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Structural Characterization of the Lipids A of Three *Bordetella bronchiseptica* Strains: Variability of Fatty Acid Substitution

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The structures of lipids A isolated from the lipopolysaccharides (LPSs; endotoxins) of three different pathogenic *Bordetella bronchiseptica* strains were investigated by chemical composition and methylation analysis, gas chromatography-mass spectrometry, nuclear magnetic resonance, and plasma desorption mass spectrometry (PDMS). The analyses revealed that the LPSs contain the classical lipid A bisphosphorylated β-(1→6)-linked D-glucosamine disaccharide with hydroxytetradecanoic acid in amide linkages. Their structures differ from that of the lipid A of *Bordetella pertussis* endotoxin by the replacement of hydroxydecanoic acid on the C-3 position with hydroxydodecanoic acid or dodecanoic acid and the presence of variable amounts of hexadecanoic acid. The dodecanoic acid is the first nonhydroxylated fatty acid to be found directly linked to a lipid A glucosamine. The lipids A were heterogeneous and composed of one to three major and several minor molecular species. The fatty acids in ester linkage were localized by PDMS of chemically modified lipids A. *B. pertussis* lipids A are usually hypoacylated with respect to those of enterobacterial lipids A. However, one of the three *B. bronchiseptica* strains had a major hexaacylated molecular species. C-4 and C-6′ hydroxyl groups of the backbone disaccharide were unsubstituted, the latter being the proposed attachment site of the polysaccharide. The structural variability seen in these three lipids A was unusual for a single species and may have consequences for the pathogenicity of this *Bordetella* species.

Bordetella bronchiseptica is a respiratory tract pathogen of mammals causing a whooping cough syndrome milder than that due to *Bordetella pertussis* in humans. This bacterium causes bronchopneumonia in rabbits and guinea pigs, kennel cough in dogs, and atrophic rhinitis in piglets (13). Recently, it has been described as responsible for bronchopneumonia in human immunosuppressed patients (1, 27, 28) and in at least two immunocompetent patients, one of whom was exposed to infected animals (14). Its endotoxin, a mixture of lipopolysaccharides (LPSs), which are a major component of the bacterial outer membrane, has been implicated as a virulence factor. *B. bronchiseptica*, like *Bordetella parapertussis*, produces smooth-type LPSs. Their O chains have identical structures (11). *B. pertussis* LPSs, which are of the rough type, have been analyzed, and their structures have recently become available (4, 6, 25). Structural variability in parts of LPS other than the O chains may thus relate to pathogenicity or to host specificity. We report here the detailed structural characterization of the lipid A moieties of three different strains of *B. bronchiseptica* grown under the same culture conditions and show that they have greater variability than that usually encountered in a single species.

MATERIALS AND METHODS

Mass spectrometry. Plasma desorption mass (PDM) spectra were obtained with a Depil TOF 21 mass spectrometer (10). Flight distance was 90 cm, acceleration voltage was +15 or −15 kV, and time bins were 1 or 2 ns/channel with a multistop time digital converter. For positive-ion spectra, the instrument was calibrated on H^+ (m = 1.00728) and Na^+ (m = 22.989); for negative-ion spectra, H^- (m = 1.00789) and $(C_2H)^-$ (m = 25.00789) were used. Counting time varied between 15 min and 4 h. Lipid A samples (50 μg) (22) were dissolved in 25 μl of chloroform-methanol-water (16:8:1 [by volume]) and desalted with a few grains of Dowex 50 $(Me)_2NH^+$, and 8 μl was deposited on an aluminized Mylar disk previously electrosprayed with nitrocellulose (20). *m/z* values were determined by centroid analysis. Resolution in the mass range of the spectra was usually within 1 mass unit of the calculated mass of the $(M - H)^-$ or $(M + Na)^+$.

Bacterial strains. Strains CIG 16 (NRCC 4175), 4098 (NRCC 4170), and Bemis (NRCC 4650) of *B. bronchiseptica* were from the National Research Council (NRC) collection.

LPS. *B. bronchiseptica* cells were grown as previously described (11). The cells were killed in 2% phenol before harvesting. The LPSs were extracted by the modified enzyme-phenol-water method (19). They were obtained as precipitated gels by ultracentrifugation (105,000 × *g*, 4°C, 12 h). Lipid A was prepared by hydrolysis of 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 (30:15:0.25) (7). Monophosphoryl lipid A was prepared by hydrolysis in 0.1 M HCl at 100°C for 15 min.

Thin-layer chromatography (TLC) was done on aluminum-backed silica gel plates (Merck) in the solvent chloroform-methanol-water-triethylamine (30:15:2.5:0.1) (7). Spots were visualized by charring after spraying with 5% sulfuric acid in ethanol.

Kinetics of inorganic phosphate release and formation of reducing 2-acetamido-2-deoxyglucose (GlcN). Lipids A (5 mg/ml) were hydrolyzed in 0.1 M HCl at 100°C for 5, 10, or 15 min as previously described (7) with samples of 50 and 300 μg for phosphate and *N*-acylhexosamine assays, respectively.

Determination of the anomeric form of GlcN II. Twenty milligrams of monophosphoryl lipid A was reduced with $NaBH_4$, treated with hydrazine (26), and then oxidized with 4 mg of sodium metaperiodate in 4 ml of water for 48 h at 4°C with stirring. The reaction was stopped with excess ethylene glycol, the product was reduced with $NaBH_4$, and a PDM spectrum was taken in the negative-ion mode to verify the presence of a major peak at *m/z* 529 corresponding to a phosphorylated GlcN bearing an ethylene glycol residue in glycosidic linkage and an OH C_{14} in amide linkage. After hydrazine treatment (60 h, 100°C) (17) and removal of the fatty acids, the product was peracetylated, extracted into chloroform, and injected on a gas-liquid chromatography (GLC) column (BP10 isotherm, 220°C, 0.4 kPa). Its retention time was compared with those of authentic α- and β-*N*-acetyl-glucosamine-ethylene glycol.

Identification of glycose absolute configurations. Lipids A (5 mg) were hydrolyzed with 1 ml of 4 M HCl at 100°C for 2 h. After cooling and extraction of fatty acids with chloroform, the mixture was concentrated to dryness under reduced pressure, and water was added and evaporated from the residue repeatedly until it became neutral. After N acetylation, the residue was treated with trifluoracetic acid–*R*-(−)-2-butanol according to reference 12 and analyzed by GLC on a BP10 (Scientific Glass Engineering) gas chromatography column with a program of 160°C (1 min) to 220°C, 5°C min^{-1}, at 0.6 kPa.

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Chemical analyses. Published methods were used to assay hexosamines (31), phosphate (8), pyrophosphate (32), and 4-amino-4-deoxyarabinose (35).

Fatty acids were analyzed after hydrolysis of the LPSs or lipids A with 4 M HCl for 2 h at 100°C; neutralization was followed by treatment with 2 M NaOH for 2 h at 100°C (15), extraction with hexane, methylation of the extract by diazomethane, and identification by GLC retention time on a BP10 column with a temperature gradient of 160 to 240°C, 4°C/min. Gas chromatography-mass spectrometry analyses were performed as previously described (26). Ester-linked fatty acids were likewise characterized after hydrazinolysis (26) of the lipid A with an internal standard of octadecanoic acid for quantitation. Secondary acylation of amide-linked fatty acids was identified by the method of Kraska et al. (23) as modified by Wollenweber and Rietschel (37) and by gas chromatography-mass spectrometry analysis of the diazomethane-methylated products. Synthetic acyloxyacyl fatty acids, provided by D. Charon, were used as reference. Methylation analysis was performed by the method of Ohno et al. (29): silicic acid-catalyzed diazomethane methylation followed by hydrolysis at 100°C for 8 h in 4 M HCl, N acetylation, reduction by sodium borohydride, peracetylation, and identification of the products by gas chromatography coupled with mass spectrometry.

Substitution groups on lipid A. PDM spectra in the negative-ion mode were examined for evidence of groups other than GlcN, phosphate, and fatty acids. Ninhydrin detection on TLC plates was used for 4-amino-4-deoxyarabinose and phosphorylethanolamine.

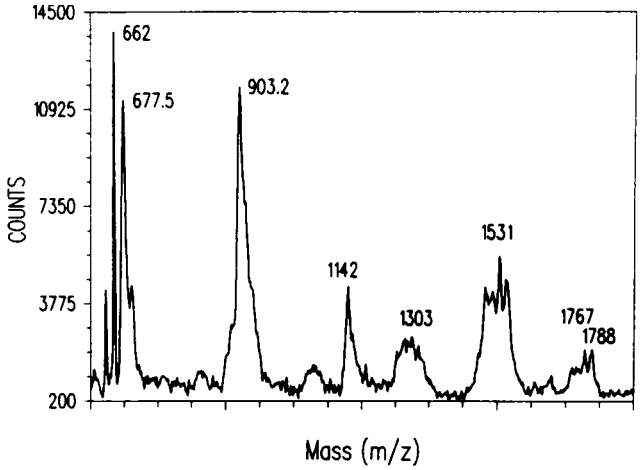

FIG. 1. Positive-ion PDM spectrum of lipid A from *B. bronchiseptica* 4650.

RESULTS AND DISCUSSION

Chemical composition of lipids A. The lipids A were shown to contain glucosamine (GlcN) and phosphorus in a 0.9:1 ratio. The GlcNs were found to be in the D configuration. No pyrophosphate or additional sugars were detected. Phosphorylethanolamine was looked for in PDM spectra, but none was found, nor was there any evidence of other substituents except for fatty acids. TLC showed heterogeneity in the three preparations, slight differences in R_f, and no ninhydrin-positive material.

The determination of the total fatty acid composition of strain 4170 lipid A revealed that tetradecanoic acid (C_{14}), 3-hydroxydodecanoic acid (3-OH C_{12}), 3-hydroxytetradecanoic acid (3-OH C_{14}), and hexadecanoic acid (C_{16}) occurred in the proportions 1:0.65:2.5:0.2. Taking into account the heterogeneity of these lipid A preparations and the tendency toward underestimation of the short-chain fatty acids (37), these proportions translated to 1 U of C_{14} to 1 U of 3-OH C_{12} to 2 or 3 U of 3-OH C_{14} to 1 or 0 U of C_{16}, depending on the molecular species (see below). Strain 4650, on the other hand, contained the same fatty acids in the proportions 1:0.68:2.47:0.44. Strain 4175 contained a fifth fatty acid, dodecanoic acid (C_{12}), in addition to the four others. They appeared in the proportions 1:0.25:2.44:0.2:0.45.

Determination of the anomeric configuration of the glucosamines. The kinetics of release of inorganic phosphate indicated that (i) half of the phosphate present in each of the three preparations was in glycosidic linkage and (ii) the rates of hydrolysis were consistent with the presence of terminal α-D-GlcN*p* phosphate residues (7). Oxidation and reduction of the de-O-acylated lipids A followed by removal of the remaining phosphate and OH C_{14} and identification of the product by GLC showed that GlcN II was in the β configuration.

Nuclear magnetic resonance (NMR) analysis. Strain 4170 lipid A was de-O-acylated by hydrazinolysis, and the product was examined by ^1H and ^{31}P NMR. The proton spectra indicated a β-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 by 3-hydroxy fatty acyl groups. Resonances for the two anomeric protons were observed at 5.35 ($J_{1,2} = -3$ Hz; $J_{H,P} = \sim 8$ Hz) and 4.64 ($J_{1,2} = 8$ Hz) ppm. The double-doublet pattern of the lower-field signal was consistent with glycosidic phosphorylation of the α-D-GlcN residue, whereas the higher-field signal was indicative of the β-D-GlcN residue. As expected, the ^{31}P NMR spectrum (pH ~8) of the O-deacylated lipid A showed two signals at 0.9 and 3.43 ppm, which could be assigned to the phosphomonoester groups at C-1 of the α-D-GlcN residue and C-4′ of the β-D-GlcN residue, respectively. The two other lipids A, from strains 4175 and 4650, gave similar results.

Linkage of fatty acids in *B. bronchiseptica* lipids A. Acid hydrolysis of the products obtained after removal of ester-linked fatty acids by hydrazine treatment showed that the amino group of both glucosamines of the disaccharide backbone was substituted by hydroxytetradecanoic acid in all three lipids A. Mild alkali treatment of the lipids A or LPSs, which liberates esterified substituents on the C-3 and C-3′ positions, gave (i) hydroxydodecanoic acid and hydroxytetradecanoic acid from strain 4170 lipid A; (ii) dodecanoic acid, hydroxydodecanoic acid, and hydroxytetradecanoic acid from strain 4175 lipid A; and (iii) hydroxydodecanoic acid, hydroxytetradecanoic acid, and hexadecanoic acid from strain 4650 lipid A. The Kraska method revealed that the tetradecanoic acid acylated one of the two hydroxytetradecanoic acids in amide position in all three strains. The tetradecanoic acid was also completely liberated by hydrazinolysis of the three lipids A. These data were confirmed by mass spectrometry of the de-O-esterified lipids that gave negative-ion signals at *m/z* 952 in all three cases (two GlcN, two phosphates, and two hydroxytetradecanoates).

Distribution of the fatty acids on the D-glucosamine residues. The monophosphoryl lipid A of strain 4650 gave a positive-ion PDM spectrum (Fig. 1) with peaks in the lower field at *m/z* 1,142, 903, 677, and 662. The peak at *m/z* 1,142 was interpreted to correspond to an ion containing one glucosamine, one phosphate, two hydroxytetradecanoic acids, one tetradecanoic acid, and one hexadecanoic acid (M_r minus H_2O = 161, 80, two times 226.36, 210.36, and 238.41, respectively). Examination of the fragmentation pattern observed under the same conditions with well-studied lipids A led us, like others (3, 36), to conclude that these signals correspond to the GlcN II (nonreducing) part of the lipid A. Therefore, it was concluded that the hexadecanoic acid esterified the hydroxytetradecanoic acid at C-3′. The absence of hexadecanoic acid in other molecular species gave rise to the peak at *m/z* 903. The *m/z* 677 and 662 signals could correspond to ions lacking both hexadecanoic and hydroxytetradecanoic acids with the split occurring on either side of the oxygen atom joining them to the C-3′. It undoubtedly represents the GlcN II moiety of the lipid A molecular species that gave rise to the peak at *m/z* 1,361,

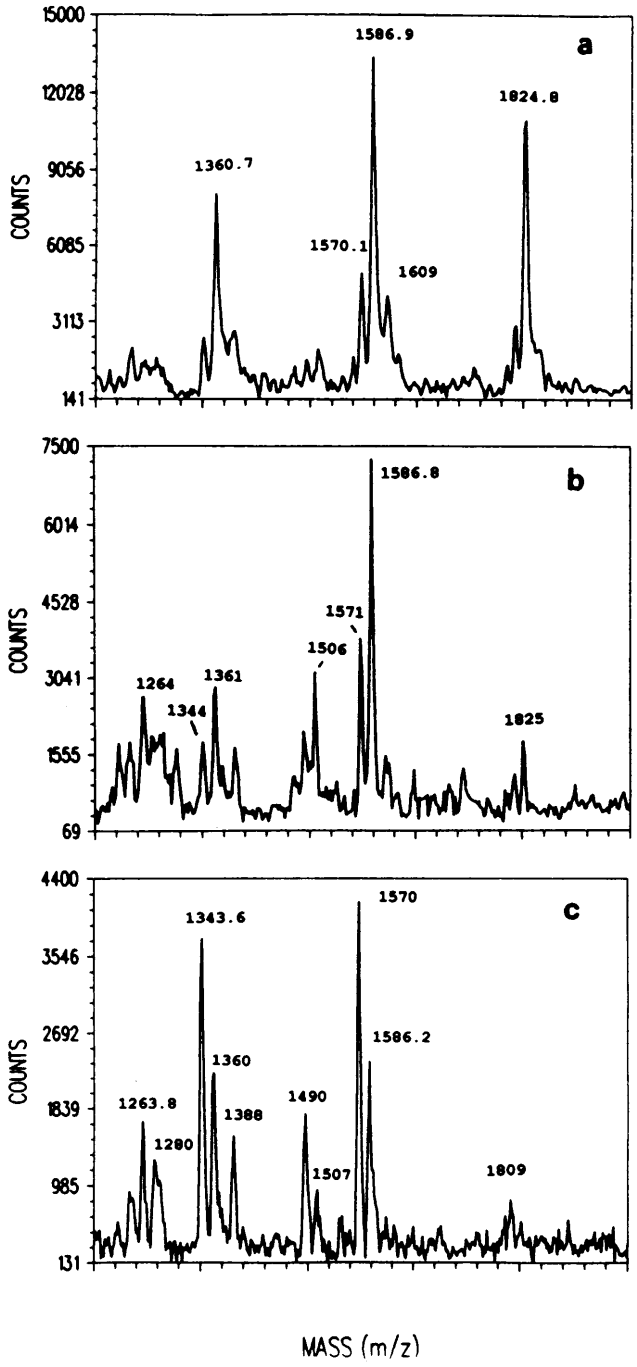

FIG. 2. Negative-ion PDM spectra of lipids A from *B. bronchiseptica* strains: 4650 (a), 4170 (b), and 4175 (c).

lacking hexadecanoic and hydroxytetradecanoic acids, in the negative-ion spectrum. In the upper field of the positive-ion spectrum, small peaks at *m/z* 1,303, 1,531, and 1,767 represent monophosphoryl $(M + Na)^+$ adducts of the major peaks (*m/z* 1,361, 1,587, and 1,825) present in the negative-ion spectrum (Fig. 2a). The peak at *m/z* 1,788 has a second sodium atom. Analysis of the lipids A of strains 4170 and 4175 under the same conditions showed only peaks at *m/z* 903 and 677, confirming that the hexadecanoic acid is present in a large amount only in the 4650 strain.

Localization of unsubstituted hydroxyl groups in lipid A backbone. Methylation analysis on the lipids A afforded the following methylation products: 1,3,5,6-tetra-*O*-acetyl-2-deoxy-2-acetamido-4-*O*-methyl-glucitol, 1,3,4,5-tetra-*O*-acetyl-2-deoxy-2-acetamido-6-*O*-methyl-glucitol, and 1,3,4,5,6-penta-*O*-acetyl-2-deoxy-2-acetamido-glucitol in the proportions 4:2:10, respectively. By analogy with other lipids A, the 4-*O*-methyl derivative presumably originated from the reducing GlcN I, and the 6-*O*-methyl-glucosamine originated from the nonreducing GlcN II. The peracetylated glucosaminitol was undoubtedly the product of undermethylation, as might be expected considering the mild conditions used for methylation. The *B. bronchiseptica* lipids A, therefore, are unsubstituted on carbons 4 and 6′. The latter is, in all probability, the site of attachment of the polysaccharide core moiety.

Analysis of lipid A heterogeneity by TLC and PDM spectrometry. TLC of the lipid A preparations from the three *B. bronchiseptica* strains showed one to three major, and a few minor, spots (not shown). No free amino groups were detectable by ninhydrin, indicating that no phosphorylethanolamine, aminodeoxyarabinose, or other unsubstituted amino groups were present in the preparations. This was confirmed by PDM spectrometry in the negative-ion mode (21). The spectra in Fig. 2 indicated the same heterogeneity as that observed on TLC. The negative-ion spectrum of strain 4650 lipid (Fig. 2a) had three main molecular-ion $(M - H)^-$ signals at *m/z* 1,361, 1,587, and 1,825. The compositions of their corresponding molecular species were attributed on the basis of the overall chemical composition. For strain 4170 (Fig. 2b), a major peak at *m/z* 1,586 and smaller peaks at *m/z* 1,361, 1,506, 1,571, and 1,825 were found. The peaks at *m/z* 1,264 and 1,506 correspond to partial dephosphorylation of the molecular ions at *m/z* 1,344 and 1,586, respectively. For strain 4175 (Fig. 2c), major peaks were found at *m/z* 1,570 and 1,344, doubled by peaks at *m/z* 1,586 and 1,361, respectively, showing that either OH C_{12} or C_{12} was present but not C_{12} O C_{12}, to give a C_{12}/OH C_{12} substitution heterogeneity. Other signals at *m/z* 1,264, 1,489, and 1,506 represent monodephosphorylated analogs of those at *m/z* 1,344, 1,570, and 1,586, respectively. The small peak at *m/z* 1,388 comes from a tetraacyl species unsubstituted at the C-3 position.

The ensemble of these results generated the structures presented in Fig. 3 for the main molecular species. All three strains share the basic lipid A structure consisting of two GlcN, two phosphates, two OH C_{14}, and one C_{14}. The major differences among them reside in the presence of a molecular species in strain 4175 substituted at C-3 with C_{12} instead of OH C_{12} and, in strain 4650, the secondary acylation at C-3′. The main differences from *B. pertussis* lipid A were the latter acylation at position C-3′ in one of the strains and the replacement of 3-OH C_{10} at the C-3 position by 3-OH C_{12} or C_{12}. It should be noted, however, that a very minor molecular species containing 3-OH C_{12} was previously detected in *B. pertussis* lipid A (6).

Differences in secondary acylation have been seen in the lipids A of other bacteria. For example, in *Salmonella* and *Enterobacter* lipids A, C_{14} at their C-3′ is present or absent or replaced by 3-OH C_{14} (5, 16). Other secondary fatty acids, like the C_{12} at their C-2′, are conserved, as are primary acylating fatty acids. Within some individual genera, such as *Neisseria* and *Haemophilus*, both primary and secondary acylations seem to be conserved (21). This is not the case with *Bordetella* lipids. The primary acylation at their C-3 and C-3′ positions has been shown to be variable from one species to another (reference 6 and unpublished data).

In the present investigation, we observed variation at the C-3

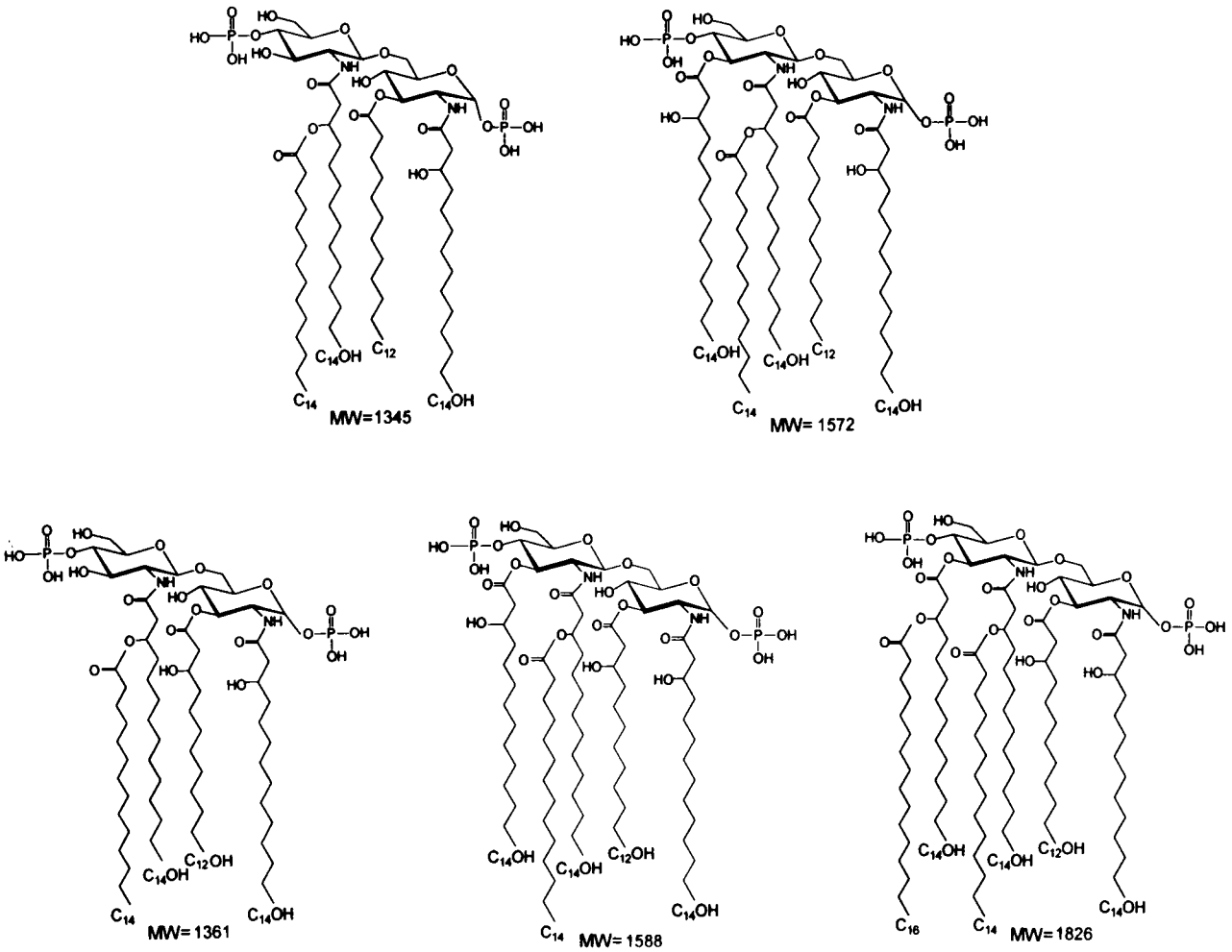

FIG. 3. Schematic representation of the major lipid A species in the three strains of *B. bronchiseptica*.

and C-3′ positions among strains of a single species with the appearance for the first time of a nonhydroxylated fatty acid substituting the diglucosamine backbone. This raises the question of the origin of the C_{12}: insertion during biosynthesis or, after biosynthesis of the lipid A, by dehydration and reduction of OH C_{12}. If the biosynthetic pathway in *Bordetella* resembles that in *Escherichia coli*, in which C-3 substitution is an early step (30), the first possibility would require (i) that the acylating enzyme be specific for chain length but not hydroxylation and (ii) that the succeeding enzymes accept the C_{12}-bearing intermediates as substrate. For the second possibility, dehydratases and reductases are known to exist for the reduction of hydroxylated precursors of phospholipid fatty acids (9). Whether they exist and function in the appropriate cell compartment remains to be seen. A third possibility is that, in *Bordetella*, substitution at the C-3 position is a late reaction which would not incur the second problem mentioned above and would render the heterogeneity similar to that mentioned for *Salmonella* and *Enterobacter* lipids A.

The number of fatty acids as well as their position and chain lengths have been shown to play a major role in toxicity (2, 24, 34). A study of the structure-activity relationships among these *Bordetella* LPSs should help clarify their specific biological differences. The core structures of the three strains, which also show some diversity, are under study.

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