Comparative chemical and biological characterization of the lipopolysaccharides of gastric and enterohepatic helicobacters

Hynes, Sean; Ferris, John A.; Szponar, Bogumila; Wadström, Torkel; Fox, James G.; O’Rourke, Jani; Larsson, Lennart; Yaquian, Elisa; Ljungh, Åsa; Clyne, Marguerite; Andersen, Leif P.; Moran, Anthony P.

Published in:
Helicobacter

DOI:
10.1111/j.1083-4389.2004.00237.x

Published: 2004-01-01

Citation for published version (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Comparative Chemical and Biological Characterization of the Lipopolysaccharides of Gastric and Enterohepatic Helicobacters

Sean O. Hynes,*† John A. Ferris,* Bogumila Szponar,†# Torkel Wadström,† James G. Fox,‡ Jani O’Rourke,§ Lennart Larsson,† Elisa Yaquian,† Åsa Ljungh,† Marguerite Clyne,¶ Leif P. Andersen** and Anthony P. Moran*

*Department of Microbiology, National University of Ireland, Galway, Ireland; †Department of Medical Microbiology, Dermatology and Infection, Lund University, Lund, Sweden; ‡Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA, USA; §School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia; ¶Children’s Research Centre, Our Lady’s Hospital for Sick Children, Crumlin, Dublin, Ireland; **Department of Infection Control, National University Hospital (Rigshospitalet), Copenhagen, Denmark

ABSTRACT

Background. The lipopolysaccharide of Helicobacter pylori plays an important role in colonization and pathogenicity. The present study sought to compare structural and biological features of lipopolysaccharides from gastric and enterohepatic Helicobacter spp. not previously characterized.

Materials and methods. Purified lipopolysaccharides from four gastric Helicobacter spp. (H. pylori, Helicobacter felis, Helicobacter bizzozeronii and Helicobacter mustelae) and four enterohepatic Helicobacter spp. (Helicobacter hepaticus, Helicobacter bilis, ‘Helicobacter sp. flexispira’ and Helicobacter pullorum) were structurally characterized using electrophoretic, serological and chemical methods.

Results. Structural insights into all three moieties of the lipopolysaccharides, i.e. lipid A, core and O-polysaccharide chains, were gained. All species expressed lipopolysaccharides bearing an O-polysaccharide chain, but H. mustelae and H. hepaticus produced truncated semirough lipopolysaccharides. However, in contrast to lipopolysaccharides of H. pylori and H. mustelae, no blood group mimicry was detected in the other Helicobacter spp. examined. Intraspecies, but not interspecies, fatty acid profiles of lipopolysaccharides were identical within the genus. Although shared lipopolysaccharide-core epitopes with H. pylori occurred, differing structural characteristics were noted in this lipopolysaccharide region of some Helicobacter spp. The lipopolysaccharides of the gastric helicobacters, H. bizzozeronii and H. mustelae, had relative Limulus amoebocyte lysate activities which clustered around that of H. pylori lipopolysaccharide, whereas H. bilis, ‘Helicobacter sp. flexispira’ and H. hepaticus formed a cluster with approximately 1000–10,000-fold lower activities. H. pullorum lipopolysaccharide had the highest relative Limulus amoebocyte lysate activity of all the helicobacter lipopolysaccharides (10-fold higher than that of H. pylori lipopolysaccharide), and all the lipopolysaccharides of enterohepatic Helicobacter spp. were capable of inducing nuclear factor-Kappa B(NF-κB) activation.

Conclusions. The collective results demonstrate the structural heterogeneity and pathogenic potential of lipopolysaccharides of the Helicobacter genus as a group and these differences in lipopolysaccharides may be indicative of adaptation of the bacteria to different ecological niches.

Keywords. Helicobacter spp., Helicobacter pylori, gastric helicobacters, enterohepatic helicobacters, lipopolysaccharide, lipid A.

Currently, there are more than 20 species ascribed to the Helicobacter genus. Chronic infection of various hosts with gastric helicobacters such as Helicobacter pylori, Helicobacter felis, Helicobacter mustelae and Helicobacter bizzozeronii has been associated with a spectrum of pathologies such as gastritis, mucosa-associated lymphoid tissue lymphoma [1–4] and, in the
case of H. pylori, peptic ulcer disease and gastric adenocarcinoma in humans [5,6]. Other related bacteria such as Helicobacter hepaticus, Helicobacter bilis, Helicobacter pullorum and ‘Helicobacter sp. flexispira’ colonize the enterohepatic niche of animals and humans, and may play a role in enterohepatic diseases such as hepatitis and gastroenteritis [7,8].

Outer membrane-derived lipopolysaccharides (LPSs) of most Gram-negative bacteria are powerful stimulators of the host immune response. However, the LPS of H. pylori has low immunological activity and, thereby, contributes to the chronicity of infection [9–11]. The molecule is composed of three moieties, lipid A, core and O-polysaccharide chain, and lipid A is central to the biological and immunological activities associated with LPS. H. pylori lipid A is underphosphorylated (lacking phosphate at position 4′) and has both 3-hydroxyhexadecanoic and 3-hydroxyoctadecanoic acids [12,13]. From a structural-bioactivity standpoint, the lower activity of H. pylori LPS is attributable to this underphosphorylation and the presence of these longer chain fatty acids [10,11]. In contrast, H. mustelae, a gastric colonizer of ferrets, has a lipid A structure which is phosphorylated at both the 1 and 4′ positions and contains 3-hydroxytetradecanoic and 3-hydroxyhexadecanoic acids [14], and is considered to have higher biological activity. Furthermore, H. pylori and H. mustelae mimic Lewis (Le) and blood group A antigens, respectively, in their O-polysaccharide chains [15,16]. A number of roles have been proposed for this mimicry, including camouflage, whereby bacterial expression of Le antigens mimics that of the gastric mucosa, thereby aiding initial colonization, and adhesion of H. pylori to the gastric epithelium [10].

However, because of their fastidious nature and the large amount of biomass required, few structural data are available regarding the LPSs of most Helicobacter spp., in contrast to H. pylori and H. mustelae [17]. Therefore, in the present study we initially assessed the structural characteristics of LPSs from gastric and enterohepatic helicobacters using electrophoretic, serological and chemical techniques, and also assessed relative biological activities. Our aim was to determine whether LPS structural differences existed between selected Helicobacter spp., which might be of potential relevance to their niche colonization and/or pathogenesis.

Materials and methods

Bacterial strains and culture conditions

Bacterial isolates were obtained from the American Type Culture Collection (ATCC), National Collection Type Cultures (NCTC) or the Culture Collection of the University of Gothenburg (CCUG), unless otherwise stated. The following gastric isolates of Helicobacter spp., with host in parentheses, were used in the present study: H. pylori NCTC 11637 (human), H. pylori CCUG 17874 (human) and H. pylori 119/95 (human) were from the Department of Gastroenterology, Lund University Hospital, Sweden; H. mustelae NCTC 12198 (ferret), H. felis ATCC 49179 (cat) and H. bizzozeronii R53 (human) were from the Department of Clinical Microbiology, Rigshospitalet, Copenhagen [1,18]. Isolates of enterohepatic Helicobacter spp. were also utilized, including: H. pullorum CCUG 33837 (chicken), H. pullorum CCUG 33839 (human), H. pullorum CCUG 33840 (chicken), H. pullorum NCTC 12827 (human), H. bilis CCUG 38995 (mouse), Helicobacter canis CCUG 33835 (dog), and H. hepaticus CCUG 33637, CCUG 44776 and CCUG 44777 (all from mice), and an isolate designated ‘Helicobacter sp. flexispira’ K0210 (dog) was obtained from M.-L. Hanninen, University of Helsinki, Helsinki, Finland.

All Helicobacter spp. were routinely grown under microaerobic conditions (10% O2, 5% CO2 and 85% N2) generated using an Anoxomat® instrument (MART Microbiology BV, Licthenvoorde, the Netherlands) or a GasPak system (Oxoid, Basingstoke, UK) on blood agar at 37°C [19]. Supplementation of basal medium with 0.1% charcoal was used to improve yields of the majority of enterohepatic species as reported previously [20].

LPS extractions

Crude LPS was extracted from biomass using the hot phenol-water method, and subsequently purified by enzymatic treatments (RNase A, DNase II and proteinase K) and by ultracentrifugation as described previously [19]. The LPS preparations were essentially free from proteins (< 0.1%) and nucleic acids (< 0.1%) [19]. Alternatively, because of more limited availability of biomass compared to the other Helicobacter spp., LPSs from isolates of enterohepatic Helicobacter spp. were extracted using a mini-phenol-water extraction technique [21], followed by application.
of the enzymatic purification steps above. No protein contamination was detected when the mini-LPS extracts were examined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis with silver staining or Coomassie blue staining [19]. Using a commercial colorimetric protein assay (Bio-Rad, Hercules, CA), the purity of these samples was estimated at not less than 99.5%. These LPS preparations were used for studying fatty acid profiles and for analysis of relative biological activities (see below).

**Electrophoresis and immunoblotting**

The macromolecular nature of the LPSs was analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis with a discontinuous buffer system [19] coupled with periodate oxidation-silver staining [22]. In addition, LPSs were electroblotted onto nitrocellulose membranes (Amersham, Buckinghamshire, UK) and probed by Western blotting as described previously [23] with a panel of mouse monoclonal antibodies specific for 

<table>
<thead>
<tr>
<th>Antigen</th>
<th>ID Laboratories, Ontario, Canada</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lex, Leα, Leγ, Leβ</td>
<td>Blood group A</td>
</tr>
</tbody>
</table>
| and group B antigens | Total hexosamine content | were estimated colorimetrically [25–27]. Sugar compositional analysis was performed by the alditol acetate method [19]. Methylation was performed using the NaOH/CH₃I/Me₂SO procedure [12]. Alditol acetates and methylated derivatives were identified by gas-liquid chromatography-mass spectrometry using a Hewlett-Packard 5880 gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a DB-5 fused-silica capillary column (30 m × 0.25 mm) and mass selective detector (model 5971A) with a temperature programme of 160°C (1 minute) to 260°C at 3°C/minute.

**Chemical analysis of LPSs**

Organic phosphate, 2-keto-3-deoxy-octulosonic acid and total hexosamine content were estimated colorimetrically [25–27]. Sugar compositional analysis was performed by the alditol acetate method [19]. Methylation was performed using the NaOH/CH₃I/Me₂SO procedure [12]. Alditol acetates and methylated derivatives were identified by gas-liquid chromatography-mass spectrometry using a Hewlett-Packard 5880 gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a DB-5 fused-silica capillary column (30 m × 0.25 mm) and mass selective detector (model 5971A) with a temperature programme of 160°C (1 minute) to 260°C at 3°C/minute.

**3-Hydroxy fatty acid analysis**

Mass spectrometric analysis of fatty acid profiles of LPSs was carried out as described previously [28]. Following methanolation and derivatization, samples were applied to a Saturn 2000 ion-trap gas-liquid chromatography-mass spectrometry instrument (Varian, Middelburg, the Netherlands) equipped with a fused silica capillary column. Analyses were in the electron-impact mode whereby the derivatized acids were measured by monitoring ion, m/z 131. Methyl esters of 3-hydroxy fatty acids with 10, 12, 14, 16 and 18 carbon chains were purchased from Larodan Lipids (Malmö, Sweden) and used as external standards. In addition, individual deuterated 3-hydroxy fatty acids (50 ng) were added to the methanolysates before extraction and used as internal standards for quantifying each of the individual fatty acids.

**Limulus amoebocyte lysate assay**

The *Limulus* amoebocyte lysate assay was performed using an endpoint chromogenic test (Charles River, Charleston, SC) according to the manufacturer’s instructions. LPSs from various *Helicobacter* spp. were examined, except for *H. felis* LPS which was not tested extensively because of a lack of available material. Determination of the relative biological activities of the LPSs in the assay was performed according to the protocol of Pece et al. [29]. *Escherichia coli* O111:B4 LPS was used as the assay standard. The relative activity of test preparations was determined based on the absorbance results (at 405 nm) from a series of dilutions (from 100 pg/ml to 100 ng/ml) of the LPSs under test. The absorbance values of duplicate samples from a triplicate set of experiments were read against a standard curve, and the results related to purified *H. pylori* LPS as a relative standard.

**Electrophoretic mobility shift assay**

An adherent human gastric adenocarcinoma cell line (AGS) was obtained from the European Collection of Cell Cultures (Porton Down, UK) and grown in Hams F-12 medium (Sigma) containing 2 mmol/l L-glutamine (Sigma) and 10% foetal bovine serum (Gibco, Grand Island, NY). Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Confluent AGS cell monolayers were exposed to
LPS extracts at a concentration of 10 µg/ml and incubated under standard conditions for 30 minutes. Thereafter, nuclear proteins were extracted as described by Keates et al. [30] and stored at −80°C. The protein concentration of extracts was determined by a commercial colorimetric assay (Bio-Rad, Hercules, CA).

NF-κB gel shift oligonucleotide, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega Corp., Madison, WI), labelled with [32P]dATP (Amersham, Uppsala, Sweden), was used. Binding reactions (20 µl) contained 0.1 ng (about 15,000 cpm) of double-stranded probe; 12 µg of extracted protein; 2 µg of poly(dI-dC); 10 mmol/l 2-mercaptoethanol; and 1% Ficoll. Nuclear extracts were preincubated in reaction buffer, labelled probe was added, and the incubation was continued for a further 30 minutes at room temperature. Equal concentrations of the reaction mixtures were then loaded on a nondenaturing 6% polyacrylamide gel which was run at 120 V for 2 hours at room temperature. Subsequently, the gel was dried and exposed to radiography film for 6–18 hours at −70°C.

Results

LPS yields and LPS electrophoretic profiles

Except for H. felis (2.5% yield), the yields of LPS from the majority of Helicobacter spp. examined were near optimal, between 4.6 and 7.7% of cellular dry weight. LPS profiles of selected Helicobacter strains, representative of the profiles of tested strains of individual Helicobacter spp., compared to that of E. coli O111:B4 (lane 1), are shown in Fig. 1. The profile of H. felis LPS (Fig. 1, lane 2) contained diffusely staining high-molecular-weight (Mr) bands of similar mobility to those of H. pylori LPS (Fig. 1, lane 3) and is indicative of LPS bearing O-polysaccharide chains [19,23]. Although H. bizzozeronii LPS possesses a slower migrating high-Mr band indicative of LPS with an O-chain (Fig. 1, lane 4), this was much less intensely staining than low-Mr LPS, reflecting the predominance of the latter LPS. In contrast, the LPS of H. mustelae had two fast-migrating low-Mr bands (Fig. 1, lane 5) consistent with the semirough nature of this LPS as determined previously in chemical studies [15]. Likewise, H. hepaticus LPS (Fig. 1, lane 6) had a similar profile but the low-Mr bands had a slightly slower mobility, reflecting differences in size of repeat units. These distinctive semirough LPS profiles were found in all the strains of H. mustelae and H. hepaticus examined. H. bilis, ‘Helicobacter sp. flexispira’ and H. pullorum had a profile consistent with those of LPSs with O-polysaccharide chains (Fig. 1, lanes 7–9) but their high-Mr bands were of slower mobility than those of H. pylori (Fig. 1, lane 3), indicating the occurrence of longer O-chains in the former LPSs. In addition to differences in banding patterns, variations in staining intensities and contrast of LPS profiles are potentially reflective of differences in sugar composition of the individual LPSs.

Serological characterization of LPSs

Control H. pylori LPSs (H. pylori A1 expressing Lex, Ley, Leα and Leβ; H. pylori A2 expressing Leα, Leβ and blood group A), which had been used in previous serological characterization studies [31], gave the expected reactions in immunoblotting. As would be expected, H. pylori strains NCTC 11637, CCUG 17874 and 119/95 expressed Leα and Leβ. However, Western blotting using an antiblood group A monoclonal antibody detected two low-Mr bands of
Lipopolysaccharides of Helicobacter spp.

<table>
<thead>
<tr>
<th>Bacterial lipopolysaccharide</th>
<th>Polyclonal antisera raised against H. pylori strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. pylori NCTC 11637</td>
<td>+++ +++++ +++++ +++++</td>
</tr>
<tr>
<td>H. mustelae NCTC 12198</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td>H. felis ATCC 49179</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td>H. bizzozeronii R-53</td>
<td>+++ – – – –</td>
</tr>
<tr>
<td>H. hepaticus CCUG 33637</td>
<td>++ + + + +</td>
</tr>
</tbody>
</table>

+++ strong reaction; ++, moderate reaction; +, weak reaction; –, no reaction.

Table 1: Cross-reactivity of anti-H. pylori core-LPS antisera with LPSs from various Helicobacter spp.

**H. mustelae** LPS (data not shown) corresponding to those observed in silver-stained gels (Fig. 1, lane 4) showing the presence of blood group A mimicry in LPS with one and two repeat units, consistent with previous chemical findings [15]. In contrast, no other helicobacter LPS examined had serologically detectable Le antigen mimicry. The other linkages in the **H. felis** LPS were terminal Glc and Gal, 2-linked Man and Gal, 4-linked GlcNAc, but the occurrence of Fuc as a 3,4-linked branched unit and 4-linked GlcNAc, thereby confirming the absence of Le antigen mimicry. The other linkages in the **H. felis** LPS resembles that of **H. pylori** and helps explain the serological cross-reactions observed above. Fuc was also present in **H. bizzozeronii**, **H. bilis** and ‘*Helicobacter sp. flexispira*’ LPSs and even occurred as 3-linked Fuc in **H. bizzozeronii**. Nevertheless, other structural features that would be consistent with the presence of Le antigen mimicry was absent, in agreement with the serological findings above. Similar to **H. pylori**, **H. mustelae** and **H. felis** LPSs, that of **H. hepaticus** contained Fuc, Glc, Gal, GlcNAc and LD-Hep, although in different molar ratios (1 : 3 : 1.5 : 1.5 : 1.5), but did not contain detectable Man or DD-Hep. However, N-acetylglactosamine (GlcNAc) was also detected in **H. hepaticus** LPS, further emphasizing structural differences between these LPSs.

### Chemical characterization of LPSs

Similar to **H. pylori** and **H. mustelae** [15,19], 2-keto-3-deoxy-octulosonic acid was detectable colorimetrically in the LPSs of all the *Helicobacter* spp. examined. Moreover, the identity of this sugar was confirmed by gas-liquid chromatography mass-spectrometry analysis of its peracetylated methyl ketoside derivative [19]. Although the molar quantities of 2-keto-3-deoxy-octulosonic acid varied slightly between the LPSs of various bacterial species (data not shown), variation in the extent of LPS phosphorylation was more significant. For example, the lipid A component of **H. felis** LPS had slightly higher phosphorylation compared with that published previously for **H. pylori** lipid A [12] (298 vs. 217 nmol/mg), whereas that of **H. bizzozeronii** was less phosphorylated (100 nmol/mg).

More detailed analysis of **H. felis** LPS showed it contained L-fucose (Fuc), D-glucose (Glc), D-galactose (Gal), D-mannose (Man), N-acetyl-D-glucosamine (GlcNAc), D-glycero-D-manno-heptose (DD-Hep) and L-glycero-D-manno-heptose (LD-Hep) (molar ratios 1 : 3.3 : 2.7 : 2.8 : 3.3 : 0.8 : 0.1). Thus, the LPS of **H. felis**, besides having the sugars present in **H. pylori** and **H. mustelae** [10,11,15,32], contains Man. Sugar linkage analysis revealed the absence of terminal Fuc and 3,4-linked GlcNAc, but the occurrence of Fuc as a 3,4-linked branched unit and 4-linked GlcNAc, thereby confirming the absence of Le antigen mimicry. The other linkages in the **H. felis** LPS were terminal Glc and Gal, 2-linked Man, 3-linked Glc and Gal, 4-linked Gal, 2-linked DD-Hep, 2,7-linked DD-Hep, 2-linked LD-Hep and 3-linked LD-Hep.

### 3-hydroxy fatty acid profiles

The 3-hydroxy fatty acid profiles of three species of helicobacters, **H. pylori**, **H. mustelae** and **H. pullorum**, have been assessed previously...
In the present study, 3-hydroxyhexadecanoic and 3-hydroxytetradecanoic fatty acids were detected in LPSs of *H. mustelae*, 3-hydroxyhexadecanoic and 3-hydroxyoctadecanoic acids in *H. pylori*, and 3-hydroxytetradecanoic and 3-hydroxyhexadecanoic acids in *H. pullorum* (Table 2), findings which are consistent with the previous observations on 3-hydroxy fatty acid profiles [12,14,19,33], thereby demonstrating the validity of our approach.

Because of their fastidious nature and the acknowledged difficulties normally encountered in their culture [20], multiple strains of non-*pylori* *Helicobacter* spp. were available only for *H. pullorum* and *H. hepaticus* (Fig. 2). Intraspecies fatty acid profiles from each set of strains

**Table 2** Percentage distribution of 3-hydroxy fatty acids in LPSs of *Helicobacter* spp.

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>Percentage of 3-OH fatty acids (nmol/mg) with carbon chains:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C12</td>
</tr>
<tr>
<td><strong>Gastric</strong></td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> CCUG 17874</td>
<td>–</td>
</tr>
<tr>
<td><em>H. bizzozeronii</em> R53</td>
<td>–</td>
</tr>
<tr>
<td><em>H. felis</em> ATCC 49179</td>
<td>–</td>
</tr>
<tr>
<td><em>H. mustelae</em> NCTC 12198</td>
<td>–</td>
</tr>
<tr>
<td><strong>Enterohepatic</strong></td>
<td></td>
</tr>
<tr>
<td><em>H. pullorum</em> CCUG 33837</td>
<td>–</td>
</tr>
<tr>
<td><em>H. pullorum</em> CCUG 33839</td>
<td>–</td>
</tr>
<tr>
<td><em>H. pullorum</em> CCUG 33840</td>
<td>–</td>
</tr>
<tr>
<td><em>H. hepaticus</em> CCUG 44776</td>
<td>46.1</td>
</tr>
<tr>
<td><em>H. hepaticus</em> CCUG 44777</td>
<td>37.0</td>
</tr>
<tr>
<td><em>H. canis</em> CCUG 33835</td>
<td>–</td>
</tr>
<tr>
<td><em>H. bilis</em> CCUG 38995</td>
<td>–</td>
</tr>
<tr>
<td>'Helicobacter sp.' flexispira' K0210</td>
<td>–</td>
</tr>
</tbody>
</table>

*Figure 2* Gas-liquid chromatography-mass spectrometry chromatographs of the 3-hydroxy fatty acid profiles of LPSs from *H. hepaticus* strains (A) CCUG 33637, (B) CCUG 44776, and (C) CCUG 44777; and *H. pullorum* strains (D) NCTC 12827, (E) CCUG 33837, (F) CCUG 33839, and (G) CCUG 33840; and external standards.
of *H. pullorum* and *H. hepaticus*, respectively, were comparable, although some variation occurred between the relative abundance of the fatty acids within each species (Table 2). This relative homogeneity in fatty acid profiles suggests that this is a highly conserved characteristic within these species, implying importance in survival in the respective ecological niches.

3-Hydroxytetradecanoic and 3-hydroxyhexadecanoic acids are important components of the LPSs of *Helicobacter* spp. from both the gastric and enterohepatic environments. Excluding *H. bizzozeronii*, one or both of these components were found in the LPSs from all of the species tested (Table 2). However, their relative abundance and the presence of other 3-hydroxy fatty acids varied considerably between species. Human gastric isolates of *H. pylori* and *H. bizzozeronii* expressed 3-hydroxyoctadecanoic acid as the predominant molecular species (Table 2), but it was not observed in the other species examined, suggesting its importance for the bacterial outer membrane in the human gastric environment. Except for *H. bilis* and ‘*Helicobacter* sp. *flexispira*’ LPS, which expressed only 3-hydroxytetradecanoic acid, and *H. bizzozeronii*, which expressed only 3-hydroxyoctadecanoic acid, all the other species possessed 3-hydroxyhexadecanoic acid. Also, the *H. hepaticus* isolates examined had a distinctive profile which included a high percentage of a shorter chain fatty acid (3-hydroxydodecanoic acid) which was not seen in the other species.

**Biological activities of Helicobacter LPSs**

The LPS of *H. pylori* has previously been shown to have low biological activities (i.e. pyrogenicity, mitogenicity, lethality, *Limulus* amoebocyte lysate activity, neutrophil priming, cytokine induction, etc.), compared to LPSs from various enterobacteria, including *E. coli* [9,11,29,34,35]. Low-level phosphorylation and the occurrence of longer fatty acids present in *H. pylori* lipid A have been implicated in this low bioactivity [12,13]. Therefore, based on the differences observed in phosphorylation and the fatty acid profiles between LPSs of *Helicobacter* spp., it was of interest to test the relative biological activities of these LPSs. *H. pylori* LPSs (NCTC 11637, CCUG 17874 and 119/95) had a *Limulus* amoebocyte lysate activity of 1 × 10^5 endotoxin units (EU)/mg, about 100-fold lower than the activity of *E. coli* LPS (1.2 × 10^7 EU/mg), and comparable to that observed previously [29]. The LPSs of the gastric helicobacters, *H. felis* ATCC 49179, *H. bizzozeronii* R53 and *H. mus-telae* NCTC 12198, had relative *Limulus* amoebocyte lysate activities which clustered around that of *H. pylori* LPS (1 × 10^5, 1 × 10^6 and 1.5 × 10^4 EU/mg, respectively). The lowest activities of helicobacter LPSs were observed for enterohepatic *H. bilis* CCUG 38995 (5 × 10^3 EU/mg), *H. canis* CCUG 33835 (4.0 × 10^3 EU/mg) and the three *H. hepaticus* isolates (CCUG 33637, CCUG 44776 and CCUG 44777) (7 × 10^2 EU/mg for each) which formed a cluster with approximately 2000- to 10,000-fold lower activities relative to *E. coli* LPS. In contrast, ‘*Helicobacter* sp. *flexispira*’ K0210 had a 3-fold higher activity (3 × 10^5 EU/mg) than *H. pylori* LPS. However, the relative *Limulus* amoebocyte lysate activities of LPSs from two isolates of *H. pullorum* (CCUG 33837 and NCTC 12827) approximated that of *H. pylori* LPSs (1 × 10^5 EU/mg), whereas the LPSs of two other *H. pullorum* isolates (CCUG 33839 and CCUG 33840) had the highest activities of the *Helicobacter* LPSs examined (1.0 × 10^6 and 4.0 × 10^6 EU/mg, respectively), 10- to 40-fold higher than that of *H. pylori* LPS. Thus, our results suggest that LPS of this particular enterohepatic species can have higher biological activity than that of *H. pylori*, consistent with the relative toxicity of *H. pullorum* previously observed in cell culture [36].

In addition, differing helicobacter LPSs, from various gastric and enterohepatic species, were capable of inducing NF-κB activation (Fig. 3), in a similar manner to that previously described for *H. pylori* [37,38], and specifically for a secreted or shed protein of *H. pylori* [39].

**Figure 3**  Electrophoretic mobility shift assay demonstrating NF-κB activation of adenocarcinoma cell line cells (10^6) treated with lipopolysaccharides (10 µg/ml each, except ‘*Helicobacter* sp. *flexispira*’ which had 5 µg/ml) of *Helicobacter* spp., with no lipopolysaccharide; 2, *E. coli* O55:B5 (Sigma) as a positive control; 3, *H. pylori* 119/95; 4, *H. bizzozeronii* R53; 5, ‘*Helicobacter* sp. *flexispira*’ K0210; 6, *H. pullorum* CCUG 33839.  

Discussion

To date, few isolates of each Helicobacter spp. have been available for detailed study because of the fastidious conditions required for their isolation and culture. Nevertheless, the present study is the first to structurally compare the LPSs of both gastric and enterohepatic Helicobacter spp. Collectively, the results of the present study demonstrate differences in the macromolecular nature and architecture of LPSs from H. pylori and non-H. pylori helicobacters, as well as between gastric and enterohepatic Helicobacter spp.

Despite extensive subculture of the strains examined, all the Helicobacter spp. expressed an O-polysaccharide chain, although this may be composed of only one or two repeat units as in H. mustelae and H. hepaticus LPSs. However, in contrast to H. pylori and H. mustelae [15,16,40], no Lewis or ABO antigen mimicry was detected using serological analyses of the LPSs of the other Helicobacter spp. and this was further supported by our chemical investigations. The differing nature and structure of the LPSs in the various Helicobacter spp. emphasize the potentially differing roles of LPS in the pathogenesis of a given bacterial species. For example, a role has been suggested for LPS in adhesion and colonization by H. pylori [10], and both species which express blood group antigen mimicry are also the only gastric species which closely associate with host epithelium [41].

Despite lacking molecular mimicry in their O-polysaccharide chains, the non-H. pylori helicobacters examined did have epitopes present that were cross-reactive with anti-H. pylori core-LPS antibodies. Such common epitopes suggest the occurrence of generally conserved structural features in the core of helicobacter LPSs which may aid chronic infection of the gastrointestinal tract. However, the relative extent and strength of cross-reactivity between the LPSs and these antibodies varied, notably with H. hepaticus and H. bizzozeronii, again indicating the occurrence of strain-specific features. The presence of LPS species-specific structural motifs, as observed in particular in H. felis, H. bizzozeronii and H. hepaticus, may be indicative of adaptations of their outer membranes, allowing these bacteria to colonize specific hosts or niches and requires further, more detailed, investigation.

Although H. felis has been extensively used in models of helicobacter pathogenesis [2,42], we have established that the chemical composition, and hence structure, of H. felis LPS differs significantly from that of H. pylori, despite some structural similarities in the core region. This may have important implications when interpreting the results of H. felis animal model studies compared to natural infection by H. pylori in humans, as LPSs are important immunomodulating and immunostimulating agents [10]. Also, H. hepaticus has been frequently utilized in animal models to study inflammatory bowel disease [43]. The results of the present study suggest that this species has an LPS with certain differing properties to LPS of other enterohepatic helicobacters expressing fucosylated LPS and both 3-hydroxydodecanoic and 3-hydroxyhexadecanoic acids. Furthermore, in contrast to most other helicobacters, but similarly to H. mustelae, H. hepaticus expresses low-molecular-weight LPS. The complete genome sequence of this organism has shown the presence of genes encoding fucosyltransferases and glycoysltransferases for LPS synthesis potentially under the control of phase variation mechanisms [44]. Nevertheless, the presence of a fucosyltransferase may not necessarily imply the expression of Lewis antigens, as indicated from our serological analyses. Likewise, although fucose was present in H. bizzozeronii LPS, expression of Lewis antigens was not detected serologically. Confirming the absence of Lewis antigens, detailed structural analysis of H. bizzozeronii LPS has shown the substitution of the core region by fucose but absence of Lewis antigen mimicry (A. P. Moran, unpublished data).

Previously, it has been hypothesized that 3-hydroxy fatty acids are normally highly conserved within a genus and could be used for taxonomy [45]. However, similar to Yersinia and Bordetella [46,47], work by Therisod et al. [14] has shown that the fatty acid profile of H. mustelae lipid A containing 3-hydroxyhexadecanoic and 3-hydroxytetradecanoic does not correspond to the 3-hydroxyoctadecenoic acid profile of H. pylori lipid A. The results from the present study expand on this finding by showing interspecies variability in 3-hydroxy fatty acid profiles of LPSs from enterohepatic and gastric species within the Helicobacter genus. The consistent intra-species fatty acid profiles of H. pylori, H. hepaticus and H. pullorum might argue for this characteristic to be dependent upon the host niche. Human gastric isolates of H. pylori and H. bizzozeronii expressed 3-hydroxyoctadecanoic
acid as the predominant molecular species, but this was not observed in the other species examined, suggesting its importance for the bacterial outer membrane in the human gastric environment. Nevertheless, strains of *H. pullorum* from both human and chicken isolates had similar profiles. Our present study has demonstrated that 3-hydroxytetradecanoic acid and 3-hydroxyhexadecanoic acid are important components of the LPS of *Helicobacter* spp. in the gastric and enterohepatic environments. Notably, *H. bilis* and *Helicobacter sp. flexispira* had an identical profile with a single peak of 3-hydroxytetradecanoic acid. Importantly, the heterogeneous *Helicobacter sp. flexispira* group has been shown to include misidentified and closely related *H. bilis* and *Helicobacter trogontum* strains [48]. Furthermore, based on structural studies, it has been demonstrated that the extent and pattern of phosphorylation and acylation of the lipid A component of *H. pylori* LPS contribute to the lower immunological activity of the latter [9,11,29,34,35] and, as LPS is an essential constituent in the outer membrane of Gram-negative bacteria, the observed lower activity has been hypothesized to aid the chronicity of *H. pylori* infection [10]. Thus, differences in phosphorylation of lipid A, and hence LPS, of the other *Helicobacter* spp., whether to a greater or lesser extent to that seen in *H. pylori* may, along with other inflammatory bacterial products, influence the extent of the immunological response and chronicity of infection in their natural hosts. Such a hypothesis is supported by previous comparative structural data on *H. pylori* and *H. mustelae* lipid A [12–14]. In the present study, no correlation among 3-hydroxy fatty acid profiles, phosphorylation levels and biological activities of LPSs of the other non-*H. pylori* helicobacters was apparent. This may indicate that other structural characteristics, such as the substitution pattern of the phosphate groups and the distribution and substitution pattern of acyl chains, play an important role in contributing to the potency of the respective LPSs. However, to resolve this question will require detailed structural analysis of the respective LPSs.

Interestingly, various helicobacter LPS preparations from gastric and enterohepatic species were found to be capable of inducing NF-κB activation, in a similar manner to that described previously for *H. pylori* [37,38]. However, in addition, *H. pylori* can induce NF-κB activation by a secreted or shed protein of the bacterium [39]. Nevertheless, contamination of the samples by a protein complexed with the LPSs cannot be ruled out and the ability of the LPSs of non-*H. pylori* helicobacters to induce this factor awaits further elucidation. On the other hand, it has been established previously that enterohepatic helicobacters are capable of inducing NF-κB, a known factor in the carcinogenesis model of *H. pylori* [30]. Consistent with this, studies have already shown that *H. hepaticus* in certain animal models can induce carcinogenesis in the liver [49].

The small number of strains from each species examined in the present study reflected their availability for study, as well as the fastidious nature of certain helicobacters, which creates technical difficulties in culturing significant quantities of biomass. Despite this, we have gained structural and biological insights into the LPSs of non-*H. pylori* helicobacters and, collectively, the results demonstrate the structural heterogeneity and pathogenic potential of LPSs of the *Helicobacter* genus as a group. Moreover, the differences observed in LPS structure among species may be indicative of the adaptation of the bacterial outer membrane to the different ecological niches of the bacterial species examined.

These studies were supported by the Irish Health Research Board and the Millennium Research Fund (grants to A.P.M), by a Swedish Research Council grant 16×04723 (to T.W.), and by the Fysiografen Society, Lund University, and a student fellowship from Enterprise Ireland (to S.O.H.). We thank Heidi Annuk for assistance.

**References**


organisms, including the first culturable case. APMS 1997;105:746–56.
Lipopolysaccharides of Helicobacter spp.

323

© 2004 Blackwell Publishing Ltd, Helicobacter, 9, 313–323