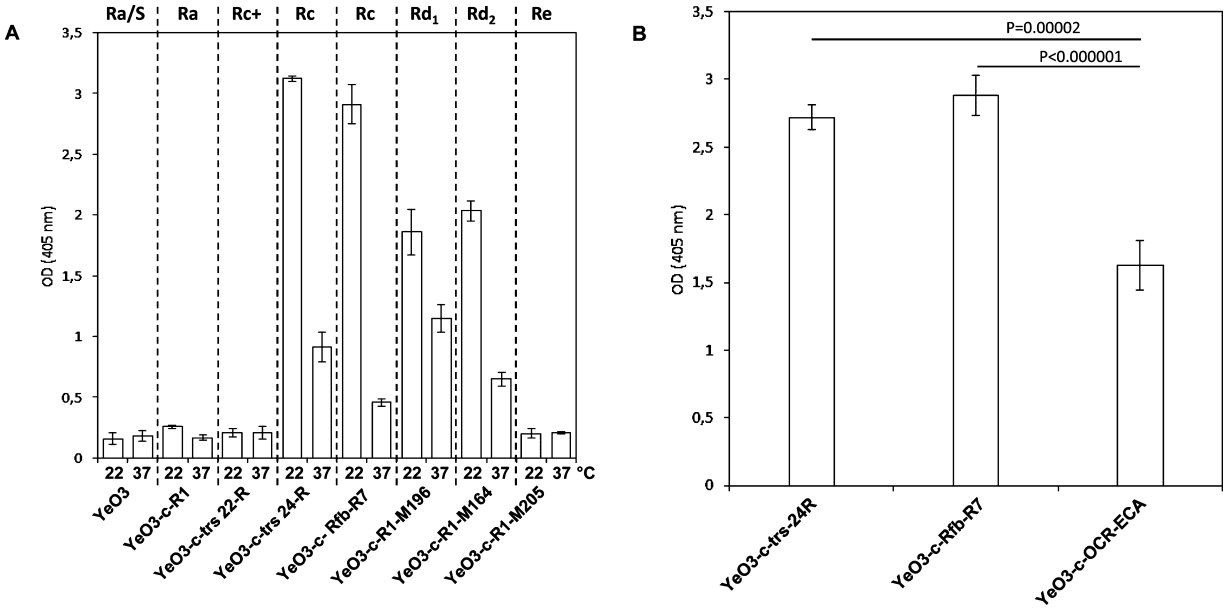

Fig. 4. Analysis of MBL binding to different chemotype LPSs isolated from the *Y. enterocolitica* O:3 LPS mutant strains cultivated at 22 °C and 37 °C. Microtiter plate wells were coated with LPS and then incubated with 1:50 diluted human serum. Bound MBL was detected with MBL-specific mAb HYB 131-01. Panel A: MBL binding to *Y. enterocolitica* LPS of various chemotypes. Panel B: Lack of ECA reduces MBL binding to Rc chemotype LPS (data for LPS from bacteria cultivated at 22 °C are presented). The ECA extension in the strain name indicates the ECA-negative mutant. The statistical significance of the differences in the ELISA OD_{405} values obtained from the binding of MBL to wells coated with LPS from different bacteria were compared with the Student's *t* test. Data are presented as mean ± SD from at least three independent experiments.

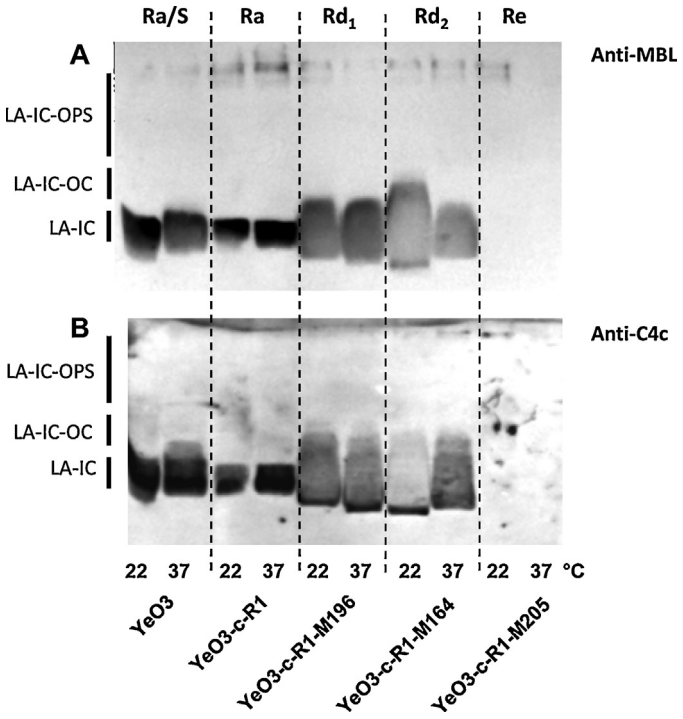

Fig. 5. Interaction of MBL with purified LPS from different *Y. enterocolitica* O:3 LPS mutants. The strains and growth temperature are indicated below and the LPS chemotypes above the images. The locations of LA-IC, LA-IC-OC and LA-IC-OPS in the gel are indicated at the left. After SDS–PAGE the LPSs were transferred to PFDV membranes. Panel A: Affinity blotting to detect binding of human serum MBL to purified LPS. The PFDV membrane was incubated with human serum and the bound MBL was detected by mAb HYB 131-01 (anti-MBL). Panel B: Activation of the complement lectin pathway demonstrated as deposition of C4c under conditions preventing classical pathway activity.

Lectin pathway activation

To study whether the MBL interaction with *Y. enterocolitica* LPS could activate the complement lectin pathway, the deposition of C4c was analyzed. Indeed, the interaction of serum MBL with the LPSs resulted in the activation of the complement lectin pathway (Fig. 5B). As expected, due to lack of MBL binding to the Re chemotype LPS, no C4c deposition took place to it. Here, however, we have to take into account the possibility that complement activation could occur upon recognition of LPS by other pattern-recognizing molecules, *e.g.*, ficolins. This, on the other hand, is not very likely as we could observe only a very weak reactivity of ficolin-2 (L-ficolin) but not of ficolin-1 (M-ficolin) or ficolin-3 (H-ficolin) (tested at 1:10 serum dilution) with Ra/S, Ra and Re chemotype LPSs (data not shown).

Discussion

In this work we provide evidence that human serum MBL interacts with *Y. enterocolitica* and, specifically, the target structure for MBL is the IC heptose region of the LPS. The minimal structure required for the recognition in LPS exposed on bacterial surface was the Hep*p*–Hep*p* disaccharide but the most efficient binding was observed when all four Hep*p* residues were present (Fig. 3, panels C and D). On the other hand, a single heptose residue was sufficient for MBL binding when isolated LPS was used (Figs. 4A and 5).

Based on our findings, it seems that the Hep*p* residues are less accessible for the MBL in LPS incorporated into the cell envelope than in purified LPS. The difference in the amount of purified LPS coated on immunoplate well (ELISA) and present on bacterial surface (Western blot, flow cytometry) may affect the observed reactivity as well. Furthermore, in *Y. enterocolitica* the MBL binding was shown to be affected by the three LA-IC substitutions, namely OPS, OC and ECA. The OC substitution had the strongest blocking effect. Only marginal MBL binding to OC-positive LPS was seen for bacteria missing both ECA and/or OPS (see strains YeO3-c-ECA and YeO3-c-R-ECA, Figs. 2C and 3C), however, in OC-ECA-positive strain the lack of OPS did not influence the blocking (see strain YeO3-c-R1, Figs. 2A and 3B). On the other hand, single OPS or ECA-substitution of LA-IC was not able to block MBL binding (see strains YeO3-c-OCR and YeO3-c-OC-ECA, Fig. 3C), however, simultaneous presence of both OPS and ECA did (see strain YeO3-c-OC, Figs. 2C and 3C).

Although the most effective LPS ligands of MBL are mannose-containing OPS as in the case of *K. pneumoniae* O:3, *Escherichia coli* O:8 and O:9, interaction of MBL with the core oligosaccharide may lead to serious biological consequences (early-phase endotoxic shock, death) as was demonstrated by Swierzko et al. (2003) in a murine model. A Ra chemotype LPS-reactive factor was identified and later recognized as MBL that interacted with Ra-chemotype *Salmonella* LPS resulting in complement activation (Ihara et al., 1982, 1991; Ji et al., 1988, 1993; Matsushita et al., 1992). The MBL target sugar residues were identified within both the OC and IC regions of LPS, including 2-acetamido-2-deoxy-D-glucopyranose (D-Glc*p*NAc) in *Proteus vulgaris* O25 LPS (Swierzko et al., 2003), and D-Glc*p*NAc and L,D-Hep*p* in *Escherichia coli* (Kawasaki et al., 1989), respectively. Similarly, the OC D-GlcNAc or the IC heptoses were demonstrated to be responsible for the interaction of *Salmonella enterica* serovar Typhimurium rough mutants with MBL (Devyatyarova-Johnson et al., 2000). In contrast to our findings, Dumestre-Perard et al. (2007) demonstrated for *Salmonella* Minnesota that MBL recognizes S, Ra, Rc and Rd chemotype LPS.

The more efficient binding of MBL to LPS of the ECA-positive but OC- and OPS-negative strain YeO3-c-OCR when compared to LPS of the YeO3-c-OCR–ECA strain might suggest that ECA could be an additional MBL target. On the other hand, MBL did not bind to ECA-positive LA-IC-OC bacteria (Fig. 3B), or to isolated ECA_{PG} where ECA is linked to phosphatidylglycerol, in contrast to the ECA_{LPS}, where ECA is attached to IC of LPS (data not shown) suggesting that ECA rather may modify MBL binding to the IC than to be specifically recognized.

The core-lipid A region of LPS plays an important role in the interaction between pathogenic bacteria and the membrane and serum proteins of the human or animal hosts and thereby influences the rate of bacterial elimination (Weiss, 2003). However, the LPS core region is often masked by long-chained OPS. Interestingly, Wuorela et al. (1993) showed that monocytes may modify LPS of ingested *Yersinia* and present it on the monocyte surface, mainly as a rough form. The structure and composition of LPS in *Yersinia* both at the LA-IC and the OPS level is variable and depends on the growth conditions of the bacteria with temperature playing a prominent role. For example, in *Yersinia pestis* the acylation status of LA is temperature-dependent; tetra-acylated at 37 °C and penta- and hexa-acylated at 25 °C (Knirel et al., 2005a,b). Moreover, at lower temperature the content of 4-amino-4-deoxy-L-arabinopyranose (Ara*p*4N) was increased (Knirel et al., 2005a,b). Similar findings were also made with *Y. enterocolitca* (Reines et al., 2012). The enteropathogenic *Yersinia* have also a tendency to down-regulate OPS expression *in vitro* at 37 °C suggesting that under such conditions the IC heptoses might be exposed to MBL binding.

In this work we noticed more efficient MBL binding to LPS isolated from bacteria grown at 22 °C reflecting perhaps slight temperature-dependent differences in IC exposure. It is worth noting that neither Ail nor plasmid-encoded virulence factors seemed

to play any role in interaction of MBL with *Yersinia* cells. In conclusion, our results demonstrate that MBL interacts with a variety of *Y. enterocolitica* LPS molecules lacking OC oligosaccharide. These might include both S type LPS (possessing OPS, OC-negative) and LPS molecules processed by immune cells, however the *in vivo* significance of MBL reactivity reported here remains to be elucidated.

Conflict of interest statement

The authors declare no conflict of interest.

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