Novel variation of lipid A structures in strains of different *Yersinia* species[1]

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Abstract The *Yersinia* genus includes human and animal pathogens (plague, enterocolitis). The fine structures of the endotoxin lipids A of seven strains of *Yersinia enterocolitica*, *Yersinia ruckeri* and *Yersinia pestis* were determined and compared using mass spectrometry. These lipids differed in secondary acylation at C-2′: this was dodecanoic acid (C_{12}) for two strains of *Y. enterocolitica* and *Y. ruckeri*, tetradecanoic acid (C_{14}) in two other *Y. enterocolitica* and hexadecenoic acid ($C_{16:1}$) in *Y. pestis*. The *enterocolitica* lipids having a mass identical to that of *Escherichia coli* were found to be structurally different. The results supported the idea of a relation between membrane fluidity and environmental adaptability in *Yersinia*.

Key words: Endotoxin; Lipid A; Structure; Plasma desorption mass spectrometry; *Yersinia*

1. Introduction

Three species of *Yersinia* cause disease in humans: *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis*. Although cases of plague caused by *Y. pestis* are now rare in the west, they can occur by contact with infected domestic or wild animals, the disease being transmitted via aerosol contamination or insect bite. *Y. enterocolitica* and *Y. pseudotuberculosis* differ considerably in invasiveness. Infections are acquired by ingestion of contaminated food or water. They infect a variety of animals as well as humans. *Y. enterocolitica* infections are common in children and outbreaks in schools are not rare [1]. *Yersinia ruckeri* is the etiologic agent of enteric redmouth disease in salmonid fish [2].

Being Gram-negative bacteria, these organisms have endotoxins, the heterogeneous but related lipopolysaccharides (LPS) constituting major components of their outer membrane. Both the lipid and glycosidic domains can be heterogeneous. Some endotoxins are not toxic but among those that are, the lipid moiety, called lipid A, has been recognized to be the principal toxic component.

Core and O-chain structures of several *Y. enterocolitica* LPS have been established [3–7] but little detailed information is available on their lipid A structures. Partial structures have been described for lipids A isolated from *Y. pestis* LPS, although no precise assignment was given for the position of ester-linked fatty acids [8,9].

Other lipids A [10,11] were described only in terms of fatty acid composition. This and the fact that the major lipid A of one strain of *Y. enterocolitica* was found to have the same molecular mass as *Escherichia coli* [12] might be the reason that it has been assumed to have the same structure [13]. It has also been shown that two different strains of *Y. enterocolitica* give different spectra [12].

We have recently determined [14] that all the fatty acid substituents of a lipid A containing the classical bisphosphorylated diglucosamine backbone could be localized using plasma desorption mass spectrometry (PDMS) of the native and alkali-treated lipid A in the negative-ion mode with the positive-ion mode serving a confirmatory role. In this communication, we determine and compare the lipid A structures of four strains of *Y. enterocolitica*, two strains of *Y. ruckeri* and one strain of *Y. pestis*.

2. Materials and methods

2.1. Bacterial strains and cultures

Smooth-type strains of *Y. enterocolitica* O:11,23; O:11,24; O:3 (previously referred to as O:11,71 in [12]) and O:9; and *Y. ruckeri* O:1 and O:2 were from the National Research Council (NRC) collection of Canada. They were grown at 37°C as reported earlier [11] and the cells were killed in 2% phenol before harvesting. The rough-type, hypovirulent *Y. pestis* EV 40 was provided by Drs. R. Fontanges and O. Creach (CRSSA, Lyon, France) and grown at 28°C as described [8].

2.2. LPS

The LPS of *Y. enterocolitica* and *Y. ruckeri* were extracted by the modified enzyme-phenol-water method [11]. They were obtained as gels by ultracentrifugation (105 000×*g*, 4°C, 12 h) and purified by extraction of phospholipids, treatment with proteases and nucleases, and centrifugation until TLC and UV spectra showed no detectable contaminants [15]. LPS II from *Y. pestis* EV 40 was prepared as described [8]. All lipids A were 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, centrifugation and extraction of the pellet with chloroform-methanol-water 12:6:1 [16].

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[1] This paper is dedicated to the memory of Linda Beynon (NRC, Ottawa, Ont., Canada).

Abbreviations: GlcN, glucosamine; C_{14}OH, hydroxytetradecanoic acid; C_{12}, dodecanoic acid; C_{14}, tetradecanoic acid; C_{16}, hexadecanoic acid; $C_{16:1}$, hexadecenoic acid; ara-4N, aminoarabinose

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2.3. Fatty acid analysis

For total composition, fatty acids were released as described in [17]. Mild alkali treatment of lipid A was effected at 37°C, 15 min with 0.25 M NaOH [14]. The released fatty acids were extracted with ethyl acetate, methylated with diazomethane and identified by gas chromatography (GC)/mass spectrometry (MS) on an HP5 column (30 m× 0.32 mm) with a program from 150°C to 300°C at 6°/min.

2.4. MS

PDM spectra were obtained as previously described [12]. Resolution was ±1 U. The fragmentation pattern is annotated according to [18]. Fatty acids were characterized by electron impact MS on a Finnigan Mat 95S mass spectrometer.

3. Results

Interpretation of the negative-ion spectra of the isolated lipids A in this study is based on earlier results showing that molecular ions in the high-mass region (*m/z* values greater than about 1000) gave signals proportional to the abundance of the corresponding lipid A species present in the preparation. An exception was found when the preparation contained a lipid A having a residue of aminoarabinose (ara-4N), a portion of which was cleaved [19]. Signals in the lower-mass region are assumed to be fragment-ions [14].

3.1. Lipids A containing a major molecular species of mass 1770 U

3.1.1. Y. enterocolitica O:11,23. According to the negative-ion spectrum, the lipid A preparation derived from strain O:11,23 was heterogeneous (Fig. 1a). The dominant peak, corresponding to a hexaacyl molecular species, appeared at *m/z* 1770, i.e. 28 U less than that of *E. coli* lipid A [14], to which *Y. enterocolitica* is closely related. On the basis of the overall chemical composition, the major peak could correspond to a molecular-ion species containing two glucosamines (GlcN) (161.16×2), two phosphates (80×2), four hydroxytetradecanoic acids (C_{14}OH) (226.36×4) and two dodecanoic acids (C_{12}) (182.31×2). The ion at *m/z* 1798 would have the same composition except for the substitution of one C_{12} by a C_{14}. Smaller peaks were found at *m/z* 2008, a heptaacyl species containing hexadecanoic acid (C_{16}) (1770+238.41 U), 1689 (1770−phosphate), 1586 (1770−C_{12}), 1570 (1798−C_{14}OH), 1388 (1570−C_{12}) and 1361 (1770−$C_{14}OC_{12}$ and/or 1798−$C_{14}OC_{14}$). The three fragment-ion peaks at *m/z* 694 (Z_1), 710 (Y_1) and 739 (X_1) attributed to the GlcN I moiety were seen in the spectrum of synthetic *E. coli* lipid A [14], indicating that part of the molecule was identical. The alkali-released fatty acids were determined by GC/MS to be C_{12}, C_{14}OH and $C_{14}OC_{12}$. In Fig. 2, the profiles of fatty acids released are presented in comparison with *E. coli*. In the latter, in addition to the major $C_{14}OC_{14}$ peak, there appears a minor peak corresponding to $C_{14}OC_{12}$. This indicates some heterogeneity at the C-3′ position and thus explains the presence of the previously observed peak at *m/z* 1770 in many spectra of this genus [20]. In addition, the negative-ion spectrum of the residual *Yersinia* lipids (not shown) gave peaks at *m/z* 1134 and *m/z* 1054 (the monophosphoryl analog). These peaks were interpreted to correspond to a molecular-ion species containing two GlcN, one or two phosphates, two C_{14}OH

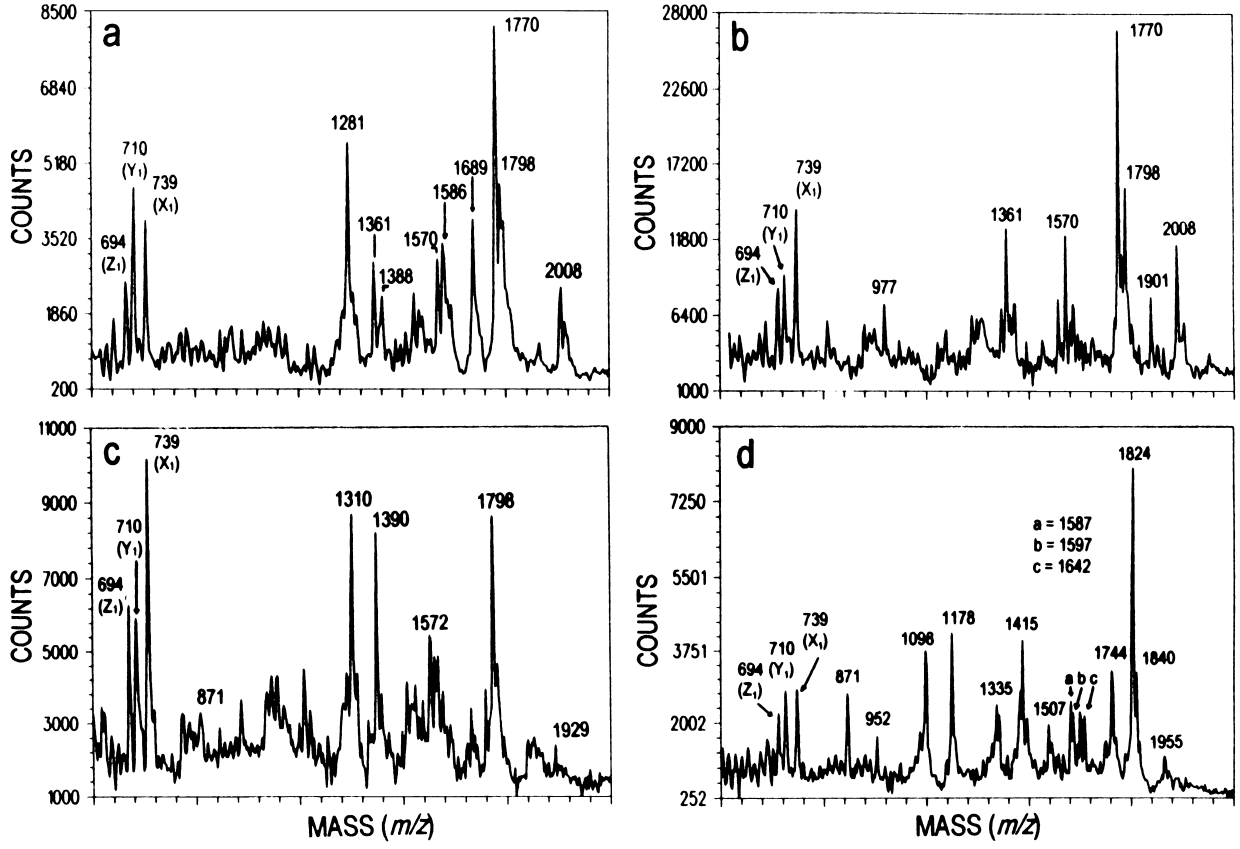

Fig. 1. (a) Negative-ion spectrum of lipid A from *Y. enterocolitica* O:11,23. (b) Negative-ion spectrum of lipid A from *Y. ruckeri* O:2. (c) Negative-ion spectrum of lipid A from *Y. enterocolitica* O:3. (d) Negative-ion spectrum of lipid A from *Y. pestis*.

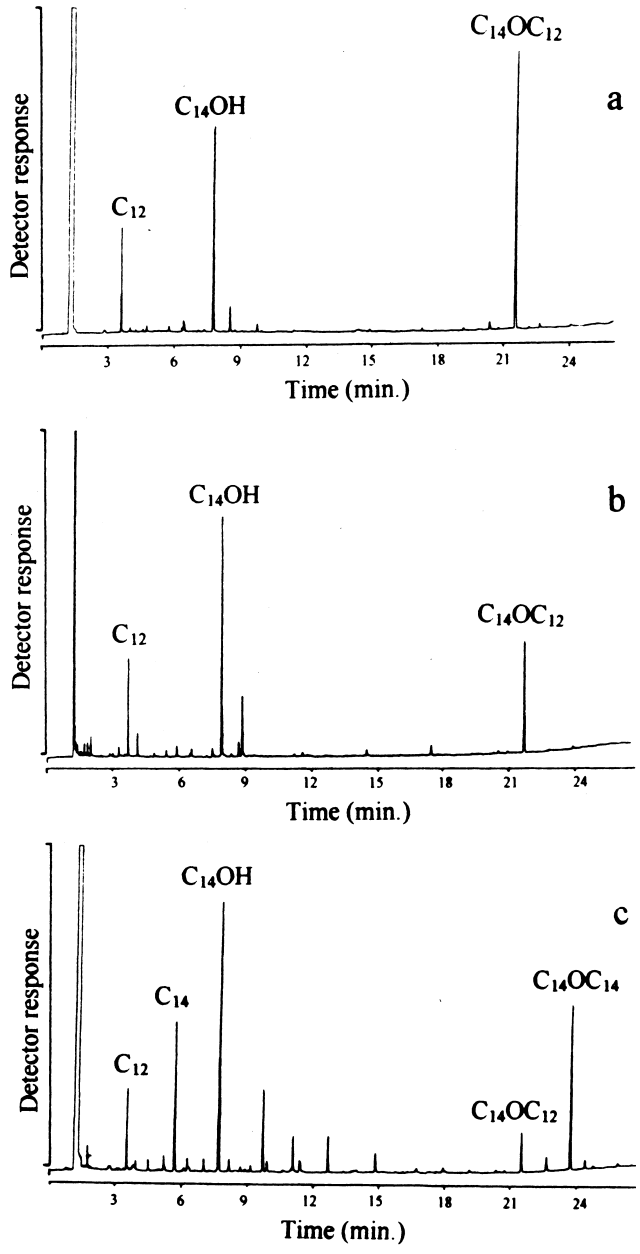

Fig. 2. GC profiles of fatty acids released by mild alkali treatment of lipids A from *Y. enterocolitica* strains O:11,23 (a), O:3 (b) and *E. coli* (c). $C_{14}OC_{12}$ present in the *E. coli* sample is due to heterogeneity at C-3′ [20].

and one C_{12}, and corresponding to the loss of the summed masses of two $C_{14}OH$ and one C_{12} of which one $C_{14}OH$ would have come from GlcN I.

The positive-ion spectrum (not shown) confirmed the distribution of fatty acids between the two GlcN, *m/z* 1058 corresponding to the GlcN II moiety to which can be attributed one GlcN, one phosphate, two $C_{14}OH$ and two C_{12}. The other large fragment-ion peak was at *m/z* 860 (1058−OC_{12}, the one linked to the amide $C_{14}OH$) [14]. These data corroborated the evidence of the negative-ion spectrum that the difference in mass of 28 U, between the lipids A of this *Yersinia* and of *E. coli*, was in the GlcN II moiety and confirmed that there were two C_{12} units on GlcN II.

3.1.2. Lipid A of Y. enterocolitica O:11,24. The native heterogeneous lipid A was analyzed and found to have the same basic structure as *Y. enterocolitica* O:11,23 except for the ara-4N-containing molecular species at *m/z* 1901 (1770+131) [12].

3.1.3. Lipids A of Y. ruckeri O:1 and O:2. These two lipids A gave similar negative-ion spectra with a major peak at *m/z* 1770. The *Y. ruckeri* O:2 lipid (Fig. 1b) gave additional signals at *m/z* 1901 (1770+131) and at *m/z* 2008 (1770+238) due to the presence of ara-4N and C_{16}, respectively. Also present were the molecular-ion peaks at *m/z* 1798, 1570 and 1361 as seen in Fig. 1a. The three fragment-ion peaks at *m/z* 694 (Z_1), 710 (Y_1) and 739 (X_1) were again attributed to the GlcN I moiety. One of these peaks (X_1) had an analog corresponding to a ion 238 U heavier at *m/z* 977, indicating that the C_{16} was located on this GlcN. The absence of signals in this region corresponding to the addition of 131 U suggested that the ara-4N residue was a substituent of GlcN II, not GlcN I. After alkali treatment, liberating ester-linked fatty acids at C-3 and C-3′, the spectrum of the residual product still had the C_{16}-containing molecular species, leading to the conclusion that it acylated the GlcN I amide-linked $C_{14}OH$.

The native and alkali-treated lipids of both strains of *Y. ruckeri* gave the same data as described for *Y. enterocolitica* O:11,23 and O:11,24, indicating that its basic structure was the same with identical fatty acid substitution.

3.2. Lipids A having a major molecular species of mass 1798 U

3.2.1. Y. enterocolitica O:3 and O:9. According to the negative-ion spectrum, these lipids were heterogeneous (Fig. 1c). The major molecular species of both was at *m/z* 1798 and was assumed to correspond to a hexaacyl molecular species similar to other enterobacterial lipids A [12] consisting of two GlcN, two phosphates, four $C_{14}OH$, one C_{14} and one C_{12} on the basis of the overall chemical composition. A small peak often seen in spectra of enterobacterial lipids A was at *m/z* 1572 (1798−$C_{14}OH$) [12]. However, the absence of a signal at *m/z* 1361, a tetraacyl species lacking one unit each of $C_{14}OH$ and C_{14}, and the presence of large signals at *m/z* 1390 (1572−182) and 1310 (1390−80) were unexpected since they do not appear in spectra of *Escherichia-Salmonella* lipids A [20]. The three fragment-ion peaks at *m/z* 694 (Z_1), 710 (Y_1) and 739 (X_1) attributed to the GlcN I were also present here. After mild alkali treatment, the negative-ion spectra (not shown) had their major peak at *m/z* 1162 which would be consistent with a composition of two GlcN, two phosphates, two $C_{14}OH$ and one C_{14}, i.e. with a loss in mass of 635 U. GC/MS identified the released fatty acids as C_{12}, $C_{14}OH$ and $C_{14}OC_{12}$. Fig. 2 compares the GC profiles of fatty acids released by weak alkali from the lipids A of the two groups of *Y. enterocolitica* and of *E. coli*.

The positive-ion spectrum (not shown) gave a prominent signal at *m/z* 1086, like that of *E. coli* lipid A representing the intact GlcN II part of the molecule with its phosphate ester and four fatty acids. The *m/z* 859 signal indicated the loss of OC_{14} (226 U = 210+O) instead of OC_{12} as observed with *E. coli* lipid A [14]. This confirmed the evidence of the negative-ion spectra and GC/MS data that the esterifying fatty acid at C-2′ was a C_{14} and the secondary acyl group at C-3′ a C_{12}. These results explained the presence in the negative-ion spectrum of *m/z* 1390 and 1310 corresponding to *m/z* 1798 and its monophosphoryl analog at *m/z* 1718,

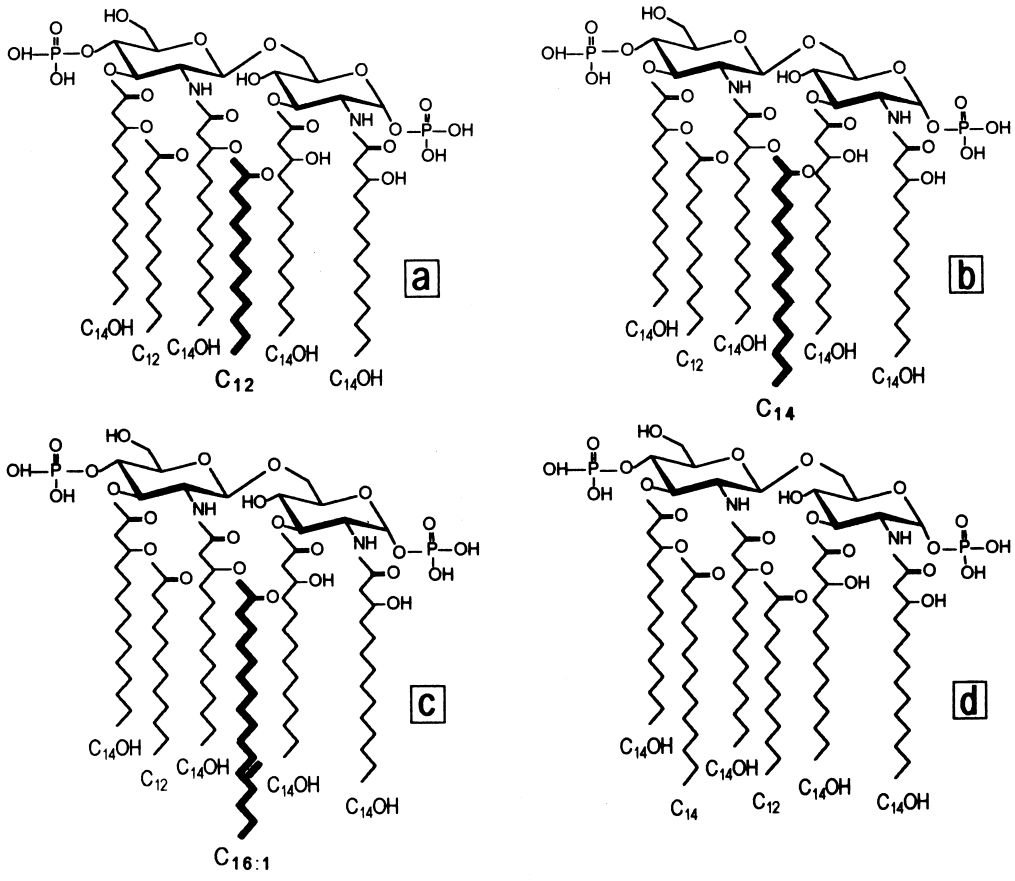

Fig. 3. Schematic representation of the major lipid A component of (a) *Y. enterocolitica* O:11,23 and O:11,24; *Y. ruckeri* O:1 and O:2. (b) *Y. enterocolitica* O:3 and O:9. (c) *Y. pestis*. (d) *E. coli*. The variable part of the structures is presented in bold. The configuration of the double bond of the $C_{16:1}$ was not determined.

respectively, minus $C_{14}OC_{12}$ (226+182), and the absence of *m/z* 1361.

3.2.2. Lipid A of Y. pestis containing a major molecular species of mass 1824 U. A detailed composition and partial characterization of this lipid A structure was described earlier [8,9]. On the basis of the overall chemical composition, the major peak in the negative-ion spectrum could correspond to a hexaacylated molecular species containing two GlcN, two phosphates, four $C_{14}OH$, one hexadecenoic acid ($C_{16:1}$) and one C_{12} (Fig. 1d). Smaller peaks were found at *m/z* 1955 (1824+ara-4N), 1642 (1824−C_{12}), 1597 (1824−$C_{14}OH$), 1587 (1824−$C_{16:1}$), 1415 (1642−$C_{14}OH$), 1178 (1415−$C_{16:1}$) and 952 (1178−$C_{14}OH$). Peaks indicating some dephosphorylation were at *m/z* 1744, 1507, 1335, 1098 and 871. The three fragment-ion peaks at *m/z* 694 (Z_1), 710 (Y_1) and 739 (X_1) attributed to the GlcN I moiety were present as in the spectra of all the other *Yersinia* and enterobacterial lipids A. This suggested that the $C_{16:1}$ was in the GlcN II moiety and treatment of the lipid with weak alkali released C_{12}, $C_{14}OH$ and $C_{14}OC_{12}$ but no $C_{16:1}$. This latter fatty acid was therefore assumed to be present in secondary position at C-2′. In accord with this assignment was the appearance of a peak at *m/z* 1188 in the negative-ion spectrum (not shown) of the residual alkali-treated lipid A. This was attributable to a molecular species consisting of two GlcN, two phosphates, two $C_{14}OH$ and one $C_{16:1}$. Confirmation was obtained in the positive-ion spectrum (not shown) by a prominent peak at *m/z* 1112 which corresponded to the expected mass of the GlcN II moiety on the basis of the mass found for the GlcN I moiety in the negative-ion spectrum.

Thus, the different *Yersinia* lipid A structures differed essentially in the secondary acylation of the GlcN II amide-linked fatty acid. The lipids A of the different species and strains tested fell into three classes on the basis of structure. In all the negative-ion spectra, three fragment-ion peaks at *m/z* 694 (Z_1), 710 (Y_1) and 739 (X_1) were observed and attributed to a GlcN I moiety. This part of the molecule, identical to the *Escherichia*-type lipid A, is therefore well conserved. The $C_{14}OC_{12}$ substituent at position C-3′ was also present in all the lipids A (Fig. 3).

4. Discussion

Of the seven lipids analyzed, two contained a unit of C_{14} esterifying the amide-linked fatty acid on its GlcN II: *Y. enterocolitica* O:3 and O:9. Their major molecular-ion species signalled at *m/z* 1798, whereas those of *Y. enterocolitica* O:11,23 and O:11,24 and of *Y. ruckeri* O:1 and O:2 were at *m/z* 1770. In the latter lipids A, the C_{14} was replaced by a second C_{12}. The third category consisted in the *Y. pestis* lipid A with a major molecular-ion species signalling at *m/z* 1824 and corresponding to a lipid A ion with a $C_{16:1}$ unit

replacing the C_{14} or C_{12} esterifying the GlcN II amide fatty acid in the other two classes. MS of this fatty acid gave a fragmentation pattern corresponding to that of Δ^{11}-$C_{16:1}$. The spectrum of the Δ^{9}-$C_{16:1}$, found in other bacteria, was slightly different [21]. The configuration of this fatty acid was not determined. In the *Y. pestis* lipid A spectrum, the major peak at *m/z* 1824 was followed by a minor one at *m/z* 1840, suggesting that a small portion of the hexaacyl lipid A has an additional hydroxyl group. Any differences between the quantities of non-polar fatty acids (including $C_{16:1}$) found by us and those found by others [8,22,23] are most likely due to different analytical methods, a difference in strain (in one case), differences in culture conditions, or simply the kind of variability that can occur in different preparations obtained under identical conditions. It should be noted that when the molecular ion at *m/z* 1770 is dominant in the other *Yersinia*, there is also an usually small *m/z* 1798 peak. The proportions are reversed in the spectra containing the major molecular ion at *m/z* 1798.

In earlier work with *Y. enterocolitica* and *Salmonella* species grown at 37°C or 40°C, there was little or no $C_{16:1}$, whereas cells grown at 10°C or 12°C contained considerable $C_{16:1}$ with a concomitant decrease in another fatty acid [24,25]. In more recent work, *E. coli* grown at 12°C was shown to substitute about 80% of its C_{12} with $C_{16:1}$, and when grown at 30°C, $C_{16:1}$ was no longer detectable [26]. The appearance of $C_{16:1}$ at the lower temperature was explained by the activation of the *LpxP* gene. In this report, all of the *Yersinia* species were grown at 37°C except *Y. pestis* which was grown at 28°C. This difference in temperature is too small to give a cold shock. At 28°C, only a small amount of $C_{16:1}$ might be expected compared to C_{12} or C_{14}, i.e. the spectrum would show a weak signal at *m/z* 1824 and a strong signal at *m/z* 1770 or 1798, which were not seen at all [20]. The other *Yersinia* lipids A grown at 37°C did not give a visible peak at *m/z* 1824.

Since the structure of the *Yersinia* lipid A skeleton has been reported to be identical to that of other enterobacteria [27] and follows the same fragmentation rules in PDMS, the ensemble of these results generated the hexaacyl lipid A structures presented in Fig. 3. The structure of *E. coli* lipid A is shown for comparison.

Yersinia is very closely related to *Escherichia, Salmonella* and *Shigella*. It would therefore not have been surprising to find that, as a group, they would share a common lipid A structure. However, the presence of the tetraacylated molecular ion at *m/z* 1390 and not at *m/z* 1361 as with *Escherichia*-type lipids A was the clue that it was different. PDMS analysis in both positive-ion and negative-ion modes of both native and alkali-treated lipids established the structural differences.

Differences in secondary acylation have been seen to occur in the lipids A of some *Salmonella typhimurium* and *Enterobacter agglomerans* lipids A in which C_{14} at the C-3′ position is present or absent or replaced by C_{14}OH [12,28]. The most striking example of variability reported until now was provided by *Bordetella* lipids A, which were found to have not only a species-related variability [29], but also some strain-related variability. Within the *Bordetella bronchiseptica* species, even a single strain exhibited different molecular species [30]. Non-conservation would now appear to characterize the lipids A of the *Yersinia* genus also. However, most examples of species-related variability lie among ester-linked acyl groups at positions C-3 and C-3′. In the present case, the variability observed concerns acylation of the amide-linked C_{14}OH at position C-2′. A different biosynthetic step is therefore involved.

The number and nature of fatty acids as well as their position have been shown to be specifically related to lipid A toxicity [31–33]. A recent comparison of outer membrane permeability in different strains of *Y. enterocolitica* (environmental and pathogenic), *Y. pseudotuberculosis* and *Y. pestis* showed increasing fluidity and permeability to a hydrophobic agent in that order, which also correlated with their invasiveness [34]. It was concluded that differences in the lipid A structure of their LPS were a factor in this phenomenon. The presence of $C_{16:1}$ in *Y. pestis* supports the idea that its outer membrane is more fluid and permeable. Investigations involving modifications of fatty acid composition in a bacterial model, by using the expression of unrelated acyl-transferases [35], would aid in defining the role of structure in relation to biological activities.

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