

# Structural characterization of *Bordetella parapertussis* lipid A

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**Abstract** *Bordetella parapertussis* like *B. pertussis*, is a causal agent of whooping cough but is not a strictly human pathogen. Because its endotoxin, a major structural component of the Gram-negative outer membrane, is an important virulence factor, we have analyzed the structure of its toxic lipid domain, in one rough and two smooth bacterial strains. Chemical analyses and mass spectra obtained before and after recently developed mild-alkali treatments revealed that the lipids A have the common bisphosphorylated  $\beta$ -(1 $\rightarrow$ 6)-linked D-glucosamine disaccharide with hydroxytetradecanoic acid in amide linkages. All three strains have two major molecular species: a tetraacyl and a pentaacyl species. The rough strain is richer in a minor hexaacyl species. Acylation at the C-2, C-3, and C-3' positions was different from that of the *B. pertussis* lipid A. The C-2 position carries a secondary hexadecanoic acid, the C-3 position is free, and the C-3' position is substituted with hydroxydecanoic acid (not at C-3 as in *B. pertussis*), and the rough strain hexaacyl species carries a second secondary hexadecanoic acid. Like the lipid A of *B. pertussis*, the hydroxytetradecanoic acid at the C-2' position was substituted by tetradecanoic acid.—El Hamidi, A., A. Novikov, D. Karibian, M. B. Perry, and M. Caroff. Structural characterization of *Bordetella parapertussis* lipid A. *J. Lipid Res.* 2009. 50: 854–859.

**Supplementary key words** *B. parapertussis* • endotoxin • lipopolysaccharide • structure

The best-studied member of the *Bordetella* genus has understandably been *B. pertussis*, the pathogen responsible for whooping cough. Its endotoxin, an immunomodulator of the bacterial surface, has been antigenically defined and the structures of the constituent lipopolysaccharides (LPS) established (1–11). *B. parapertussis*, the other agent of whooping cough is not a strictly human pathogen and can often be isolated from late-stage pertussis patients as well as from infected sheep (6, 9, 12, 13). Like *B. bronchiseptica*, it has a smooth-type LPS, and the O-chains of these two species are similar homopolymers of 1,4 linked 2,3-diacetamido-2,3-dideoxy- $\alpha$ -L-galactopyranosyluronic acid with different substituents on the nonreducing terminal sugar, one having an alanine, and the other an O-methyl lactic acid residue

(9, 14). This epitopic difference explains the early observed serological differences between the two species (15).

The ester-linked fatty-acid of *Bordetella* lipids A are highly variable (5, 16). In *B. bronchiseptica*, this variability, is found in the nature and the localization of its fatty acids and may be related to the multiplicity of its hosts (16).

The lipid A molecules can be modified via acylation; deacylation; secondary fatty acid hydroxylation; and /or phosphate-group substitutions with aminoarabinose, galactosamine, or phosphoethanolamine (17–19). We recently reported substitution with glucosamine on the phosphates of *B. bronchiseptica* and *B. pertussis* lipids A, causing on other examples a significant modulation of host responses to infection (11, 20).

The *Bordetella* genus shows a remarkable ability to modify lipid A structures by late-steps in their biosynthesis. This may represent adaptations of a putative ancestor of *B. bronchiseptica*, an animal pathogen, to diverse niches or hosts, leading to the relatively recent appearance of the human pathogens (21).

We report here, for the first time, the detailed structures of lipids A of one rough- and two smooth-type strains of *B. parapertussis*, structures obtained with the critical help of a method recently established to distinguish between primary and secondary esters (20). This structure modifies an earlier one proposed in a congress poster (22) also reproduced by others (23). It is in accord with the reported genetic and biochemical characteristics of *Bordetella* acyl transferases, which so far have not been shown to esterify lipid A backbones with unhydroxylated fatty acids (23).

## MATERIALS AND METHODS

### Bacterial strains

*B. parapertussis* smooth-type strains NRCC4364 and 4425, and the rough-type strain 4424 were from the National Research Council (NRC) collection (Canada).

Abbreviations: C<sub>12</sub>, dodecanoic acid; C<sub>14</sub>, tetradecanoic acid; C<sub>16</sub>, hexadecanoic acid; C<sub>10</sub>-OH, hydroxydecanoic acid; C<sub>12</sub>-OH, hydroxydecanoic acid; C<sub>14</sub>-OH, hydroxytetradecanoic acid; GlcN, D-glucosamine; LPS, lipopolysaccharide.

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*B. pertussis* strain 1414 was grown at the Institut Mérieux (Lyon, France). *B. parapertussis* cells were grown as described (9) and the cells killed in 2% phenol before harvesting.

### LPS and lipid A preparation

The LPSs were extracted by the enzyme-phenol-water method (24) and sedimented by ultracentrifugation (105,000 g, 4°C, 12 h). Lipid A was prepared by mild, detergent-facilitated hydrolysis of LPS and purified as before (25). Alternatively, lipid A was obtained by direct hydrolysis of the lyophilized bacteria (26). Briefly, 10 mg of lyophilized bacteria were suspended in 400 µl of isobutyric acid and 1 M ammonium hydroxide (5:3, v:v), heated 2 h at 100°C with stirring, cooled to 4°C, and centrifuged (as before). The supernatant was diluted with water (1:1, v:v) and lyophilized. The material obtained was then washed twice with 400 µl of methanol and centrifuged (2,000 g for 15 min). Finally, the insoluble lipid A was extracted once in a 100 to 200 µl mixture of chloroform/methanol/water (3:1.5:0.25, v:v:v).

### Identification of glucose absolute configurations

Lipids A (4 mg) were hydrolyzed with 0.5 ml of 4 M HCl at 100°C for 2 h. After cooling and extraction of fatty acids with chloroform, residual solutions were brought to neutrality by repeated evaporation under reduced pressure. After N-acetylation, the residue was treated with trifluoroacetic acid - R(-)-2-butanol, peracetylated, and analyzed by gas chromatography on a BP10 capillary (Scientific Glass Engineering) column using a program 160°C (1 min) to 220°C, 5°C min<sup>-1</sup> at 0.6 kPa (27).

### Sequential liberation of ester-linked fatty acids by mild alkali treatment

Sequential liberation of ester-linked fatty acids by mild alkali treatment was used to establish the lipid A acylation patterns (20). For the first-step liberation 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. To liberate the secondary ester-linked fatty acids, lipid A was suspended in 41% methylamine and stirred for 5 h at 37°C. The solutions were dried under a stream of nitrogen, the residues taken up in a mixture of chloroform/methanol/water (3: 1.5: 0.25, v:v:v) followed by MALDI/MS analysis. In this case, kinetics (15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h) were done to follow the complete process in parallel with the *B. pertussis* lipid A taken as a reference.

### Mass spectrometry

Two different techniques were used in this work.

#### Plasma desorption mass spectrometry

Plasma desorption mass spectra were obtained with a Depil TOF 21 mass spectrometer as described, and lipid A samples were prepared as before (16). Spectra were recorded in the positive and negative-ion modes.

#### MALDI/MS

MALDI/MS was done in the linear mode with delayed extraction using a Perseptive Voyager STR (PE Biosystem, France) time-of-flight mass spectrometer (I.B.B.M.C., Orsay, France). A suspension of lipid A in chloroform/methanol/water (3:1.5:0.25, v/v/v) (1 mg/ml) was desalted with a few grains of Dowex 50W-X8 (H<sup>+</sup>), 1 µl was deposited on the target, mixed with one µl of the matrix suspended at 10 µg/µl in the same solvent or in 0.1 M aqueous citric acid (28) and dried. Analyte ions were desorbed from the gentisic acid (2,5-dihydroxybenzoic acid) matrix

with pulses from a 337 nm nitrogen laser. Spectra were obtained in the negative-ion mode at 20 kV.

### Chemical analyses

Hexosamines were assayed as in (29) and phosphate, as in (30).

Fatty acids were analyzed as in (31); GC-MS was done as before using a Finnigan Mat 95S mass spectrometer (5).

### Nuclear magnetic resonance spectra

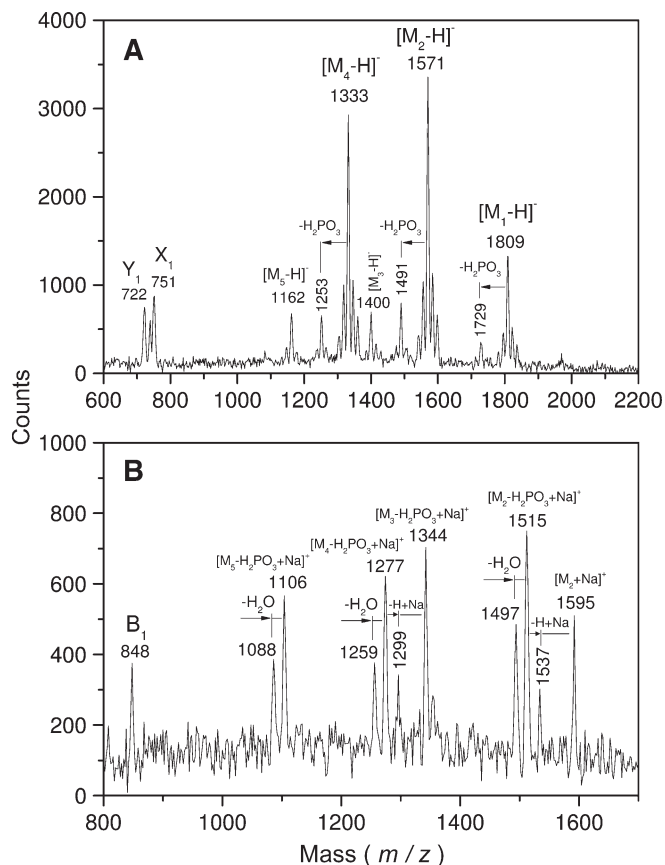
(<sup>1</sup>H and <sup>31</sup>P nuclear magnetic resonance) were obtained on a Bruker AMX-500 spectrometer using standard Bruker software as previously described (5).

## RESULTS AND DISCUSSION

Colorimetric tests showed that the lipids A contain D-glucosamine (GlcN) and phosphorus in a 0.9:1 ratio (29, 30). No pyrophosphate or additional sugars were detected. Plasma desorption mass and MALDI spectra had no signals indicating the presence of phosphorylethanolamine or any other substituents than fatty acids.

### Total fatty-acid compositions

Total fatty-acid compositions (31) of the three strains lipid A was performed by GC-MS after strong acid treatment, extraction, and esterification with diazomethane. Strain 4364 lipid A had 3-hydroxydecanoic acid (C<sub>10</sub>-



**Fig. 1.** Plasma desorption spectra of *B. parapertussis* strain NRCC 4424 lipid A. A: Negative-ion mode; B: positive-ion mode.

OH), tetradecanoic acid ( $C_{14}$ ), 3-hydroxytetradecanoic acid ( $C_{14}$ -OH), and hexadecanoic acid ( $C_{16}$ ) in the proportions 0.5:1:2:0.9. Taking into account the heterogeneity of this lipid A preparation and the tendency toward underestimation of the short-chain fatty acids (31) these proportions translated to 1 unit of  $C_{10}$ -OH, 1 unit of  $C_{14}$ , 2 units of  $C_{14}$ -OH, and 1 unit of  $C_{16}$ , for a major molecular species (see later discussion). Strain 4425 gave similar results, but the rough-strain 4424 showed a larger amount of  $C_{16}$  fatty acid with the proportions: 0.7:1: 2:1.3.

As the three lipids A gave identical results in the different experiments apart from the extra  $C_{16}$ , only one experiment of each is along this work.

Nuclear magnetic resonance analysis of the O-deacylated rough-type LPS indicated a  $\beta$ -1',6-D-glucosamine disaccharide backbone substituted at C-4' and C-1 by phosphomonoester groups and N-acylated at C-2' and C-2. Characteristic values were similar to those obtained with *B. pertussis* lipid A (5). Data not shown.

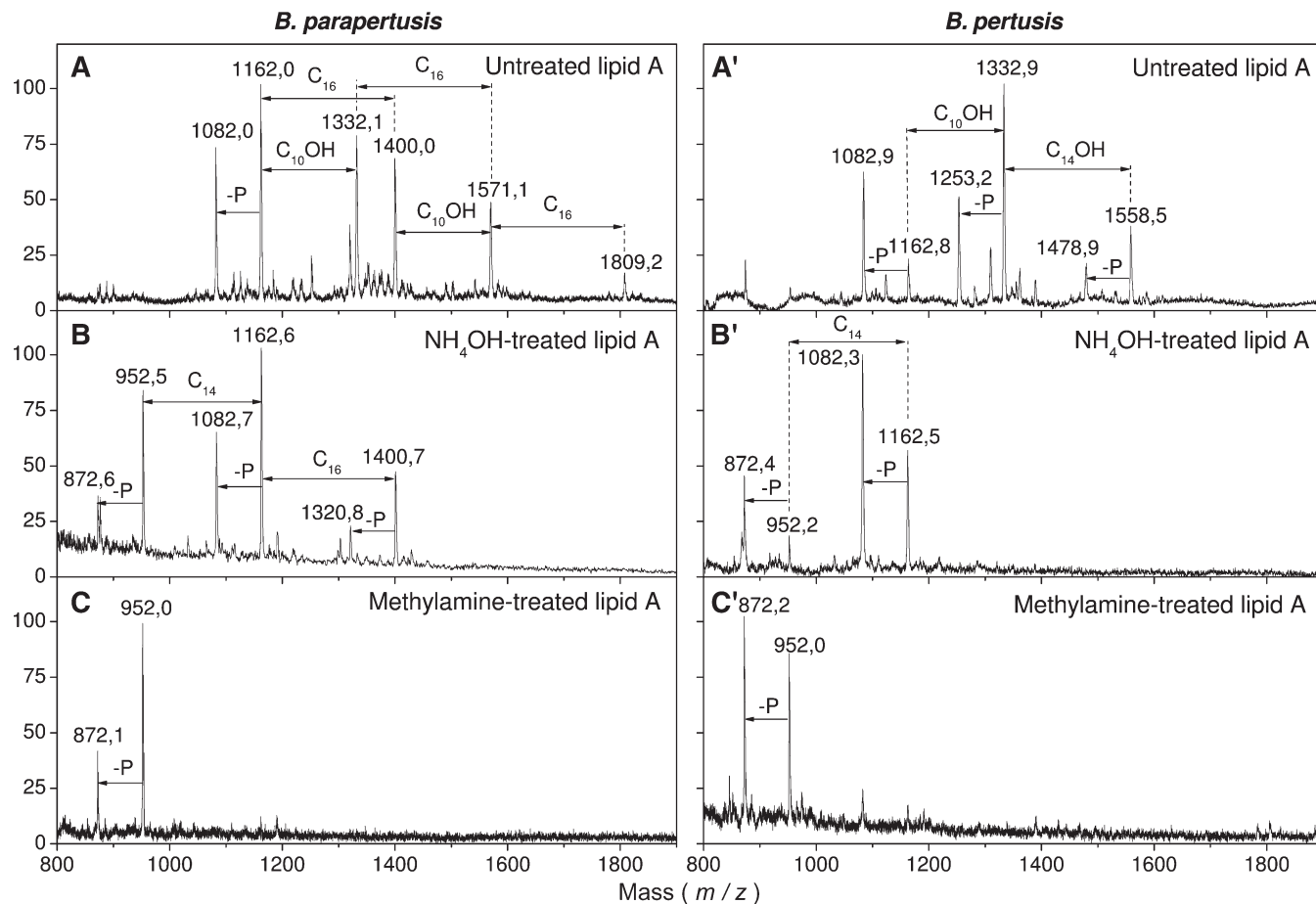
### Molecular heterogeneity and distribution of the fatty acids between the two D-glucosamine residues

A combination of the positive- and negative- ion mode plasma desorption mass spectrometry fragmentation pat-

terns was used (32–34). This method although rarely employed nowadays gives in the high mass region molecular-ion peaks that are due to the natural heterogeneity of lipid A preparations. Peak intensities are proportional to the relative abundances of the molecular species.

In the negative-ion mode the XYZ series of peaks, corresponding to well-defined fragments associated with GlcN-I, is usually observed while in the positive-ion mode, B1 fragments are associated with GlcN-II, and both modes give the fatty acid distribution between the two GlcN residues (35).

The negative-ion spectrum (Fig. 1A) of strain 4424 lipid A showed two main molecular-ion  $[M-H]^-$  signals at  $m/z$  1,333 and 1,571. Compositions of the corresponding molecular species attributed on the basis of the overall chemical composition given by colorimetric methods as well as GC-MS gave 2 GlcN, 2 phosphates, 2  $C_{14}$ -OH, 1  $C_{14}$ , 1  $C_{10}$ -OH with and without 1  $C_{16}$  ( $M_r-H_2O = 161.16, 80, 226.36, 210.36, 170.25, \text{ and } 238.4$ , respectively). Smaller peaks at  $m/z$  1,162 ( $1,333-C_{10}$ -OH), 1,400 ( $1,571-C_{10}$ -OH) 1,491 ( $1,571$ -phosphate), and 1,809 ( $1,571+C_{16}$ ) were also found. Consistent with GC-MS data, the rough-type lipid A peak at  $m/z$  1,809 was higher than that in the corresponding spectrum of each smooth type lipid A. It is thus richer in a molecular species having two residues of  $C_{16}$ .



**Fig. 2.** Matrix-assisted laser-desorption/ionisation mass spectrometry of: *B. parapertussis* lipid A NRCC4323 (A); lipid A obtained after a first primary deO-acylation step (at 50°C for 5 h with ammonium hydroxide) (B); lipid A obtained after complete deO-acylation (at 37°C for 5 h with methylamine) (C). A', B', C': same treatments for *B. pertussis* strain 1414 lipid A.

Fragmentation is annotated according to the nomenclature presented in Ref. 35. In the lower field, there were peaks at  $m/z$  751 ( $X_1$ ) and 722 ( $Y_1$ ) corresponding to the O-GlcN I moiety with and without CHO separated from GlcN II by double breakage (C5'-O', C1'-C2'). A composition of 1 GlcN, 1 phosphate, 1 C<sub>14</sub>-OH, and 1 C<sub>16</sub> was attributed to it. Lower in the spectrum, a peak at  $m/z$  512 was interpreted to be  $m/z$  751 minus C<sub>16</sub>, a confirmation of the incomplete secondary acylation at C-2. The absence of other peaks indicated the absence of substitution at C-3 and C-4.

Positive-ion mass spectrum of the same lipid A preparation is presented in Fig. 1B. A peak at  $m/z$  848 corresponded to B1 fragments associated with GlcN-II and containing 1 GlcN, 1 phosphate, 1 C<sub>14</sub>-OH, 1 C<sub>14</sub>, and 1 C<sub>10</sub>-OH. In the higher mass region, multiple peaks were observed resulting from the loss of phosphate groups (H<sub>2</sub>PO<sub>3</sub> or H<sub>3</sub>PO<sub>4</sub>) and attachments of one or two atoms of sodium by the different lipid A molecules present in the mixture. These data taken together allows to define the fatty acid distribution for the major molecular species as follows:

$m/z$  1571: GlcN-I, C<sub>14</sub>-OH, C<sub>16</sub>; GlcN-II, C<sub>14</sub>-OH, C<sub>14</sub>, C<sub>10</sub>-OH

$m/z$  1333: GlcN-I, C<sub>14</sub>-OH; GlcN-II, C<sub>14</sub>-OH, C<sub>14</sub>, C<sub>10</sub>-OH

The C<sub>14</sub> would then have to be on the C<sub>14</sub>-OH at C-2' as in *B. pertussis*, and the second hexadecanoic acid, on the C<sub>10</sub>-OH, which would be at C-3'. Interpretation for other peaks is given in the spectrum (Fig. 1A, B).

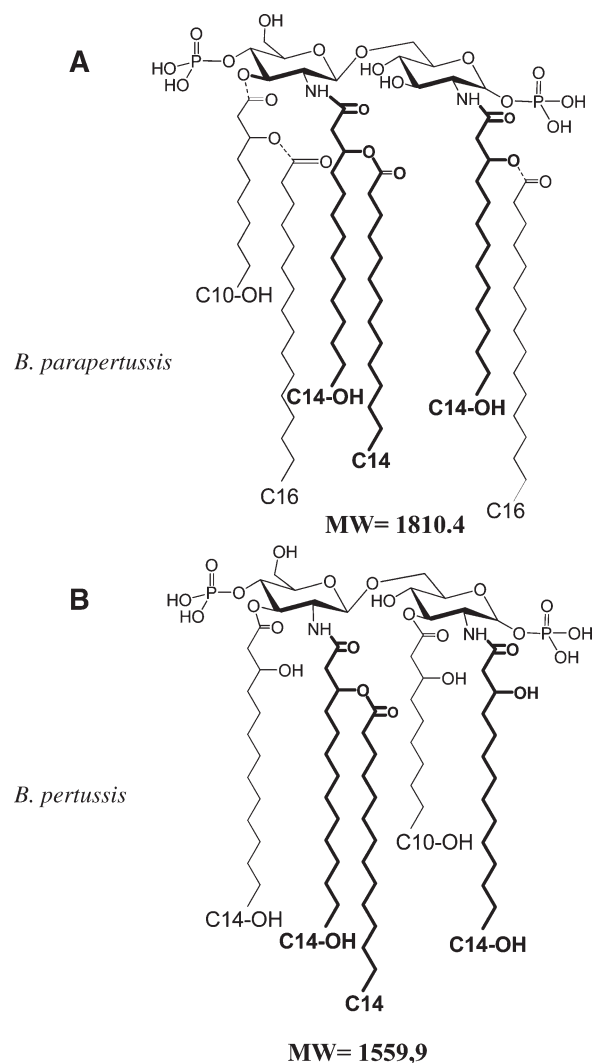
#### Liberation of primary ester-linked fatty acids

Liberation of primary ester-linked fatty acids was also applied to *B. paraptentussis* and *B. pertussis* lipids A isolated directly from bacteria (Fig. 2A, A') (26). The kinetics of *B. pertussis* lipid A deacylation showed a total release of the fatty acid at C-3 (C<sub>10</sub>-OH) in the first 15 min of treatment (not shown). The first 15 min of treatment applied to *B. paraptentussis* lipids A did not change the general aspect of the spectrum, which could be explained by a free position at C-3.

In the conditions used to liberate esterified substituents at the C-3 and C-3' positions (NH<sub>4</sub>OH 5 h at 50°C), (Fig. 2B, B'), C<sub>10</sub>-OH, C<sub>10</sub>-O-C<sub>16</sub> were completely released together with a small amount of C<sub>16</sub> in *B. paraptentussis* lipids A. After a 5 h treatment, the C<sub>14</sub> and a C<sub>16</sub> fatty acid were still present as indicated by peaks at  $m/z$  1,162 and 1,400. It was concluded that this C<sub>16</sub> was present in secondary acylation at C-2 as for the C<sub>14</sub> at C-2', which was confirmed by the fragmentation data. The appearance of two peaks, one at  $m/z$  1,162 (952+ C<sub>14</sub>) and the other at +C<sub>16</sub> demonstrated that the C<sub>14</sub> substituted all the C<sub>14</sub>-OH at C2', whereas the C<sub>16</sub> only substituted some of the C<sub>14</sub>-OH at C-2. In the same conditions C10-OH and C14-OH were liberated from *B. pertussis* lipid A.

In the conditions used to liberate secondary esterified substituents at C-2 and C-2' (methylamine 5 h at 37°C) the C<sub>14</sub> and C<sub>16</sub> fatty acids were liberated giving rise to a single molecular species at  $m/z$  952 and some of its dephosphorylated form (Fig. 2C, C').

The ensemble of these results defined the structures presented in Fig. 3 with the higher mass molecular species of each lipid A, MW 1810 for *B. paraptentussis* (Fig. 3A) and



**Fig. 3.** Structures corresponding to the higher mass molecular species present in the negative-ion spectra of *B. paraptentussis* (A) and *B. pertussis* (B). Dotted lines indicate incomplete fatty acid substitution. Bold lines represent common fatty acids in both lipid A structures.

the basic structure found in *B. pertussis* (Fig. 3B). It should be noted that the peak at  $m/z$  1,333, although identical and corresponding to the same overall composition as that seen in *B. pertussis* lipid A, differs in that the hydroxydecanoic acid at C-3 in *B. pertussis* lipid A, is at C-3' in *B. paraptentussis* (Fig. 3A). This could indicate a different biosynthetic pathway and illustrates the usefulness of spectra taken in both tension modes and the use of fragmentation patterns for detecting such differences.

The presence of C<sub>16</sub> fatty acids in lipid A structures was always associated with late biosynthetic steps, these fatty acids originating from the outer membrane phospholipids (36). In the present case, an ester-linked C10-OH and a C14-OH amide-linked fatty acid would both be substituted in secondary linkage.

C<sub>14</sub>-O-C<sub>16</sub> substitutions on C-2 of the reducing GlcN are particularly common in *Salmonella typhimurium*, and first thought to be a characteristic of Salmonellae. In Bordetellae, this PagP modification was first detected in secondary acyl-

ation at C-3' on GlcN II (37). In *B. bronchiseptica*, *pagP* expression is Bvg-dependent and impacts colonization in mice and serum resistance in vitro (37). With *B. parapertussis* we have the expression of both secondary acylations due to PagP. No C<sub>16</sub> was ever found at C-2 of the lipid A of any *Bordetella* species examined by our group.

The lipids A of some strains of *B. bronchiseptica*, *B. hinzii*, and *B. trematum* are substituted with hexadecanoic acid in secondary linkage at C3' (M. Caroff, unpublished observations). Recently, the occurrence of a novel secondary acyl chain in *B. pertussis* LPS and its negative effect on infection of human macrophages has been reported (19).

At this stage, the situation could be tentatively explained for the two human pathogens by the following scenario: the two species could follow a common biosynthetic pathway for their lipids A up to the formation of a more common symmetric GlcN disaccharide bearing a C<sub>10</sub>-OH at both C-3 and C-3'. Then, this lipid could undergo an enzymatic deO-acylation, one at C-3 for *B. parapertussis* and one at C-3' for *B. pertussis*, leading to the two different structures. DeO-acylation would then have to be asymmetric, but the presence of specific deacylases has been reported in other genera (38, 39).

The lipids A of the three strains of *B. parapertussis* differed only in degree of acylation as seems to be the case with various species of some genera such as *Haemophilus* and *Neisseria* (33). Some investigators consider the 3 *Bordetella* species that infect human beings as variants of a single species. *B. pertussis* and *B. parapertussis* still maintain the short C<sub>10</sub>-OH in primary acylation. *B. bronchiseptica* displays a high degree of heterogeneity and variability. In all mass spectra, each major peak representing a molecular species is surrounded by smaller peaks corresponding to molecular species having fatty acids differing by 2 carbons or by an oxygen atom showing the capacity of the bacteria to shift to other structures and the relaxed enzyme specificity already described (23). The relation of this flexibility and the relatively recent human niches together with the potential of late-stage alterations could explain the adaptation of earlier *Bordetella* pathogens to humans and the reemergence of resistant strains. With the present strong interest in the biosynthesis of *Bordetella* LPS by various groups, an understanding of this phenomenon may soon be achieved. ■

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