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Desulfovibrio desulfuricans isolates from the gut of a single individual: Structural and biological lipid A characterization

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ABSTRACT

The levels of sulfate-reducing bacteria (SRB), including *Desulfovibrionaceae*, in the gut increase following a fat-enriched diet. Endotoxins from gut microbiota contribute to the inflammation process, leading to metabolic diseases. Thus, we sought to characterize the lipid A structures of *Desulfovibrionaceae* lipopolysaccharides (LPS) that are associated with the microbiota inflammatory properties. LPS variants were obtained from two SRB isolates from the gut of a single individual. These LPS variants shared similar lipid A moieties with Enterobacterial LPS, but differed from one another with regard to fatty-acid numbers and endotoxic activity. This first complete structural characterization of Desulfovibrio lipid A gives new insights into previously published data on Desulfovibrio lipid A biosynthesis. LPS microdiversity within SRBs illustrates how adaptation can influence pro-inflammatory potential.

1. Introduction

Imbalances in the composition of gut microbiota have been reported to be associated with susceptibility to obesity and insulin resistance diseases [1,2]. Despite recent progress, the specific factors that cause or ameliorate these diseases largely remain to be identified [3]. Recently, different studies demonstrated that Lipopolysaccharides (LPS) from gut bacteria and their receptor on the host cells, TLR4, participated in the production of inflammatory cytokines and the complication of diabetic diseases [4].

Endotoxins are lipopolysaccharides (LPSs), the major components of the external membrane of Gram-negative bacteria. Not all LPS are toxic, but when they are, their lipophilic moiety, called lipid A, is responsible for their major characteristic toxic and beneficial properties [5,6]. Lipid A structure generally consists of a diglucosamine backbone substituted with varying numbers (usually 4–7) of ester- or amide-linked fatty acids. The number and chain lengths of the fatty acids are related to the most toxic effects of LPS [7]. The addition of a single fatty acid can be responsible for virulence properties [8]. In most cases, phosphate and/or other substituents are linked to carbons at the C-1 and C-4′ positions of the lipid A disaccharide unit [9,10].

The sulfate-reducing bacteria (SRB) are a group family of anaerobic microbes using sulfate as terminal electron acceptor for their respiration and generating large quantities of hydrogen sulfide (H_2S) [11]. SRB are ubiquitous and are present in animal and human intestines [12]. Recent studies showed a significant increase in the *Desulfovibrionaceae* family in both obese human volunteers and mice compared to lean individuals [13,14]. This suggested that the SRB are potential endotoxin producers leading to a low grade, but chronic inflammation, causing obesity and diabetes. Although partial chemical compositions of SRB LPS have been published [15], they did not lead to the complete structural characterization of their lipid A, and consequently, no relevant relation between the structures and biological activities could be reported until now.

In this study, we report the first complete description of two SRB lipid A structures explaining their relationship to biological activities. MALDI mass spectrometry and complementary biochemical analyses allowed a complete qualitative and quantitative analysis of the lipid A molecular species. The structures, found to be different in the two SRB strains, will be described in relation to their biological activities.

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2. Materials and methods

2.1. Bacterial strains

The SRB strains were isolated from the gut of a healthy human and grown in Postgate culture medium [16] in anaerobic conditions.

2.2. LPS extraction and purification by preparative thin layer chromatography

Chromatography was performed on HPTLC glass silica coated plates (Merck) and LPS extraction was performed as previously described [17]. Briefly, samples were extracted from lyophilized bacteria in a mixture of isobutyric acid: 1 M NH_4OH in a (5:3) ratio and were deposited in lines of 18 deposits of about 30 μg on the HPTLC plate. After 2 h of migration, LPS molecules were detected by non-destructive localization [17]. They were then eluted from the scraped silica with the same mixture of solvent followed by centrifugation, and lyophilisation of the extract. The different lyophilized extracts were washed with ethanol in order to remove any residual salt or solvent traces prone to quench the mass spectra.

2.3. SDS–polyacrylamide gel analysis of LPS

Fifteen percent acrylamide gel was used, and 0.5 μg of LPS was loaded onto the 4% starting gel. The LPS preparation, electrophoresis process and nitrate coloration were performed as previously described [18,19].

2.4. Triethylamine (TEA)-citrate microhydrolysis

We used the LPS microhydrolysis method [20] to analyze our SRB LPS extracts, this method allows direct analysis of LPS, and its fragments, by Matrix Assisted Laser Desorption mass spectrometry (MALDI-MS) without salt removal.

2.5. Liberation of the ester-linked fatty acids [21]

Conditions for the first-step cleavage of primary ester-linked fatty acids: Lipid A (200 μg) was suspended at 1 mg/ml in 35% ammonium hydroxide and stirred for 5 h at 50 °C. For liberation of the secondary ester-linked fatty acids, lipid A was suspended in 41% methyl-amine and stirred for 5 h at 37 °C. The solutions were dried with a stream of nitrogen, the residue were taken up in a mixture of $CHCl_3$/MeOH/water (3; 1.5; 0.25 v/v) followed by TLC and analysis by MALDI-MS.

2.6. MALDI mass spectrometry (MALDI-MS)

Analyses were performed on a PerSeptive Voyager-DE STR model time-of-flight mass spectrometer (Applied Biosystem) (IBBMC, Université de Paris XI), in linear mode with delayed extraction. Both negative- and positive-ion spectra were recorded. The ion-accelerating voltage was set at 20 kV. Dihydroxybenzoic acid (DHB) (Sigma chemical Co., St Louis) was used as a matrix. A 1 μl aliquot of the lipid A solution (1 μg/μl) in $CHCl_3$/CH_3OH/H_2O (3; 1.5; 0.25 v:v) was deposited on the target and covered with the same volume of the matrix dissolved at 10 mg/ml in the same solution [17]. Different ratios of sample to DHB were tested when necessary. *Escherichia coli* J5 lipid A was used as external standard.

2.7. Gas chromatography–mass spectrometry

Fatty acids were identified by gas chromatography (GC). A Shimadzu (GCMS-Q P2010SE-Gas Chromatograph mass spectrometer) GC/MS apparatus was used and a temperature gradient from 170 °C to 250 °C, 3 °C/min was employed on a Phenomenex ZB-5MS (0.25 × 0.25 × 30 m) capillary column. Arachidonic acid (C20), a fatty acid absent from the natural samples, was used as an internal standard.

The α- and β-anomers of 2 acetoxy-ethyl 2-peracetyl-D-GlcN, chemically synthesized standards, were separated on the same column with a gradient from 150 °C to 240 °C, 2 °C/min and were eluted at 29.81 min for the α-anomer and 31.47 min for the β-anomer [27].

2.8. Cell culture

THP-1 cell were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated (56 °C, 30 min) foetal bovine serum (PAA laboratory) and 100 IU/ml penicillin, 100 μg/ml streptomycin. Cells were incubated at 37 °C in humid air with 5% CO_2.

2.9. THP-1 stimulations and cytokines detection by ELISA

For THP-1cell stimulations, 6×10^5 viable THP-1 cell in 2 ml of culture medium were added to each well of tissue culture-treated, flat-bottomed, non-pyrogenic, polystyrene 6-well plates (Greiner-bio-one) and then stimulated with 5 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml LPS, no LPS added was the control.

THP-1 cell were cultured for 48 h and supernatants were stored for ELISA analysis. TNF-α and IL-6 levels in cell-free supernatants were determined by an ELISA kit (eBioscience). The OD of each well was read by using a microplate (96-well Maxisorp Nunc) reader at 450 nm with 540 nm correction (Multiskan EX). Each experiment was repeated at least three times.

2.10. Statistical analysis

Data groups were analyzed by 1-way analysis of variance with Biosta TGV with repeated measures. A value of $P < 0.0001$ was considered to indicate a statistically significant difference.

3. Results and discussion

It was found to be very difficult to isolate SRB LPS directly by using different classical extraction procedures [22,23]. In fact, we observed that some unknown molecules, we named "X molecules", were co-extracted with LPS, which complicated their purification from these strains. They seemed to be strain specific as their masses varied from 2000 to m/z 4000 Da in the different strains as shown by MALDI-MS. Importantly, from the first SRB LPS structure published in 1985 until now, no article has described the precise structure of SRB LPS, we assume that the presence of these contaminating "X molecules" explain the absence of any previous clear-cut analysis.

We obtained pure LPS and their biologically active moiety, lipid A, by use of preparative Thin Layer Chromatography [17] followed by the LPS microhydrolysis method [20]. The MALDI spectra obtained for the lipid A fractions from the two strains were similar to those of *E. coli* or *Salmonella*, with the presence of penta- to hexa-acyl molecular species. However, MALDI-MS comparison is never sufficient to accurately characterize lipid A structures, as we demonstrated earlier for *Yersinia* lipid A [24]. This is why complementary biochemical analyses followed by sequential release of fatty acids were performed for a full structural characterization.

3.1. Negative-ion MALDI mass spectrometry

Negative-ion MALDI mass spectra of intact SRB 1 and SRB 2 lipid A, respectively, are presented on Figs. 1 and 2. The quality of the spectra is similar, and two peaks were common to both spectra (at *m*/*z* 1360.7 and 1586.8). The two negative-ion mass spectra gave a rough idea of the degree of heterogeneity present in the preparation.

The MALDI negative-ion mass spectrum obtained for the SRB 1 strain presented in Fig. 1 was analyzed, the spectrum displayed a major molecular species at *m*/*z* 1586.8 corresponding to a penta-acyl lipid A. Two other molecular species of medium abundance were observed at plus 238 (C16:0) and minus 226 [C14:0 (3-OH)]. A minor molecular species was seen at *m*/*z* 1709.7 corresponding to Phosphoethanolamine (PEA) substituted penta-acyl lipid A. The latter is presented in Fig. 1 as a substituent of the glycosidic phosphate as found in most *E. coli* structures. We found no evidence of PEA substituting the second phosphate group, since, no peak characteristic of this position was found in the spectra.

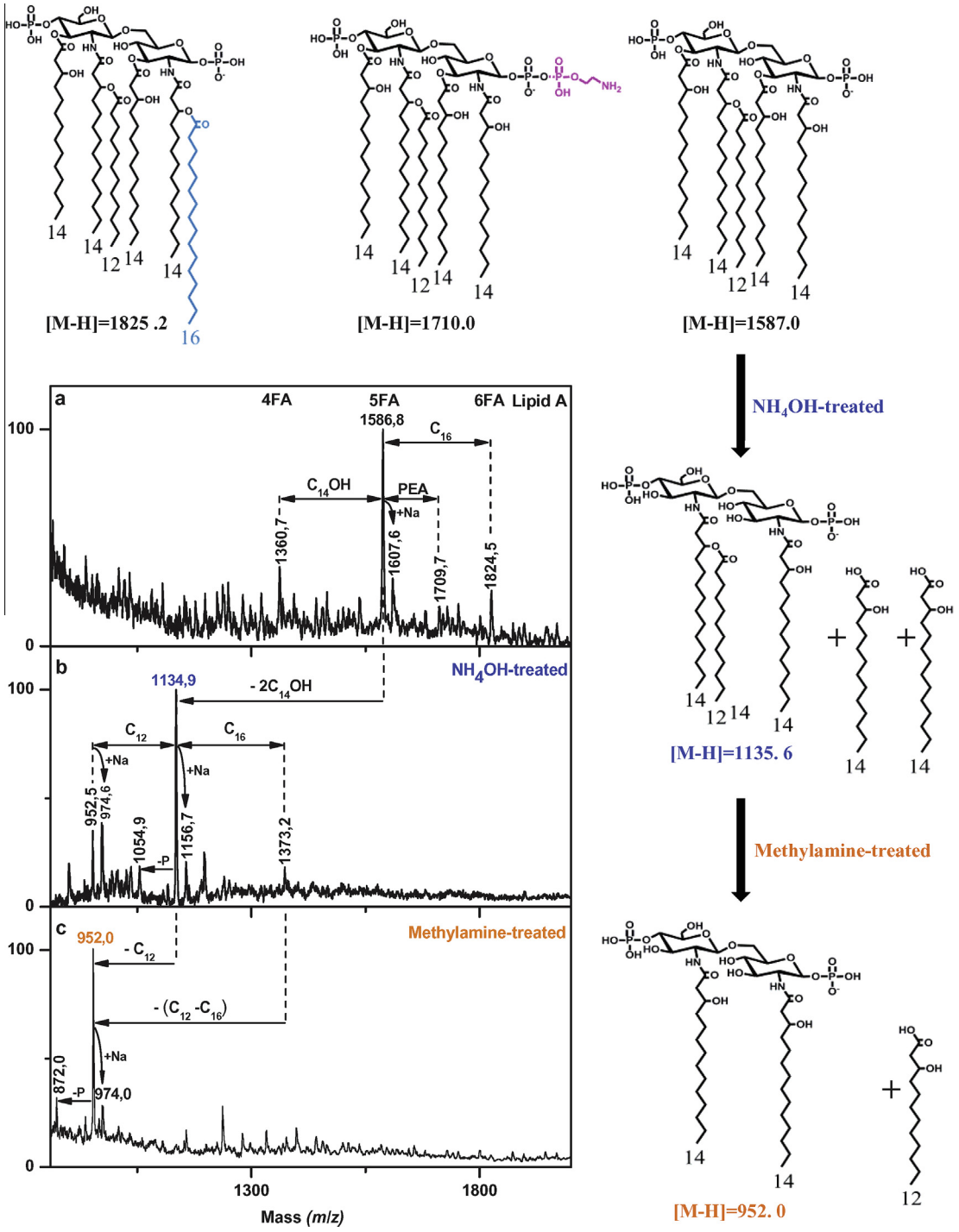

Fig. 1. Negative-ion MALDI mass spectra of SRB 1 lipid A before and after alkaline treatments and the corresponding structures. (a) Native lipid A spectrum. (b) Spectrum of the lipid A obtained after the primary de-O-acylation step. (c) Lipid A obtained after complete de-O-acylation.

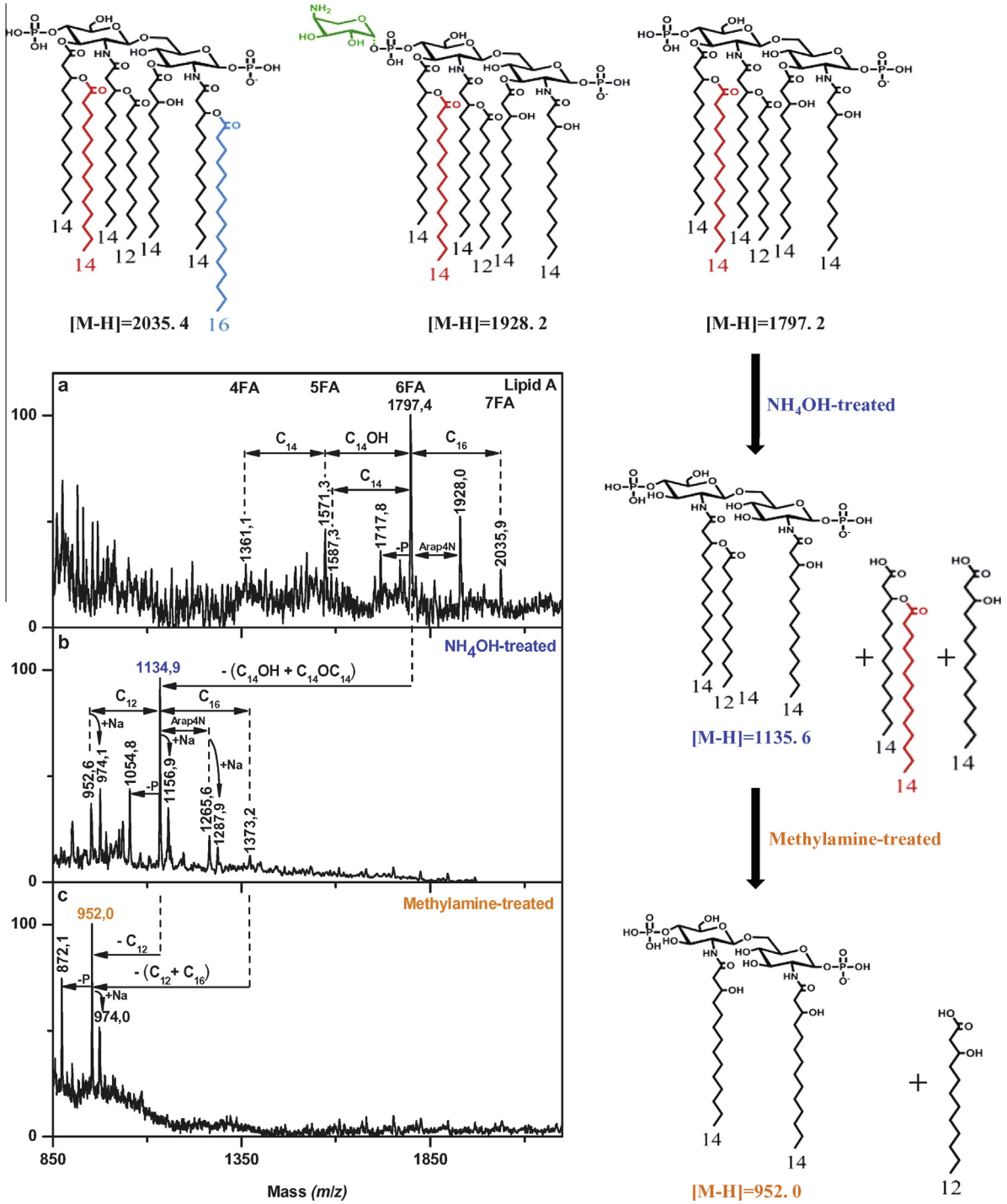

Fig. 2. Negative-ion MALDI mass spectra of SRB 2 lipid A before and after alkaline treatments and the corresponding structures. (a) Native lipid A spectrum. (b) Spectrum of the lipid A obtained after the primary de-O-acylation step. (c) Lipid A obtained after complete de-O-acylation.

The negative-ion MALDI mass spectra obtained for the SRB 2 strain was compared to that from SRB 1 (Fig. 2), the spectrum corresponding to SRB grown in the Postgate medium displayed major hexa-acyl molecular species at *m*/*z* 1797.4, corresponding to the classical hexa-acyl molecular species with additional peaks at plus 131u and a minor peak at plus 238u, corresponding respectively to 4-amino-4-deoxy-L-arabinopyranose (Arap4N) and C16:0. Minor peaks appeared at minus 210u and at minus 226u, from *m*/*z* 1797.4, corresponding to penta-acyl molecular species, lacking respectively C14:0 and C14:0 (3-OH) fatty acids.

The total fatty acid composition and configuration of GlcN were established by GC–MS as described earlier [25,26]. The presence of di-glucosamine was confirmed after total fatty acid liberation by MALDI-MS. The β-1-6 linkage between the two GlcN residues was characterized by comparison with chemically synthesized partial lipid A molecules [27]. We found identical retention time for the oxidised reduced lipid A with 2-acetoxy [^{2}H] ethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside eluted at 31.47 min and well separated from the α-form eluted at 29.81 min on the Phenomenex ZB-5MS capillary column [27].

The determination of the α-configuration of the glycosyl phosphate present in the natural lipid A was deduced from the stability of the phosphate group as demonstrated earlier on α- and β-D-glucopyranose 1-phosphate standards [28].

The fatty acid localization in the di-glucosamine core in *Desulfovibrio desulfuricans* lipid A strains was deduced from their stability upon alkaline treatment as shown in Figs. 1 and 2.

3.2. Positive-ion MALDI mass spectrometry

The distribution of fatty acids on the two GlcN residues was demonstrated by MALDI-MS fragmentation in the positive ion-mode by cleavage between the two glucosamines [29]. This showed peaks at *m*/*z* 1086 and 888 corresponding respectively to one GlcN, one Phosphate, two C14:0 (3-OH), one C14:0 and one C12:0, for the first molecular species and the same structure with loss of a OC12 (198u) for the second.

The lipid A structures corresponding to the main molecular species described for the two SRB strains deduced from all the presented data are illustrated in Figs. 1 and 2.

3.3. THP1 stimulation and IL-6/TNF-α detection

To characterize and compare the endotoxin activity of the two types of lipid A, we stimulated human monocytes (THP1) with SRB 1 and SRB 2 LPS. We tested both IL-6 and TNF-α activities, which were selected as good markers for acute inflammation and compared them to those of *E. coli* J5 LPS (Fig. 3B and C). The latter has a hexa-acyl lipid A and was also taken as a reference for its similar migration by SDS–PAGE (Fig. 3A).

As expected from previous work performed in our laboratory and in others [7,30] the hexa-acyl lipid A molecular species, like the one present in SRB 2 LPS, induced higher levels of cytokines than the others. As shown in Fig. 3B, SRB 1 LPS activated IL-6 induction at a medium level while SRB 2 LPS gave higher IL-6 production, and as expected close to that of *E. coli* J5 LPS. Similar results were observed for TNF-α production and confirmed that SRB 1 LPS was much less active than SRB 2 and *E. coli* J5 LPS (Fig. 3C).

3.4. Comparison of the current Desulfovibrio lipid A structures with the activity of LpxM and LpxL proteins described for Desulfovibrio vulgaris lipid A biosynthesis pathway

SRB 1 lipid A structure corresponded to what was expected from the biosynthesis pathway recently published for the lipid A from *D. vulgaris* lipid A, which lacks the LpxM enzyme [31] as shown in Fig. 4, where are presented the organisms most closely related to the *Desulfovibrio* species and the corresponding enzymes involved in lipid A biosynthesis. According to Opiyo et al. [31], the absence of LpxM in *D. vulgaris* results in the absence of a C14:0 fatty acid compared to the *E. coli* hexa-acyl lipid A structure. LpxH and LpxH2 are also missing in this species, corresponding to the glycosidic phosphate and pyro-phosphate moieties.

From our results, and with the two structural examples characterized for the two strains, LpxM should be missing in SRB 1 but

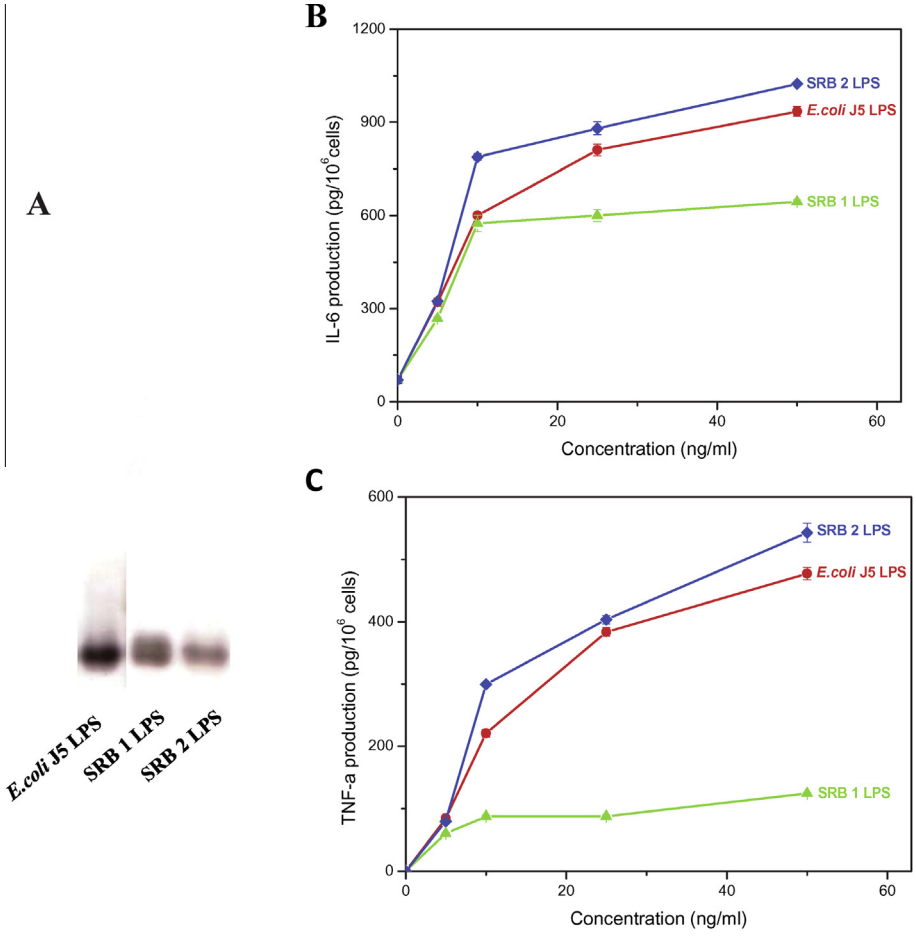

Fig. 3. Secreted cytokines IL-6 and TNF-α, after 48 h stimulation of human THP-1 (6×10^5 cells) with different doses of purified LPS from two strains of SRB and *E. coli* J5. (A) SDS–PAGE, the bands correspond to the different extracts of LPS as indicated. (B) IL-6 production. (C) TNF-α production. All experiments were performed in triplicate.

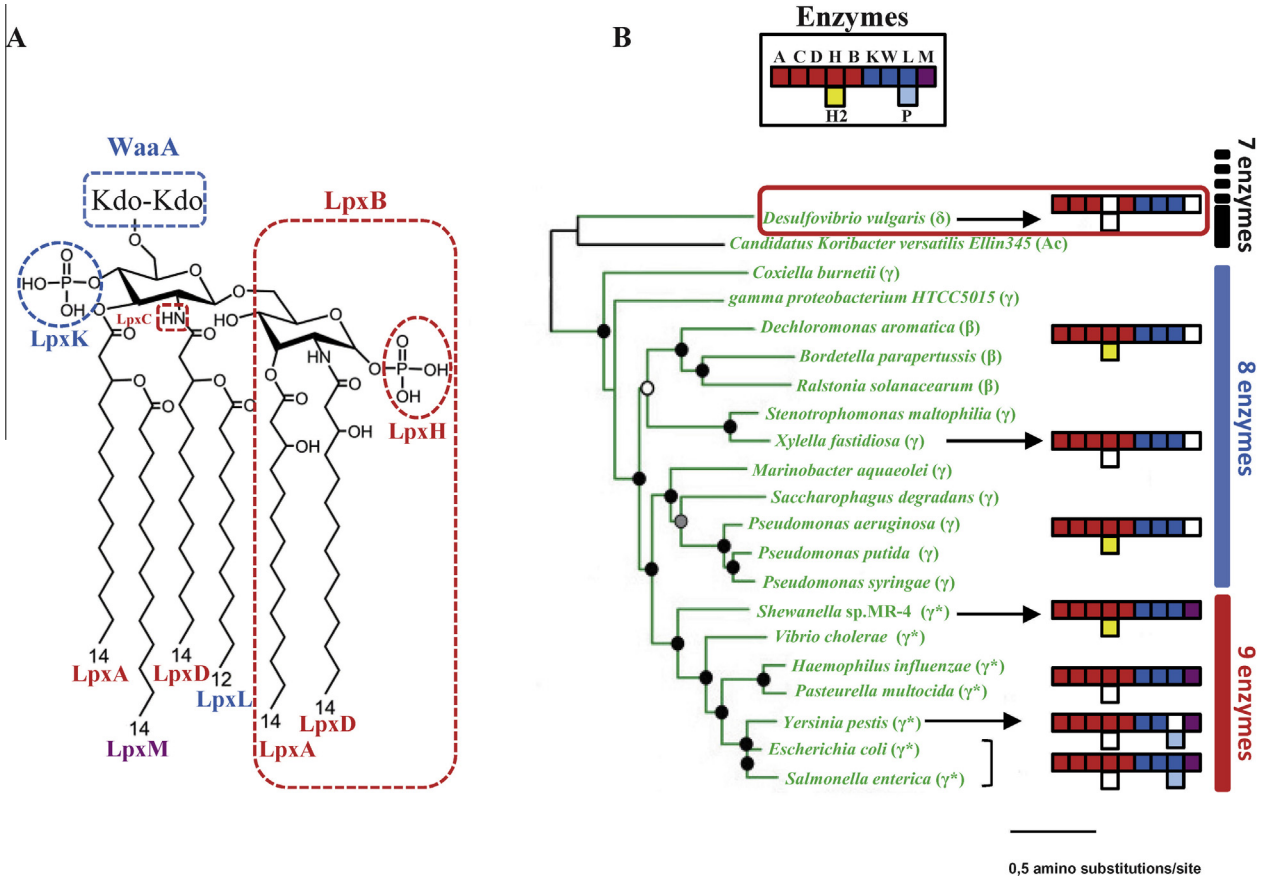

Fig. 4. (A) Structure of lipid A from *E. coli* K12. (B) Distribution of lipid A biosynthetic enzymes across bacterial genomes (modified from [31]).

not SRB 2. Consequently, the absence of LpxM in the *D. vulgaris* pathway should not be considered as a characteristic of the genus. In addition, as shown in Fig. 4 a glycosidic phosphate and a palmitate are present in the two lipid A structures. These elements result from the activation of the LpxH and LpxP in both strains while the corresponding genes are absent in *D. vulgaris*.

4. Conclusion

Since a relatively large increase of SRB was observed in the human and mouse guts after a fat enriched diet [13,14], the absence of the C14:0 fatty acid due to the deletion of LpxM should have an important impact on the resulting inflammatory process and would vary depending on the presence of hexa-acyl or penta-acyl lipid A molecular species. The implication of these data in diabetes and obesity based on LPS structure to activity relationships is comforted by this work and that of other authors [1,4].

In addition to their own LPS inflammatory capacities, SRB are able to alter the intestinal epithelial membrane by their capacity to generate H_2S. The dissociated cells could open the way, not only to other LPS molecules present in the gut, but also to bacteria. This transmembrane passage was demonstrated earlier [32].

It is interesting to note that *D. desulfuricans* shares the well-known di-phospho-di-glucosamine lipid A structure characteristic of *Enterobacteriaceae* with the same fatty acid distribution. The evolution of the lipid A enzymatic pathway described by Raetz et al. [33] is extremely well conserved in a majority of Gram-negative bacteria. However, the evolution of the biosynthetic pathway, as shown in [18], involved duplication or losses of different genes dependent upon adaptation to hosts and different niches.

In this work we present data concerning a single human gut, stressing the micro-diversity observed. The significance of our findings are that while accumulating evidence implicates a pivotal role for LPS-producers in obesity/diabetes, this is the first example that different strains of the same species, in the same habitat (isolated from a single human's gut), can actually have a different structure and consequently different pro-inflammatory capacities of their lipid A. Therefore, the observation of increasing endotoxin production in the gut microbiota of obese mice and diabetic patients needs to be monitored at the strain level.

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