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Structural analysis of *Yersinia pseudotuberculosis* ATCC 29833 lipid A

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This paper is dedicated to Yannik Hoppilliard on her 60th birthday. We all appreciate her dynamism, friendliness, and devotion to the community of mass spectrometry in France.

Abstract

The *Yersinia* genus includes human and animal pathogens (plague, enterocolitis) as well as non-pathogens. The lipopolysaccharide of the facultative pathogen *Yersinia pseudotuberculosis* has been implicated in the invasiveness of these bacteria. In this work, we have investigated the fine structure of the lipid A isolated from *Y. pseudotuberculosis* lipopolysaccharide using chemical analyses, gas chromatography/mass spectrometry, plasma desorption mass spectrometry, and matrix-assisted laser desorption mass spectrometry. Arabinose (Ara) and aminoarabinose (Ara-4N) esterified the phosphates as in *Yersinia pestis* lipid A. The acylation of *Y. pseudotuberculosis* lipid A differed from those found in *Yersinia enterocolitica*, *Yersinia ruckeri*, and *Y. pestis* lipopolysaccharides (LPSs): in the distribution of fatty acids between the two glucosamines in the fully acylated hexaacyl molecular species and by the acyloxyacyl substitution at position C-2′, where the *Y. pseudotuberculosis* lipid A has a $C_{14}OC_{16}$ making it closest to that of *Y. pestis*. (Int J Mass Spectrom 219 (2002) 549–557)

Keywords: *Y. pseudotuberculosis*; Endotoxin; Lipid A; PDMS; MALDI

1. Introduction

The Yersiniae, which belong to the *Enterobacteriaceae*, include both pathogenic and non-pathogenic bacteria. Three *Yersinia* species cause disease in humans: *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* by ingestion of contaminated food or water; *Yersinia pestis* by infected flea bite or inhalation of respiratory droplets from a pneumonic plague patient. *Y. pseudotuberculosis* infects humans and animals causing necrotic lesions in liver, spleen, and lymph nodes. *Y. pestis* has been described as "a recently emerged clone of *Y. pseudotuberculosis*" [1].

Gram-negative bacteria produce endotoxin, a mixture of related lipopolysaccharides (LPSs) found as components of their outer membranes [2]. Some endotoxins are not toxic but among those that are, the lipid moiety, called lipid A, has been recognized as the principal toxic component. The number and nature of its fatty acids as well as their structural position have been shown to be specifically related to lipid A toxicity [3–5].

The structures of the lipids A isolated from *Y. enterocolitica*, *Yersinia ruckeri*, and *Y. pestis* have been

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recently described [6]. Compared to other *Yersinia* species, *Y. pestis* and *Y. pseudotuberculosis* display increased outer membrane permeability that seems to be related to the fluidity of their LPS acyl-chains as well as to their shorter polysaccharides [7]. However, no structural data or chemical analyses were given to explain these results.

We determine here the structure of the native lipids A of *Y. pseudotuberculosis* strain ATCC 29833, and compare the results to the previously analyzed lipid A structures described for *Yersinia* species [6].

2. Materials and methods

2.1. Bacterial strains and cultures

Y. pseudotuberculosis strain ATCC 29833, strain type RF 4738 was obtained from the National Research Council collection, NRCC 4269. It was grown at 37 °C as reported earlier [8] and the cells were killed in 2% phenol before harvesting.

2.2. LPS

The LPS of *Y. pseudotuberculosis* was extracted by the modified enzyme–phenol–water method [9]. It was obtained as a precipitated gel by ultracentrifugation (105,000 × *g*, 4 °C, 12 h) and then purified by extraction with solvents to remove phospholipids and free fatty acids. It was further treated with proteases and nucleases until TLC and UV spectra showed no detectable contaminants [10]. Lipid A was prepared by hydrolyzing LPS in 20 mM Na acetate–acetic acid pH 4.5–1% Na dodecylsulfate at 100 °C for 1 h, lyophilization, removal of the detergent by extraction with acidified ethanol, followed by centrifugation, and extraction of the lipid A in the pellet with chloroform:methanol:water 12:6:1 [11].

2.3. Fatty acid analysis

To determine the total lipid composition, fatty acids were released as previously described [6]. To identify fatty acids substituting glucosamine positions at C-3 and C-3′, lipid A was treated with 0.2 M NaOH at 37 °C for 5 min [12]. The released fatty acids were extracted with ethyl acetate, methylated with diazomethane, and identified by GC on an HP5 column (30m × 0.32 mm) with a program 150–300 °C at 6 °C/min. Gas chromatography/mass spectrometry (GC/MS) was performed on a DB5ms capillary column (30 m) coupled to a Finnigan MAT 95.S.

2.4. Thin-layer chromatography (TLC)

TLC was done on aluminium-backed silica-gel plates (Merck). Products were visualized by charring (in an oven at 150 °C for 5 min) after spraying with 10% sulfuric acid in ethanol or by spraying with ninhydrin solution. 10 μg of lipid A was deposited on a TLC plate and chromatographed in the solvent mixture—chloroform:methanol:water:triethylamine (12:6:1:0.04) [11].

2.5. SDS–polyacrylamide gel of LPS

15% polyacrylamide gels were prepared and loaded with samples of 0.2–0.5 μg of the starting LPS preparation and its silica-gel fractions, electrophoresed as previously described [13], and then stained [14].

2.6. Mass spectrometry

PDM spectra were obtained as previously described [12]. They have been shown to give the distribution of fatty acids between the two glucosamines (GlcNs) as well as the heterogeneity of the sample. They also give molecular-ion intensities proportional to the relative abundances of the molecular species. This information is very useful, although plasma desorption mass spectometry (PDMS) is not as sensitive and fast as matrix-assisted laser desorption mass spectrometry (MALDI). Resolution was ±1 u and the fragmentation pattern is annotated according to [12,15]. Fatty acids were characterized by electron impact mass spectrometry (EI/MS) on a Finnigan MAT 95.S mass spectrometer. MALDI/MS was carried out in the

linear mode under delayed-extraction conditions on a Perseptive Voyager STR model (PE Biosystem, France) time-of-flight mass spectrometer (I.B.B.M.C., Orsay). Gentisic acid (2,5-dihydroxybenzoic acid) was used as a matrix: a suspension of lipid A in chloroform:methanol:water 12:6:1 (1 mg/mL) was desalted with a few grains of Dowex 50 W-X8(H^+), 1 μL was deposited on the target, mixed with 1 μL of the matrix suspended at 10 mg/mL in water or 0.1 M aqueous citric acid [16] and dried. In this case, suspension of the lipid A in water gave the same result. Analyte ions were desorbed from the matrix with pulses from a 337 nm nitrogen laser. Spectra were obtained in the negative-ion mode at 20 kV with an average of 128 pulses. For the post-source decay (PSD) analysis, the reflectron configuration with delayed-ion extraction was used to obtain the fragment–ion spectrum by metastable decomposition of a preselected ion.

3. Results

The different *Yersinia* LPSs exhibited a high degree of heterogeneity on SDS–polyacrylamide electrophoresis gels (Fig. 1). TLC analysis of the isolated lipid A also showed heterogeneity, some spots giving a positive response to ninhydrin, indicative of free amino groups (not shown).

The fatty-acid composition obtained by GC/MS indicated the presence of hexadecanoic acid (C_{16}; two equivalents), hydroxytetradecanoic acid ($C_{14}OH$; four equivalents) together with minor quantities of dodecanoic acid (C_{12}).

Interpretation of the PDMS negative-ion spectra of the isolated lipids A in this study was based on earlier results showing that molecular ions in the high-mass region (*m*/*z* values greater than about 1100 in this case) gave signals proportional in intensity to the abundance of the corresponding lipid A species present in the preparation [17]. Lipid A molecular species having a residue of aminoarabinose (Ara-4N) substituting the phosphate at C-4′ were exceptions, a portion of this residue being cleaved off [13]. Signals in the lower-mass region were assumed to be fragment ions.

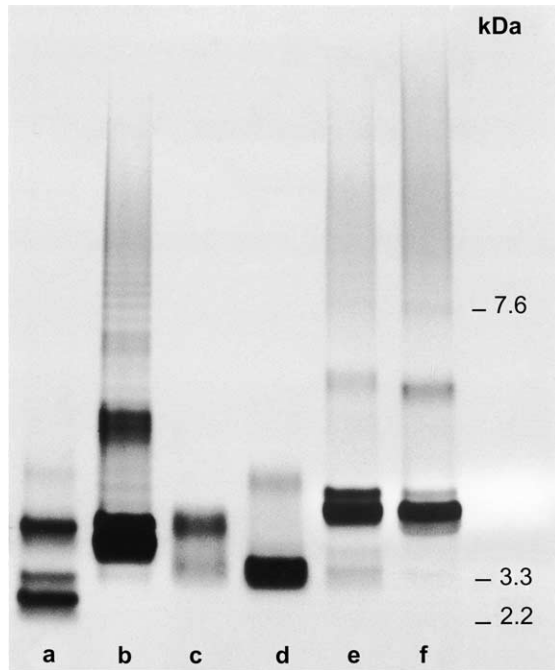

Fig. 1. SDS–polyacrylamide gel electrophoresis of various *Yersinia* lipopolysaccharides[6]: (a) *Y. ruckeri* O1, (b) *Y. ruckeri* O2, (c) *Y. pseudotuberculosis*, (d) *Y. pestis*, (e) *Y. enterocolitica* O117, and (f) *Y. enterocolitica* O3. LPS molecular weight standards are at right.

3.1. Negative-ion PDMS

The spectrum (Fig. 2B) confirmed the high heterogeneity of the lipid A preparation as deduced from TLC analysis. The dominant peak, corresponding to a tetraacyl molecular species, appeared at *m*/*z* 1405. On the basis of the overall chemical composition, this peak would correspond to a molecular-ion species containing two GlcN (161.16 × 2), two phosphates (80 × 2), and four $C_{14}OH$ (226.36 × 4). The ion at *m*/*z* 1643 would correspond to a molecular species with, in addition, a C_{16} unit (238.41), and that at *m*/*z* 1882, with a second C_{16}. A triacyl molecular species (3 $C_{14}OH$) gave a peak at *m*/*z* 1179. The peak at *m*/*z* 1417 would correspond to a tetraacyl molecular species with 3 $C_{14}OH$ and 1 C_{16}, the one at *m*/*z* 1548 has, in addition, an Ara-4N. A triacyl molecular species with Ara-4N (131.13) gave a peak at *m*/*z* 1310, and the latter with an additional $C_{14}OH$ appeared at *m*/*z* 1536. Smaller

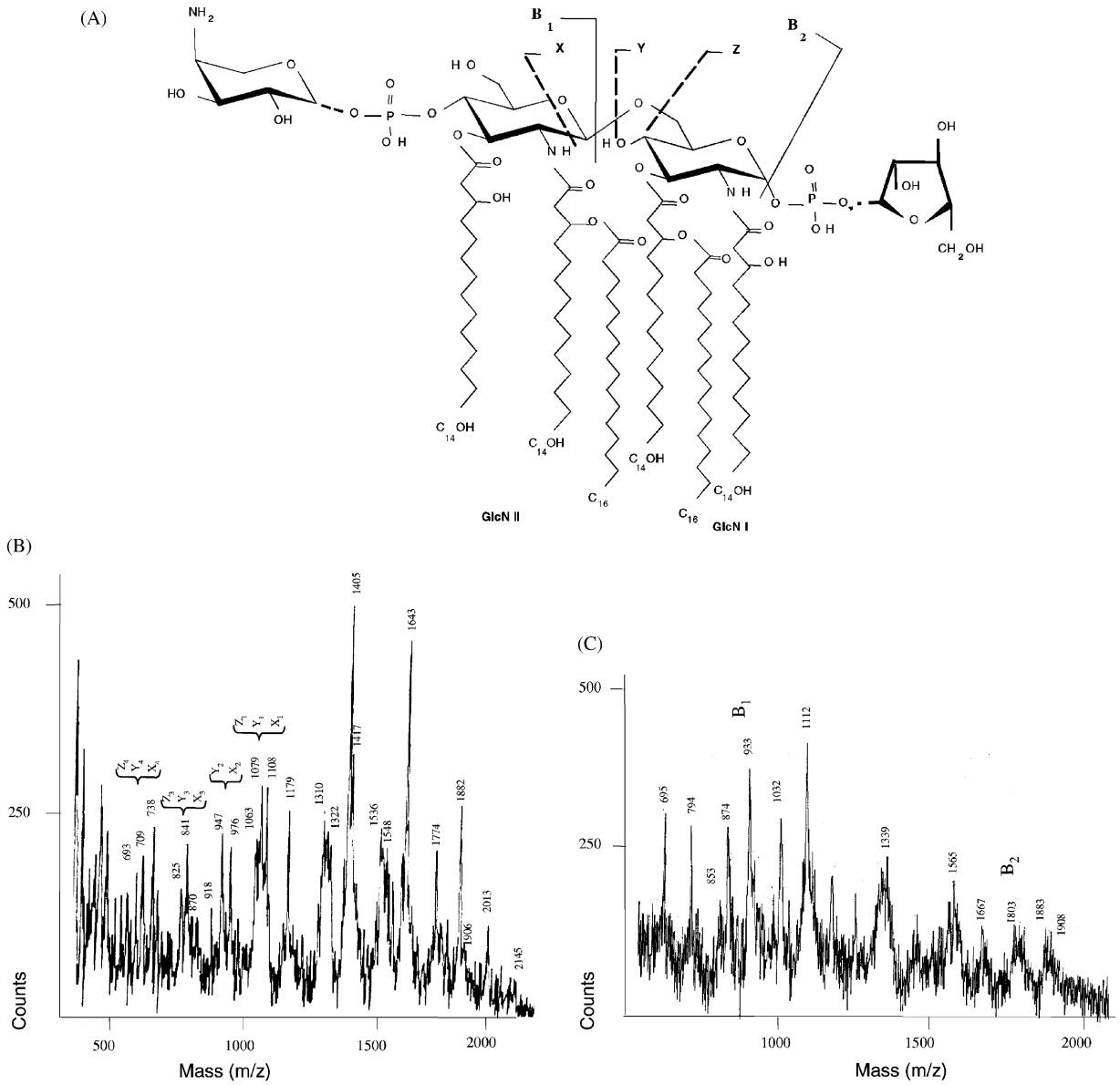

Fig. 2. Lipid A structure of *Y. pseudotuberculosis* showing fragmentation sites in positive- and negative-ion PDMS spectra (A), PDMS negative-ion spectrum (B), and PDMS positive-ion spectrum (C).

peaks found at *m*/*z* 1774 and 1906 corresponded to *m*/*z* 1643 with an Ara-4N and Ara-4N plus arabinose (Ara; 132.12), respectively. The same additions to the molecular-ion species giving a signal at *m*/*z* 1882 generated peaks at *m*/*z* 2013 and 2145. Peaks observed in the lower-mass region are fragment-ions corresponding to GlcN I. Three sets of three peaks and one set of two peaks were observed corresponding to the ions resulting from the already described fragmentations at the *X*, *Y*, and *Z* sites (Fig. 2A): 1108 (X_1), 1079 (Y_1)

and 1063 (Z_1) correspond to one GlcN, one phosphate, two C_{14} OH, one C_{16}, one Ara [12]. The series *m/z* 976 (X_2) and 947 (Y_2) were attributed to the corresponding fragments minus Ara. The series *m/z* 870 (X_3), 841 (Y_3), 825 (Z_3) were attributed to the corresponding fragments minus C_{16}, and *m/z* 738 (X_4), 709 (Y_4), and 693 (Z_4) to the fragments minus C_{16} plus Ara. The distribution of fatty acids on the two GlcNs were deduced from the masses obtained for the molecular-ion species and from the fragment-ion species corresponding to GlcN I. Each GlcN of the fully substituted lipid A had two C_{14}OH and one C_{16}. If Ara was localized on the phosphate of GlcN I, the Ara-4N had to be a substituent of the phosphate linked to GlcN II. Since the difference between the two substituents is only one atomic mass unit, it was not possible to confirm their attachment sites by PDMS.

3.2. Negative-ion MALDI

The molecular-ion peaks observed in the negative-ion mode were similar to those obtained by PDMS. The spectra (Fig. 3A) differed mainly by the presence of only two fragment-ion peaks corresponding to the GlcN I moiety: *m/z* 931.2 and 692.8 composed of one GlcN, one phosphate and two C_{14} OH, with and without C_{16}, respectively.

The accuracy of the masses observed in the MALDI compared to PDMS confirmed the presence of both Ara and Ara-4N, as shown by the peaks at *m/z* 2012.8 and *m/z* 2144.8 in the negative-ion spectrum of the native lipid A. These peaks differed from *m/z* 1882.0 by the masses of 132 (Ara) and the sum of 132 and 131 (Ara-4N), respectively. Minor peaks at *m/z* 1825.8 and 1957.1 would be homologues of the pentaacyl molecular species at *m/z* 1882 and 2012. 8, respectively, with a C_{12} replacing the second C_{16}.

3.3. Positive-ion PDMS

The spectrum (Fig. 2C) confirmed the distribution of fatty acids between the two glucosamine residues, *m/z* 933 corresponding to the GlcN II moiety (B_1) composed of one glucosamine, one phosphate, two C_{14}OH, and one C_{16} [12]. The other large fragment-ion peak was at *m/z* 695 (933 minus C_{16}). These data corroborated the analysis of the negative-ion spectrum in which each GlcN carried two C_{14}OH and one C_{16}. The molecular-ion peak of highest mass was observed at *m/z* 1908 corresponding to M (2145.8 u) minus C_{16}, *m/z* 1883 differed from M by the combined masses of Ara and Ara-4N, *m/z* 1803 (B_2) contained one phosphate less than that, and *m/z* 1565 lacked, in addition one C_{16}. *M/z* 1565 minus one and two C_{14}OH gave, respectively, *m/z* 1339 and *m/z* 1112, a major peak. The latter ion with two GlcN, one phosphate, only two C_{14}OH and one C_{16} was a strong indication of a $C_{14}OC_{16}$ in amide position. A fragment ion without C_{16} appeared at *m/z* 874 and a dephosphorylated form at *m/z* 794.

3.4. Alkali treatment

Alkali treatment, known to liberate fatty acids in ester linkage at positions C-3 and C-3′ on the GlcNs [12], liberated C_{14}OH, C_{16}, and $C_{14}OC_{16}$ as shown by GC/MS analysis. The MALDI spectrum (negative-ion mode) of the residual product (not shown) gave a signal at *m/z* 1191, corresponding to two GlcN, two C_{14}OH, two phosphates, and one C_{16}. If this C_{16} had been a substituent of an ester-linked C_{14}OH, both C_{16} would have been liberated under the alkaline conditions used. In the positive-ion spectrum, a fragment-ion at *m/z* 707 confirmed the location of the amide-linked $C_{14}OC_{16}$ on GlcN II (non-reducing). Since each GlcN carries one of the two C_{16}, and C-3 and C-3′ are the only positions liberated under the mild conditions used [12], it could be deduced that the alkali-liberated $C_{14}OC_{16}$ originated at C-3 (GlcN I).

3.5. Post-source decay (PSD)

An experiment was performed in order to localize the Ara-4N substituent. A molecular ion lacking a substituent at position C-1 (obtained by dephosphorylation during the hydrolytic process used to prepare lipid A) was selected in the negative-ion spectrum of the alkali-treated lipid A. This ion at *m/z* 1003.2 (Fig. 3B)

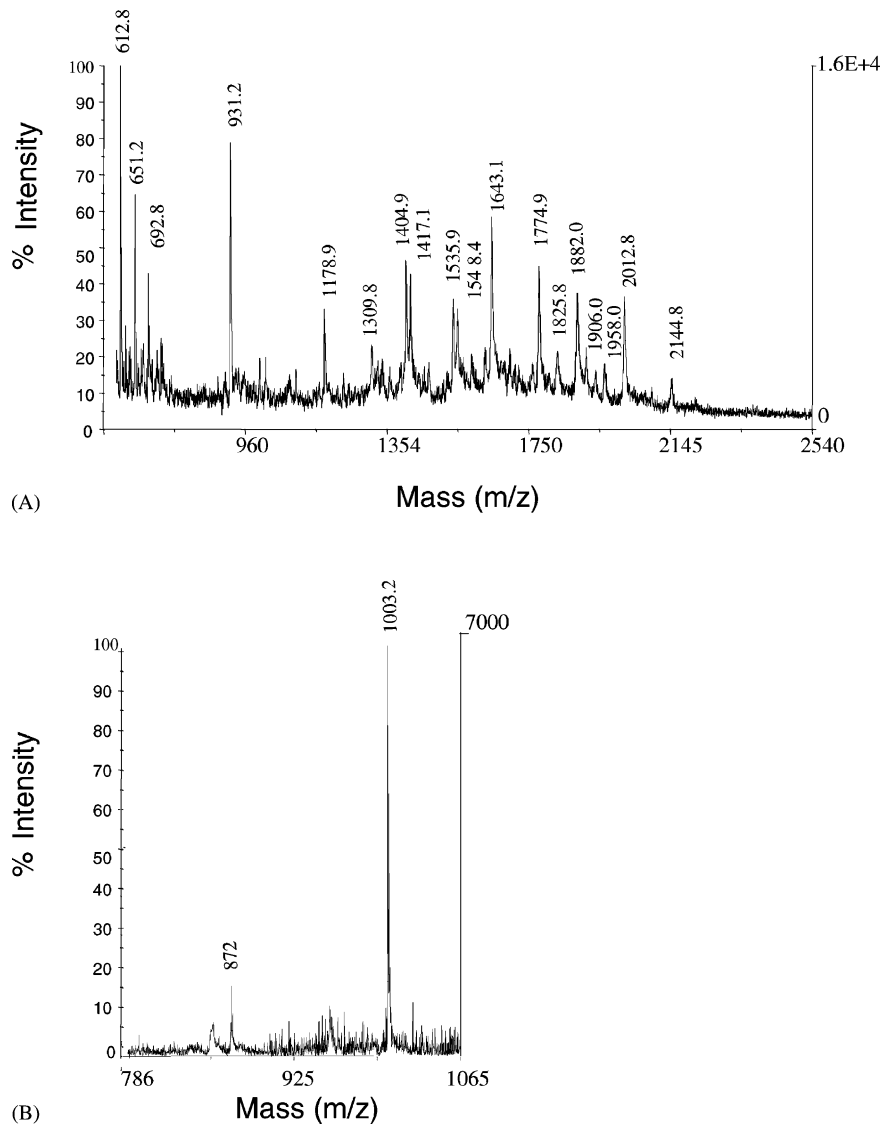

Fig. 3. MALDI negative-ion spectrum of the lipid A isolated from *Y. pseudotuberculosis* (A); PSD spectrum of peak *m*/*z* 1003.2 from the negative-ion spectrum of the alkali treated lipid A (B).

tentatively attributed to two GlcN, one phosphate, two C_{14}OH, and one Ara-4N generated a fragment-ion at *m*/*z* 872.0 (131.1 amu less), thus localizing Ara-4N at C-4′ on the unique phosphate group. This experiment led to the conclusion that Ara-4N was linked to the same position as in other Yersiniae [6]. Thus, the Ara substituent had to be located at C-1.

4. Discussion

SDS–gel electrophoresis showed that this strain of *Y. pseudotuberculosis* LPS has a short polysaccharide (Fig. 1). O-chain structures of a few other strains of this species have been determined [18,19].

The *Y. pseudotuberculosis* lipid A has the classical diGlcN backbone structure [20]. The present analysis

of the lipid A structure of *Y. pseudotuberculosis* confirms the structural variability found in the *Yersinia* lipids A and, more precisely, the differences observed in secondary acylation at position C-2′. The lipids A of the different species and strains analyzed previously fell into three groups on the basis of fatty acids amidating this position: $C_{14}OC_{12}$, $C_{14}OC_{14}$, and $C_{14}OC_{16:1}$. Fig. 4 summarizes the present results and compares them with those obtained with other species of the genus. The *Y. pseudotuberculosis* lipid A structure with a C_{16} substituent suggests that it is more closely related to *Y. pestis* lipid A which carries an unsaturated C_{16} substituent at this position. The presence of a longer chain fatty acid might partially explain the similar membrane fluidity of these species [7]. In addition to the variability observed at position C-2′, there is also a different distribution of the fatty acids between the two GlcNs of *Y. pseudotuberculosis* lipid A compared to that of the other species. The previously analyzed structures had in common with *E. coli* lipid A, an asymmetric distribution of fatty acids on the two GlcNs: four fatty acids linked to GlcN II and two to GlcN I for the fully acylated molecular species. The more unusual *Y. pseudotuberculosis* lipid A has three fatty acids on each GlcN (two ester- and one amide-linked). This distribution also exists in *Neisseria* lipid A, but, instead of giving a mirror image, as in *Y. pseudotuberculosis*, the fatty acids are placed identically on the two GlcNs [21]. Moreover, the only other lipid A we know of having a secondary acylation at the C-3 position is that of *Moraxella catarrhalis* [22]. The biosynthetic pathways leading to the *Yersinia* lipid A structures and the biological consequences of their variability will have to be explored.

It should be stressed that our present and earlier data on lipids A of *Y. enterocolitica* and *Y. ruckeri* were obtained from bacteria grown in rigorously identical culture conditions allowing more valid comparison of their structures.

An early report on the lipid A of another strain of *Y. pseudotuberculosis* [23] showed the presence of C_{12}, $C_{14}OH$, C_{16}, and $C_{16:1}$. The investigators assumed that the latter two fatty acids were contaminants because of the fluctuating amounts found in various LPS preparations. Our results suggest that they might also have been evidence of variability in fatty-acid composition in this species considering that all the preparations were extracted with solvents to eliminate contaminants. Our analysis revealed a small amount of C_{12} which probably originated in a minor $C_{14}OC_{12}$ substituent at C-3′ as shown by GC/MS analysis of the fatty acids released by mild alkali treatment. This was also confirmed by minor signals of molecular-ion species in mass spectra interpreted to contain C_{12} in place of C_{16}. The major molecular species of *Y. ruckeri* and some *Y. enterocolitica* lipids A (Fig. 4) contained C_{12} at this position.

In *Salmonella* lipid A, the C_{16} was recently reported [24] to be transferred from a phospholipid to the N-linked $C_{14}OH$ on GlcN I by an enzyme localized in the outer membrane, PagP. The gene for this enzyme has also been found in *Y. pestis* [24]. *Y. pseudotuberculosis* is now found to contain both an ester-and an amide-linked $C_{14}OH$ substituted with C_{16} and it would be interesting to compare this strain with other strains of this species, reported to be devoid of C_{16}, for the presence or absence of the transferase.

Another similarity between *Y. pestis* and *Y. pseudotuberculosis* lipids A was the presence of Ara and Ara-4N as frequent substituents of the phosphate groups located at positions C-1 and C-4′, respectively [25]. In the present study, these compounds were not isolated for chemical characterization but were localized by fragmentation in PSD analysis. The presence of ninhydrin-positive spots in TLC of the native lipid A was consistant with the interpretation of some signals in the mass spectra as Ara-4N-containing molecular ions. Moreover, the negative-ion MALDI spectrum had vicinal signals of ions differing in mass by the molecular weights of Ara and Ara-4N, 132 u and 131 u, respectively. Fragmentation of the Ara-4N linkage has already been reported in PDMS [13], and more recently in MALDI [26]. This substituent on lipid A structures is known to constitute a factor of resistance to polymyxin [27] and other cationic agents [28].

The shared presence of a long fatty acid, Ara-4N, and Ara support the recent report that *Y. pestis*

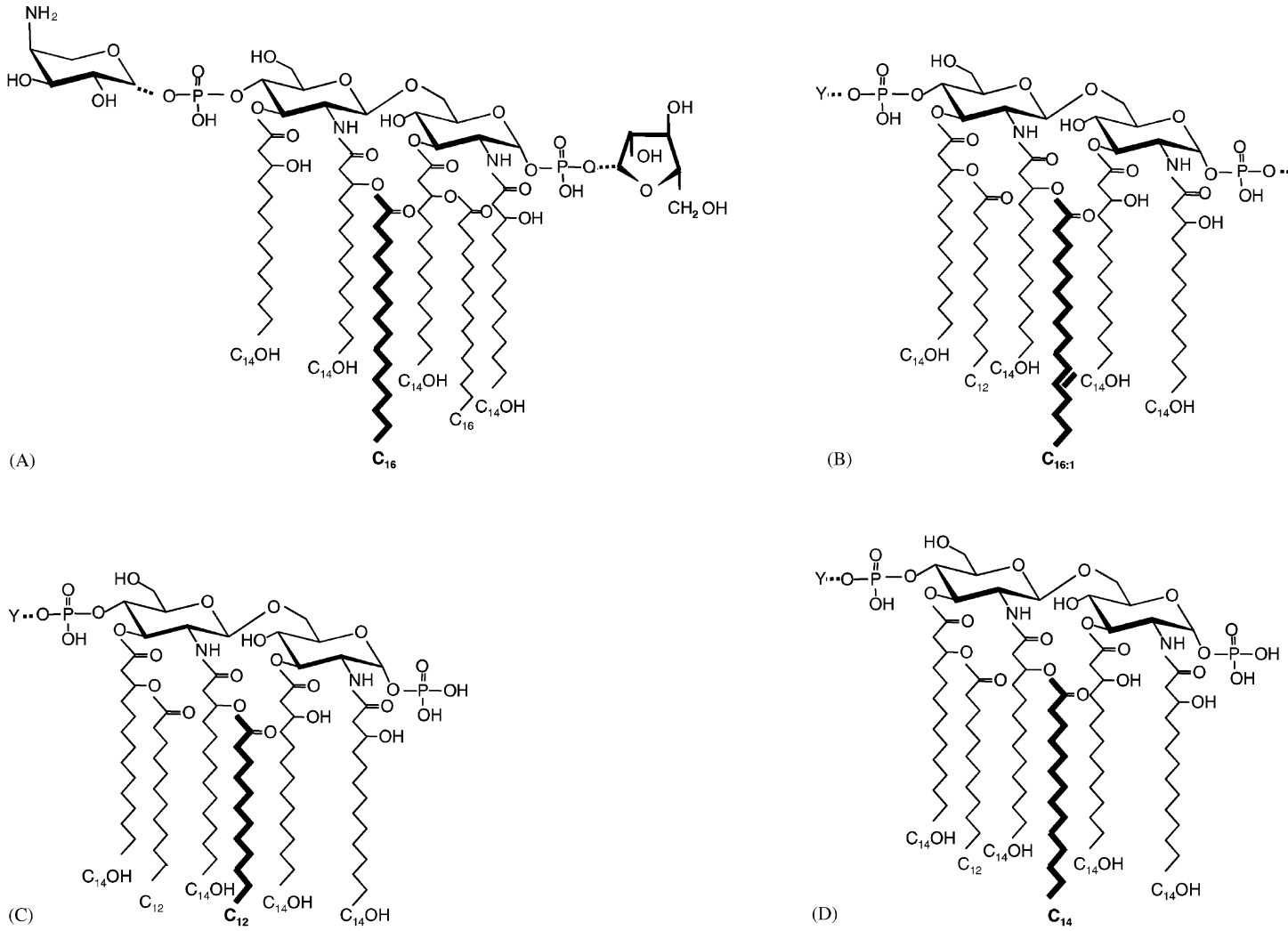

Fig. 4. Major molecular structures found in the lipids A of *Y. pseudotuberculosis*, *Y. pestis*, *Y. enterocolitica*, and *Y. ruckeri* [6]: *Y. pseudotuberculosis* (A), *Y. pestis* (B), *Y. ruckeri* and some *Y. enterocolitica* (C), and other *Y. enterocolitica* (D). X = Ara or H, Y = Ara-4N or H. The configuration of the double bond in (B) was not determined.

and *Y. pseudotuberculosis* were one species in the not-too-distant past [1]. These data and results obtained with other genera [29,30] lead to the conclusion that there is more variability among lipid A structures within a given genus than was previously thought.

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