Complex *O*-Acetylation in *Legionella pneumophila* Serogroup 1 Lipopolysaccharide. Evidence for Two Genes Involved in 8-*O*-Acetylation of Legionaminic Acid†

Oliver Kooistra,‡ Edeltraud Lüneberg,§ Buko Lindner,‡ Yuriy A. Knirel,‡,|| Matthias Frosch,§ and Ulrich Zähringer*,‡

Forschungszentrum Borstel, Zentrum für Medizin und Biowissenschaften, Parkallee 22, D-23845 Borstel, Germany, Institut für Hygiene und Mikrobiologie, Universität Würzburg, Josef-Schneider-Strasse 2, D-97080 Würzburg, Germany, and N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky Prospekt 47, 117913 Moscow, Russia

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ABSTRACT: A putative gene encoding an *O*-acetyl transferase, *lag-1*, is involved in biosynthesis of the O-polysaccharide (polylegionaminic acid) in some *Legionella pneumophila* serogroup 1 strains. To study the effect of the presence and absence of the gene on the O-polysaccharide *O*-acetylation, *lag-1* from strain Philadelphia 1 was expressed *in trans* in the naturally *lag-1*-negative OLDA strain RC1, and immunoblot analysis revealed that the *lag-1*-encoded *O*-acetyl transferase is active. O-Polysaccharides of different size were prepared from the lipopolysaccharides of wild-type and transformant strains by mild acid degradation followed by gel-permeation chromatography. Using NMR spectroscopy and MALDI-TOF mass spectrometry, it was found that *O*-acetylation of the first three legionaminic acid residues next to the core occurs in the short-chain O-polysaccharide (<10 sugars) from both strains. Hence, there is another *O*-acetyl transferase encoded by a gene different from *lag-1*. In the longer-chain O-polysaccharide, a legionaminic acid residue proximal to the core is *N*-methylated and could be further 8-*O*-acetylated in the *lag-1*-dependent manner. Only strains expressing a functional *lag-1* gene were recognized in Western blot analysis by monoclonal antibody 3/1 requiring 8-*O*-acetylated polylegionaminic acid for binding. The highly *O*-acetylated outer core region of the lipopolysaccharide is involved in the epitope of another serogroup 1-specific monoclonal antibody termed LPS-1. The *O*-acetylation pattern of the *L. pneumophila* serogroup 1 core oligosaccharide was revised using MALDI-TOF mass spectrometry. *lag-1*-independent *O*-acetylation of the core and short-chain O-polysaccharide was found to be a common feature of *L. pneumophila* serogroup 1 strains. The biological importance of conserved *lag-1*-independent and variable *lag-1*-dependent *O*-acetylation is discussed.

Legionella pneumophila is a facultative intracellular Gram-negative bacterium and the cause of legionellosis, a pneumonia with sometimes fatal progression (*1*). The reservoirs of legionellae are natural or man-made water systems, and their natural hosts are various amoebae species (*2*). *L. pneumophila* spreads and infects humans via aerosols and subsequently invades the lung where it replicates within alveolar macrophages (*3*). The serogroup-specific antigens of legionellae reside in the lipopolysaccharide (LPS)[1] of the outer membrane (*4*, *5*).

The structure of the LPS of *L. pneumophila* serogroup (Sg) 1 (strain Philadelphia 1) has been extensively studied (*6*−*10*) (Figure 1). The O-polysaccharide (OPS) of the LPS is a homopolymer of a 5-*N*-acetimidoyl-7-*N*-acetyl derivative of 5,7-diamino-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid, termed legionaminic acid (*6*, *11*, *12*). In strain Philadelphia 1, belonging to the Pontiac group (*5*, *13*, *14*), legionaminic acid is 8-*O*-acetylated (*6*, *15*), whereas in strains of the non-Pontiac group, e.g., from subgroup OLDA (*16*, *17*), it is not. The 8-*O*-acetyl group of legionaminic acid is a part of the epitope of LPS-specific monoclonal antibodies (mAbs) 2 and 3/1 (*14*, *15*). The gene encoding 8-*O*-acetyl transferase, termed *lag-1*, that is responsible for 8-*O*-acetylation of polylegionaminic acid has been identified and cloned (*18*). The presence of the *lag-1* gene in *L. pneumophila* Sg 1 strains correlates with binding of mAb 3/1. The mAb 3/1 epitope could be lost by spontaneous events (*18*, *19*) with high frequency or in a phase-variable manner (*20*) without affecting the growth behavior of the cells (*19*, *20*). Phase variation of *L. pneumophila* Sg 1 LPS, caused by a 30 kb instable genetic element of presumably phage origin (*21*), was described by our group to affect virulence (*16*). In contrast to the parental virulent wild-type strain RC1, a phase

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* To whom correspondence should be addressed. Telephone: 49-4537-188462. Fax: 49-4537-188612. E-mail: uzaehr@fz-borstel.de.

‡ Zentrum für Medizin und Biowissenschaften.

§ Universität Würzburg.

|| Russian Academy of Sciences.

[1] Abbreviations: LPS, lipopolysaccharide; OPS, O-polysaccharide; Sg, serogroup; mAb, monoclonal antibody; SPR, surface plasmon resonance; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Rha, rhamnose; Man, mannose; C3, C4, and C5, core heptasaccharides with three, four, and five *O*-acetyl groups, respectively; C4*, C4 with a modified Kdo residue.

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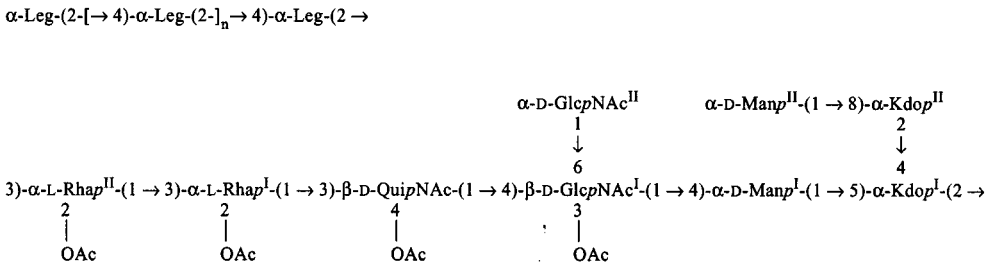

FIGURE 1: Proposed structure of the core nonasaccharide and the OPS of different chain lengths of *L. pneumophila* Sg 1 LPS. Adopted from refs *6*−*9* and *12*. QuiNAc, 2-acetamido-2,6-dideoxyglucose (*N*-acetylquinovosamine); Leg, 5-(acetimidoylamino)-7-acetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (legionaminic acid); $n < 10$, $= 15-20$, and > 30 in the short-, middle-, and long-chain OPS, respectively. In the middle- and long-chain OPS, a legionaminic acid residue proximal to the core is *N*-methylated. In Sg 1 strains of the Pontiac group, legionaminic acid is 8-*O*-acetylated. The anomeric configuration of the ketosidic linkage of the legionaminic acid residue attached to the core may be different.

Table 1: *L. pneumophila* Sg1 Strains That Were Studied

strain	origin	*lag-1*	reactivity mAb 3/1	reactivity mAb LPS-1	refs
OLDA subgroup					
RC1	clinical isolate	−	−	+	*16*, *22*
5271	RC1 transformed with *lag-1* on plasmid pCL6	+	+	+	this study
811	spontaneous phase variant from RC1	−	−	+	*16*, *22*, *23*
5215	mutant derived from RC1 by deletion of Orf 8-12	−	−	+	*22*
5097	ATCC 43109	−	−	+	*17*
137	spontaneous LPS mutant from 5097	−	−	+	*17*
Knoxville subgroup					
wild-type Corby	clinical isolate	+	+	+	P. C. Lück, personal communication
mutant Corby	spontaneous LPS mutant from wild-type Corby	−[a]	−	+	P. C. Lück, personal communication
Philadelphia subgroup					
Philadelphia 1	ATCC 33152	+	+	+	*6*−*9*, *15*
AM511	Philadelphia 1	+	+	+	*18*
CS332	spontaneous LPS mutant from AM511	−	−	+	*18*
CS338	CS332 complemented with *lag-1* on plasmid pLPS17	+	+	+	*18*

[a] The *lag-1* gene is present but mutated.

variant, termed strain 811, is avirulent and has an altered LPS structure; the OPS does not contain *N*-methylated legionaminic acid residues which were recognized by a monoclonal antibody, mAb 2625 (*22*), and lipid A is composed of different fatty acids (*23*).

The core of the LPS lacks heptose and phosphate, contains abundant 6-deoxy sugars and *N*-acetylated amino sugars, and is highly *O*-acetylated (*7*−*9*). Four *O*-acetyl groups have been identified previously at four different sugar residues by NMR spectroscopy applied to an isolated heptasaccharide fragment of the core (*8*) (Figure 1). In contrast to the OPS, no gene responsible for *O*-acetylation of the core has been identified so far in *L. pneumophila*. The epitope bound by monoclonal antibody mAb LPS-1, which is specific to *L. pneumophila* Sg 1 LPS (*24*), was shown by competitive binding assays to reside in the core oligosaccharide (*16*).

To investigate in more detail the role of the *lag-1* gene in *O*-acetylation of the LPS, we constructed a mutant that expressed *lag-1* after transformation of a *lag-1*-negative *L. pneumophila* OLDA strain with *lag-1* from strain Philadelphia 1. Investigation of the LPS structure of both mutant and parent strains, as well as of some other *lag-1*-positive and -negative *L. pneumophila* Sg 1 strains, demonstrated a complex *O*-acetylation pattern in the LPS and suggested the presence of multiple *O*-acetyl transferase-encoding genes. The data obtained also showed the role of particular *O*-acetyl groups in the epitope specificity of monoclonal antibodies to LPS.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Cultivation, and Isolation of LPS. The *L. pneumophila* Sg 1 strains that have been studied are listed in Table 1. Wild-type strain RC1 (subgroup OLDA) is a clinical isolate described previously (*16*, *17*). Mutant 5271 was generated by transforming wild-type strain RC1 with the Philadelphia 1 *lag-1* gene. For this purpose, a 3.1 kb chromosomal *Hin*dIII fragment from strain Philadelphia 1 (ATCC 33152) was ligated into plasmid vector pBC SK(+) (Stratagene). Sequencing of the resultant plasmid pCL6 confirmed that the complete *lag-1* gene with its promotor region was located on the insert. Plasmid pCL6 was employed to transform *L. pneumophila* RC1 by electroporation. The resultant transformant was analyzed by an ELISA and Western blot with mAb 3/1 for the presence of 8-*O*-acetylated polylegionaminic acid.

Wild-type RC1 and mutant 5271 were grown on buffered charcoal yeast extract agar supplemented with buffered charcoal yeast extract α-growth supplement (Merck). LPS of each strain was extracted from dried, defatted, and enzyme-digested cells by a modified phenol/chloroform/petroleum ether procedure as described previously (*6*, *25*) in yields of 9 and 16% of the bacteria dry mass, respectively.

Southern Blot Analysis. A total of 2 μg of chromosomal DNA from each strain was digested with *Hin*dIII, and restriction fragments were separated on a 0.8% agarose gel. Southern blot analysis was carried out following standard protocols (*26*). A PCR fragment of 1 kb covering the entire

lag-1 gene was generated and labeled with digoxigenin according to the instructions of the manufacturer (Boehringer Mannheim, Mannheim, Germany). Hybridization with this probe was performed at 60 °C using DIG Easy Hyb hybridization buffer (Boehringer Mannheim).

SDS−PAGE and Western Blot Analysis. SDS−PAGE was carried out with a 14% polyacrylamide gel as described previously (*16*, *17*). A total of 1 μg of LPS from each strain was applied to the gel, which was fixed and silver-stained (*27*). For Western blot analysis, 2 μg of LPS per lane was applied to a 12.5% polyacrylamide gel. Western blotting onto nitrocellulose filter membranes was carried out as described previously (*28*). Immunostaining was performed with mAb 3/1 and Sg 1-specific mAb LPS-1 (Progen) (*24*).

Surface Plasmon Resonance (SPR). SPR analyses were carried out using an automated BIACORE 3000 biosensor instrument (Biacore). mAb LPS-1 was immobilized on a research grade CM5 sensor chip in 10 mM sodium acetate (pH 4.5) using the amine coupling kit supplied by the manufacturer (Biacore). Unreacted moieties were blocked with ethanolamine. A control surface with an anti-myoglobin IgG (Biacore) was prepared in the same manner. All measurements were performed in 10 mM HEPES (pH 7.4) containing 150 mM NaCl and 0.005% (v/v) polysorbate 20 (Biacore) at a flow rate of 5 μL/min. Surfaces were regenerated by normal dissociation or with 20 μL of distilled water. Sensorgram data were analyzed using the BIAevaluation 3.0.2 software (Biacore). Binding affinity (K_D) was determined by steady-state affinity fitting based on end point values of a series of sensorgrams generated with at least five analyte concentrations ranging from 1 to 750 μM. K_D determinations using line fitting agreed well with values determined by linear regression of Scatchard plots.

Chemical Analysis of LPS. GLC was performed with a Hewlett-Packard model 5890 Series II instrument equipped with a 30 m capillary column of SPB-5 (Supelco) using a temperature gradient of 150 to 320 °C at a rate of 5 °C/min. Monosaccharides were analyzed by GLC as the alditol acetates after hydrolysis of LPS with 0.1 M HCl for 48 h at 100 °C for neutral sugars (*29*) or with 10 M HCl for 30 min at 80 °C for amino sugars (*30*). Following hydrolysis, sugars were dried by evaporation in a vacuum, reduced with $NaBH_4$, and acetylated with acetic anhydride in pyridine (30 min at 85 °C). The total amount of hexosamine was determined after acid hydrolysis (4 M HCl for 16 h at 100 °C) using the Morgan−Elson reaction as described previously (*31*). The level of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) was determined by the thiobarbituric acid assay according to the modified method (*32*). Quantification of the total amount of phosphate was carried out by the ascorbic acid method (*33*).

Degradation of LPS and Chromatography. LPS (80 mg of each strain) was degraded at 100 °C for 2.5 h with 0.1 M NaOAc/HOAc buffer (pH 4.4; 10 mg/mL LPS), and the resultant precipitate was removed by centrifugation. The lyophilized supernatant (39 mg of each strain) was dissolved in 1 mL of water and fractionated by gel-permeation chromatography on a column (2.5 cm × 120 cm) of Sephadex G-50 (S) (Pharmacia) using 50 mM pyridinium acetate buffer (pH 4.3) at a rate of 30 mL/h, monitoring with a Knauer differential refractometer and a fraction volume of 2.5 mL. The column was calibrated beforehand with a mixture of dextran (average molecular mass of 67 kDa, exclusion volume of ~160 mL), malto-oligosaccharides (average degree of polymerization of 5, elution volume of ~440 mL), and NaCl (void volume of ~490 mL). Fractions corresponding to long- and short-chain OPS, core oligosaccharide, and mono- and disaccharides contaminated with salt were collected and lyophilized. The core oligosaccharide pool was further fractionated by reversed-phase HPLC on a semipreparative column (19 mm × 300 mm; analytical scale, 3.9 mm × 300 mm) of Nova-Pak HR C18 60 Å (Waters) at a rate of 4 mL/min (analytical scale, 1 mL/min) with a gradient of 2 to 22% (v/v) acetonitrile in water for 120 min with UV monitoring at 210 nm.

NMR Spectroscopy. ^{1}H NMR and two-dimensional NMR spectra were recorded with a Bruker Avance DRX-600 spectrometer. ^{13}C NMR spectra were recorded with a Bruker Avance DPX-360 spectrometer. Standard Bruker software was used to acquire and process the NMR data. Samples were lyophilized three times from D_2O and assessed in D_2O at 27 °C. Chemical shifts were referenced to external acetone (δ_H 2.225; δ_C 31.45).

Mass Spectrometry. Negative ion mode MALDI-TOF MS was performed with a Bruker-Reflex II spectrometer in linear and reflector configurations at an acceleration voltage of 20 kV and with delayed ion extraction. The dried samples were dissolved in distilled water at a concentration of 20 μg/μL, and 2 μL of solution was mixed with 2 μL of a 0.5 M matrix solution of 3,5-dimethoxy-4-hydroxycinnamic acid (Aldrich), 2,4,6-trihydroxyacetophenone (Aldrich), or 2,5-dihydroxybenzoic acid (Aldrich) in methanol. Aliquots (0.5 μL) were deposited on a metallic sample holder and analyzed immediately after drying in a stream of air. The mass scale was calibrated externally with similar compounds of known chemical structure. The spectra that are shown represent the average of at least 50 single laser shot analyses.

RESULTS

Southern Blot Analysis. Southern blot analyses were performed to demonstrate the presence or absence of the *lag-1* gene in Sg 1 strains of different subgroups. DNA from all *L. pneumophila* strains of the Pontiac group, namely, subgroups Philadelphia, Knoxville, and France, hybridized to the probe (Figure 2), indicating that the *lag-1* gene is present in all of these strains. In contrast, strains of the non-Pontiac group, i.e., subgroups OLDA, Camperdown, Bellingham, Heysham, and Washington, did not possess the *lag-1* gene.

Construction of Mutant 5271 and Western Blot Analysis. *L. pneumophila* Sg 1 subgroup OLDA wild-type strain RC1 which does not own a *lag-1* gene was transformed with the *lag-1* gene from *L. pneumophila* Sg 1 strain Philadelphia 1. Unlike the parent strain, cells from the transformant strain 5271 bound LPS-specific mAb 3/1 as assayed by the ELISA technique. The LPS was isolated from the parent and mutant strains and analyzed by Western blot analysis with mAb LPS-1 and mAb 3/1. The LPS from both strains bound Sg 1-specific mAb LPS-1, showing the identical pattern as described previously (*16*, *17*), whereas mAb 3/1 only bound to the LPS from mutant 5271 (Figure 3, panels A and B, respectively). In contrast to LPS of wild-type *lag-1*-positive strains, like Philadelphia 1, the binding of mAb 3/1 was

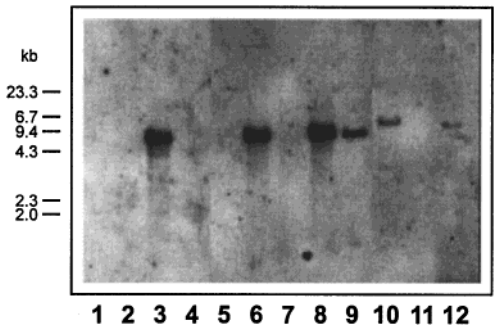

FIGURE 2: Southern blot analysis of *L. pneumophila* Sg 1 strains for determination of *lag-1* gene distribution. A PCR fragment covering the entire *lag-1* gene of strain Philadelphia 1 was labeled with digoxigenin and employed as a hybridization probe: lane 1, OLDA wild-type RC1; lane 2, OLDA wild-type 5097 (ATCC 43109); lane 3, Philadelphia 1 (ATCC 33152); lane 4, Camperdown (ATCC 43113); lane 5, Bellingham (NCTC 11404); lane 6, Cambridge (NCTC 11231); lane 7, Heysham (NCTC 12025); lane 8, Kingston (NCTC 11378); lane 9, Knoxville (ATCC 33153); lane 10, France (ATCC 43112); lane 11, Washington (NCTC 11201); and lane 12, Pontiac (NCTC 11191).

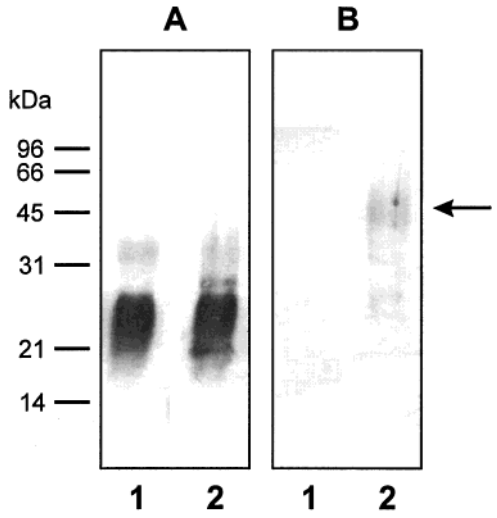

FIGURE 3: Western blot of isolated LPS from *L. pneumophila* OLDA strains RC1 (lane 1) and 5271 (lane 2) with mAb LPS-1 (A) and mAb 3/1 (B). The numbers at the left side indicate the molecular masses of standard protein markers. The arrow at the right side indicates the LPS bands from strain 5271 recognized by mAb 3/1.

limited to long-O-chain LPS species from mutant 5271, and the immunostaining was only faint (for comparison, see ref *18*).

Degradation of LPS and Fractionation of the Carbohydrate Portion. LPS each of wild-type RC1 and mutant 5271 was subjected to mild acid hydrolysis, which cleaved the ketosidic linkage of 3-deoxy-D-*manno*-oct-2-ulosonic acid residues (Kdo^{I} and Kdo^{II}) to release lipid A and a lateral $Man^{II}\rightarrow Kdo^{II}$ disaccharide, respectively (*8*, *9*) (Figure 1). In some molecules the linkage between the OPS and the core was also cleaved. As a result, after fractionation of the water-soluble material by gel-permeation chromatography on Sephadex G-50, the major core fragment representing a partially *O*-acetylated heptasaccharide was isolated (*8*, *9*).

The gel-permeation chromatography elution profile of the carbohydrate portion released from the LPS of all strains studied was similar and indicated high heterogeneity with respect to the chain length. The OPS could be fractionated

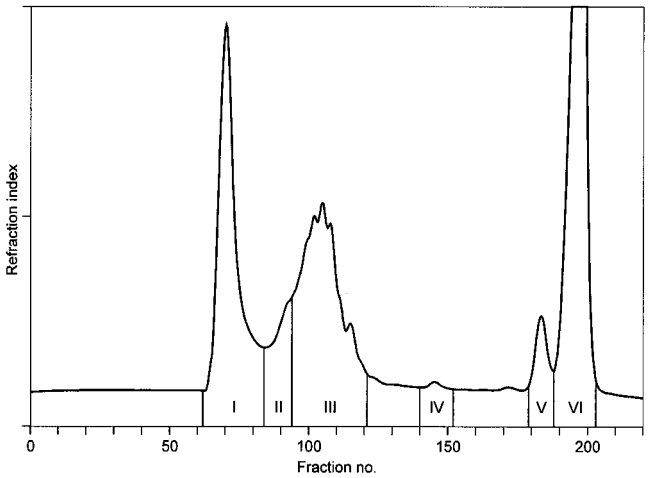

FIGURE 4: Elution profile of Sephadex G-50 gel-permeation chromatography of the carbohydrate portion from *L. pneumophila* OLDA strain RC1 LPS after mild acid hydrolysis: pools I−III, long-, middle-, and short-chain OPS, respectively; pool IV, core oligosaccharide; pool V, mono- and disaccharides; and pool VI, salts. See Experimental Procedures for details.

to short- and long-chain molecular species composed of less than 10 and more than 30 residues of legionaminic acid, respectively, and an intermediate fraction of a middle-chain OPS (Figure 4). In the majority of the molecules, the OPS was attached to the core heptasaccharide (see below).

Studies on the Core O-Acetylation Pattern of OLDA Wild-Type RC1. The MALDI-TOF mass spectrum of the core oligosaccharide from wild-type RC1 showed the presence of a penta-*O*-acetylated heptasaccharide (C5) as the major compound, whereas the expected tetra-*O*-acetylated heptasaccharide (C4) (*8*) was present in a smaller amount (Figure 5A). Accompanying pseudomolecular ions for C5 and C4 with a mass difference $\Delta m/z$ of −18 originated from anhydro artifacts of the reducing Kdo^{I} residue generated in the course of mild acid hydrolysis (*34*). Fractionation by reversed-phase HPLC gave oligosaccharides with a different number and positions of *O*-acetyl groups and with modified and non-modified Kdo^{I}. As expected, higher *O*-acetylated compounds eluted after lower *O*-acetylated compounds.

To determine the position of the *O*-acetyl groups in detail, the purified C5 with the nonmodified Kdo^{I}, as judged by MALDI-TOF MS, was studied by two-dimensional NMR spectroscopy. $^{1}H-^{1}H$ COSY, TOCSY, NOESY, and H-detected $^{1}H-^{13}C$ HMQC experiments revealed a close agreement between the ^{1}H and ^{13}C NMR chemical shifts and $^{1}H-^{1}H$ correlations and the published data (*8*).

Previously, on the basis of the NMR spectroscopic data, it has been suggested that the terminal nonreducing rhamnose residue of the core (Rha^{II}) is mono-*O*-acetylated at position 2. The appearance of Rha^{II} with an *O*-acetyl group at position 3 or 4 has been ascribed to migration accompanying the cleavage of legionaminic acid from position 3 of Rha^{II} (*8*). The present data showed that this conclusion has to be revised and that, in fact, a 2,4-di-*O*-acetylated Rha^{II} is present, whereas migration of one of the *O*-acetyl groups to position 3 might still occur during cleavage of the O-chain. Indeed, prolongation of mild acid hydrolysis (up to 80 h) significantly increased the heterogeneity of the isolated core oligosaccharide due to partial removal and migration of the *O*-acetyl groups and a higher degree of dehydration of Kdo^{I}.

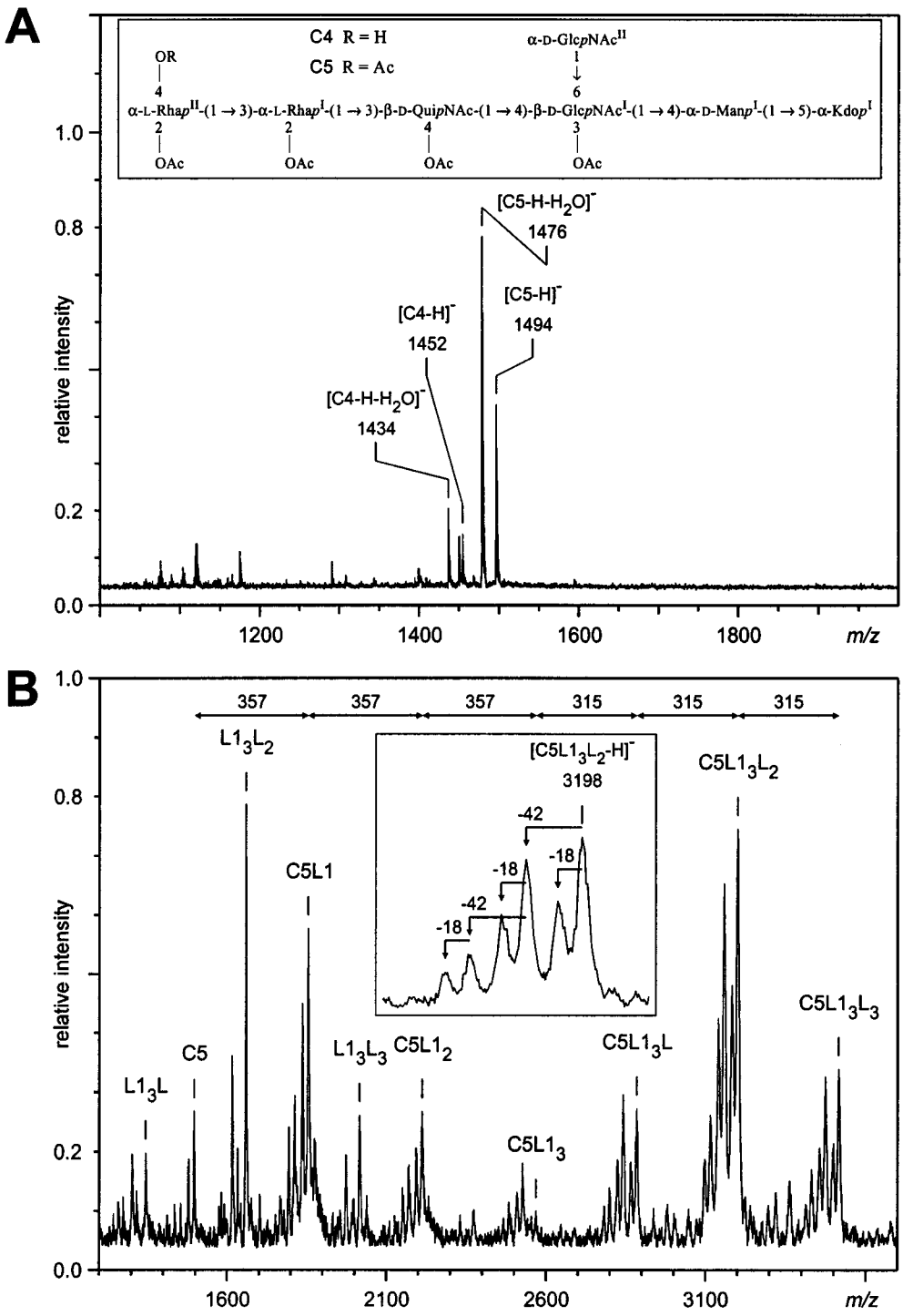

FIGURE 5: Parts of negative ion MALDI-TOF mass spectra of the core oligosaccharide (A) and the short-chain OPS (B) isolated by gel-permeation chromatography from acid-degraded LPS of *L. pneumophila* OLDA strain RC1. The spectrum in panel A was recorded in the reflector configuration using 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix. The inset shows the corresponding structures. The spectrum in panel B was recorded in the linear configuration using 2,5-dihydroxybenzoic acid as the matrix. An extension of the cluster for $C5L1_3L_2$ is shown in the inset. Mass differences $\Delta m/z$ of −18 and −42 correspond to losses of water and *O*-acetyl groups, respectively, as described for the core oligosaccharide (see the Results): C4 and C5, core heptasaccharide with four and five *O*-acetyl groups, respectively; L, legionaminic acid; and L1, 8-*O*-acetyllegionaminic acid.

It was observed that, compared to the penta-*O*-acetylated core oligosaccharide, 10−20% of the *O*-acetyl group, predominantly from position 4 of Rha^{II}, is lost during the first 2 h under the mild acid hydrolysis conditions (Figure 5A). After 48 h, the ratio of tri-, tetra-, and penta-*O*-acetylated core oligosaccharides was approximately 1:2:1 as judged from the intensity of peaks in the MALDI-TOF MS spectra.

Studies on the OPS O-Acetylation Pattern of OLDA Wild-Type RC1. In accordance with the lack of the *lag-1* gene, the OPS from wild-type RC1 was expected to consist of non-*O*-acetylated legionaminic acid. However, NMR studies of the short-chain OPS revealed a rather high degree of 8-*O*-acetylation (∼30%) of legionaminic acid. The ^{13}C NMR spectrum (Figure 6) clearly displayed signals for both 8-*O*-

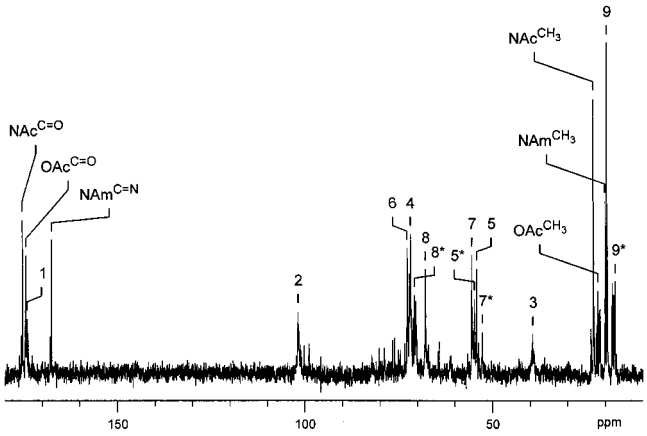

FIGURE 6: ^{13}C NMR spectrum of the short-chain OPS from *L. pneumophila* OLDA strain RC1. Numerals refer to carbons C-1−C-9 in legionaminic acid. Signals from 8-*O*-acetyllegionaminic acid are marked with asterisks. Nondesignated signals belong to core components. NAm, acetimidoylamino group.

acetylated and non-*O*-acetylated legionaminic acid residues with the same chemical shifts as reported for the initial and de-*O*-acetylated OPS from strain Philadelphia 1, respectively (*6*).

The MALDI-TOF mass spectrum of the short-chain OPS (Figure 5B) showed clusters for the OPS species of different chain lengths attached to the core heptasaccharide (C, core) carrying three to five *O*-acetyl groups (C3−C5). Separate OPS and core oligosaccharide species in the short-chain OPS fraction represent laser-induced in-source fragmentation ions originating from cleavage of the labile glycosidic linkage, and the intensity of these ions was dependent on laser irradiance (*35*). Mass differences $\Delta m/z$ of 357 between the first three consecutive OPS clusters corresponded to 8-*O*-acetyllegionaminic acid. Hence, three legionaminic acid residues next to the core are 8-*O*-acetylated (Figure 11). Mass differences $\Delta m/z$ of 315 for further OPS clusters corresponded to non-*O*-acetylated legionaminic acid.

In contrast to the short-chain OPS, the ^{1}H and ^{13}C NMR spectra of the long-chain OPS from wild-type RC1 showed no 8-*O*-acetylated legionaminic acid residues. MALDI-TOF MS analysis of the long-chain OPS was not possible, most likely, because the molecular mass was too high (>10 kDa).

Studies on the Core and OPS O-Acetylation Pattern of OLDA Mutant 5271. The MALDI-TOF mass spectrum of the core oligosaccharide from mutant 5271 was similar to that of wild-type RC1, and NMR spectroscopic analysis could not reveal any difference (data not shown).

The MALDI-TOF mass spectrum of the short-chain OPS from mutant 5271 showed the presence of OPS species of different chain lengths attached to core heptasaccharide C3, C4, or C5. The OPS formed the same clusters as observed for wild-type RC1 short-chain OPS, and again the first three, but no further, residues of legionaminic acid were 8-*O*-acetylated (data not shown).

^{13}C NMR spectroscopic analysis could not reveal any significant difference between the long-chain OPS from mutant 5271 and that from wild-type RC1. However, two-dimensional NMR spectroscopic studies, including H-detected ^{1}H−^{13}C HMQC (Figure 7) and NOESY experiments, indicated that in the long-chain OPS from mutant 5271, but not from wild-type RC1, ~10% of the legionaminic

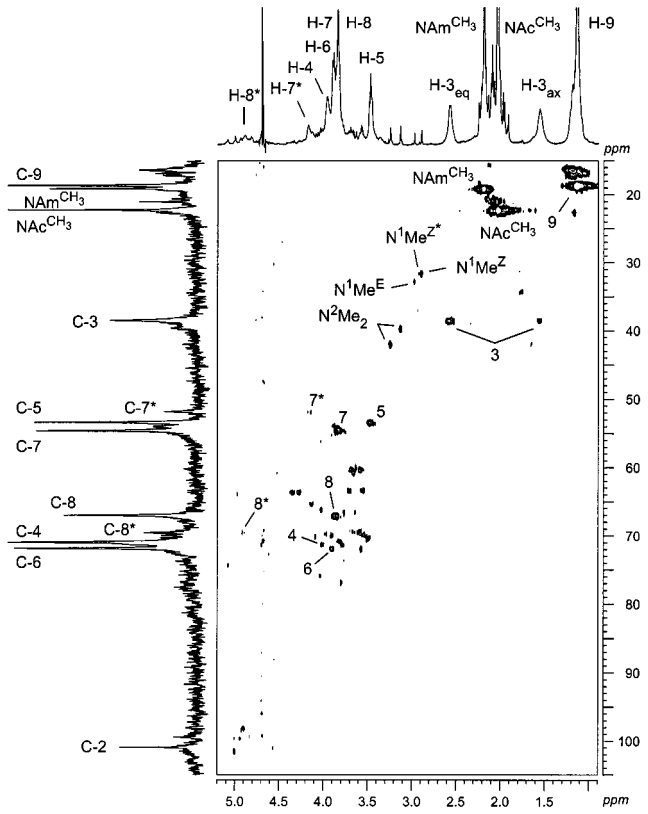

FIGURE 7: ^{1}H−^{13}C HMQC spectrum of the long-chain OPS from *L. pneumophila* OLDA strain 5271. The corresponding ^{13}C and ^{1}H NMR spectra are displayed along the F_1 and F_2 axes, respectively. Numerals refer to cross-correlations between protons and carbons of legionaminic acid or the corresponding 8-*O*-acetylated residues (marked with asterisks). Signals belonging to core components are not designated. N^{2}Me$_2$ and N^{1}Me stand for the *N*-methyl groups in 5-(*N*,*N*-dimethylacetimidoyl)amino and 5-acetimidoyl(*N*-methyl)-amino derivatives of legionaminic acid, respectively, or the corresponding 8-*O*-acetylated residues (marked with asterisks): NAm, acetimidoylamino group; and *E* and *Z*, stereoisomers of N^{2}Me$_2$ and N^{1}Me, respectively.

acid residues are 8-*O*-acetylated. Diagnostic signals were those for H-8 and C-8 which shifted downfield due to *O*-acetylation from δ_H 3.89 and δ_C 67.96 to δ_H 4.96 and δ_C 70.41 and those of the vicinal H-7 and C-7 which shifted in accordance with published data (*6*) from δ_H 3.89 and δ_C 55.52 to δ_H 4.19 and δ_C 52.80, respectively (Figure 7).

Characteristic changes in the ^{1}H NMR chemical shifts of the *N*-methyl groups (Figure 8B) indicated partial 8-*O*-acetylation of not only the major legionaminic acid derivative but also minor, *N*-methylated derivatives, 5-acetimidoyl(*N*-methyl)amino-7-acetamido- and 5-(*N*,*N*-dimethylacetimidoyl)-amino-7-acetamidolegionaminic acids, located close to the core (compare the ^{1}H NMR spectra of the non-*O*-acetylated and predominantly 8-*O*-acetylated *N*-methylated legionaminic acid residues present in the long-chain OPS of strains OLDA wild-type RC1 and Philadelphia 1 shown in panels A and C of Figure 8, respectively). The NOESY spectrum of the middle-chain OPS from strain Philadelphia 1 (Figure 9) showed NOE correlations for the *N*-methyl group of the two stereoisomers each of 8-*O*-acetylated (~75%, *E** and *Z**) and non-*O*-acetylated (~25%, *E* and *Z*) 5-acetimidoyl(*N*-methyl)amino-7-acetamidolegionaminic acid. The signals for correlating protons H-4*, H-6*, and H-7* of the 8-*O*-acetylated residue were shifted downfield by 0.2−0.3 ppm

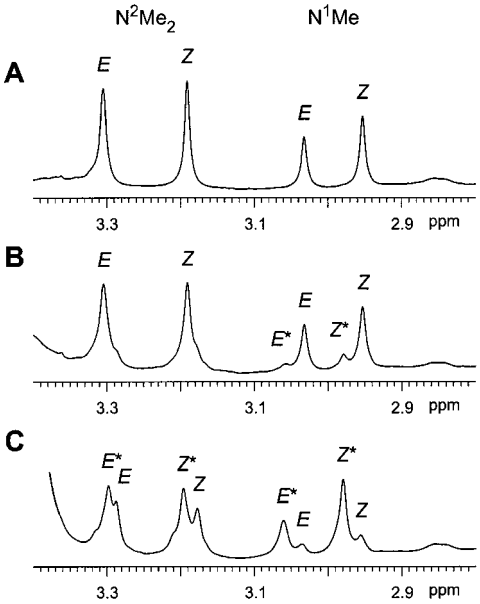

FIGURE 8: Resonance region of the *N*-methyl groups in 600 MHz ^{1}H NMR spectra of the long-chain OPS from *L. pneumophila* OLDA strains RC1 (A) and 5271 (B), and strain Philadelphia 1 (C). For the abbreviations, see the legend of Figure 7.

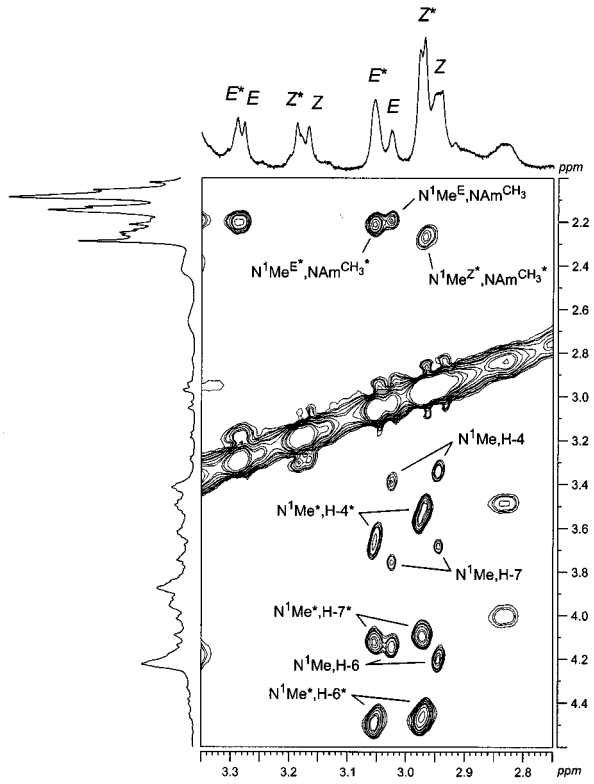

FIGURE 9: Section of a NOESY spectrum of the middle-chain OPS from *L. pneumophila* strain Philadelphia 1. The spectrum was recorded at 600 MHz and 27 °C using a mixing time of 300 ms. For the abbreviations, see the legend of Figure 7.

compared to those of the corresponding non-*O*-acetylated derivative (Figure 9). After mild alkaline de-*O*-acetylation of the OPS, only signals of the non-*O*-acetylated derivative remained, and after the following partial *O*-acetylation with acetic anhydride in pyridine and formamide (*36*), the signals of the *N*-methyl groups of the 8-*O*-acetylated derivative appeared again (data not shown). Although no NOE correlation could be detected for 5-(*N*,*N*-dimethylacetimidoyl)-amino-7-acetamidolegionaminic acid and the corresponding 8-*O*-acetylated derivative (Figure 9), it seems likely that 8-*O*-acetylation of this residue has a similar effect on the chemical shifts of the *N*-methyl groups. Therefore, it was concluded that the signals shifted downfield belong to the *N*-methyl groups of the 8-*O*-acetylated derivative.

These data together showed that the expression of the *lag-1* gene in OLDA strain RC1 resulted in partial 8-*O*-acetylation in the long-chain OPS but did not change the *O*-acetylation pattern in the short-chain OPS.

Studies on the LPS O-Acetylation Pattern of Other L. pneumophila Sg 1 Strains. To evaluate the incidence of the *O*-acetylation pattern described, LPS of some other *L. pneumophila* Sg 1 strains was investigated (Table 1). For instance, *lag-1*-positive wild-type strain Corby (subgroup Knoxville) and a spontaneously derived *lag-1* mutant were studied. Wild-type strain Corby reacted with mAb 3/1 in Western blot analysis, and as judged by NMR spectroscopy data, the degree of 8-*O*-acetylation of polylegionaminic acid was ~95%; the mutant strain carried a mutated *lag-1* gene, which led to an inactive enzyme, and did not react with mAb 3/1 (P. C. Lück, personal communication). Mass differences $\Delta m/z$ of 357 between all clusters in the MALDI-TOF mass spectrum of the short-chain OPS from the wild-type strain (Figure 10A) showed that all legionaminic acid residues are 8-*O*-acetylated. In contrast, the mass spectrum of the short-chain OPS from the mutant strain indicated that legionaminic acid residues are not *O*-acetylated (mass difference $\Delta m/z$ of 315), except for the first three residues next to the core (Figure 10B). Compared to OLDA strains (see above), core species C4* with one fewer *O*-acetyl group and with a modified Kdo^I predominated in Corby strains, which can be ascribed to a prolonged mild acid hydrolysis of LPS (4 vs 2.5 h; see above). All other strains of Sg 1 that were studied (Table 1) showed essentially the same *O*-acetylation pattern of the core oligosaccharide and the first three legionaminic acid residues in the short-chain OPS.

SPR Studies with Immobilized mAb LPS-1. To investigate the influence of *O*-acetylation on the binding behavior of mAb LPS-1, binding affinity (K_D) was determined by SPR for the binding to mAb LPS-1 of purified core oligosaccharides as well as long- and short-chain OPS from *L. pneumophila* OLDA wild-type strain RC1. Three core heptasaccharides, purified by HPLC and characterized by MALDI-TOF MS as C5, C4, and C3 (see above), bound to mAb LPS-1 with K_D values of 1.21×10^{-4} (C5), 1.24×10^{-4} (C4), and 2.64×10^{-4} M (C3). The short-chain OPS with an average molecular mass of 3.2 kDa ($C5L1_3L_2$; Figure 5B) exhibited a considerably higher affinity ($K_D = 4.17 \times 10^{-6}$ M), increased by 2 orders of magnitude compared to that of the purified core heptasaccharides. The affinity of the long-chain OPS ($K_D = 2.82 \times 10^{-5}$ M), having a calculated average molecular mass of 14 kDa ($C5L_{40}$), was also higher than that of the purified core heptasaccharides but lower compared to that of the short-chain OPS. The long-chain OPS from OLDA mutant strain 5215, which lacks the *N*-methylated legionaminic acid residue proximal to the core, bound to mAb LPS-1 with a high affinity ($K_D = 6.91 \times 10^{-6}$ M) in the same range as that of the short-chain OPS from the same strain ($K_D = 3.85 \times 10^{-6}$ M) and OLDA wild-type strain RC1 ($K_D = 4.17 \times 10^{-6}$ M).

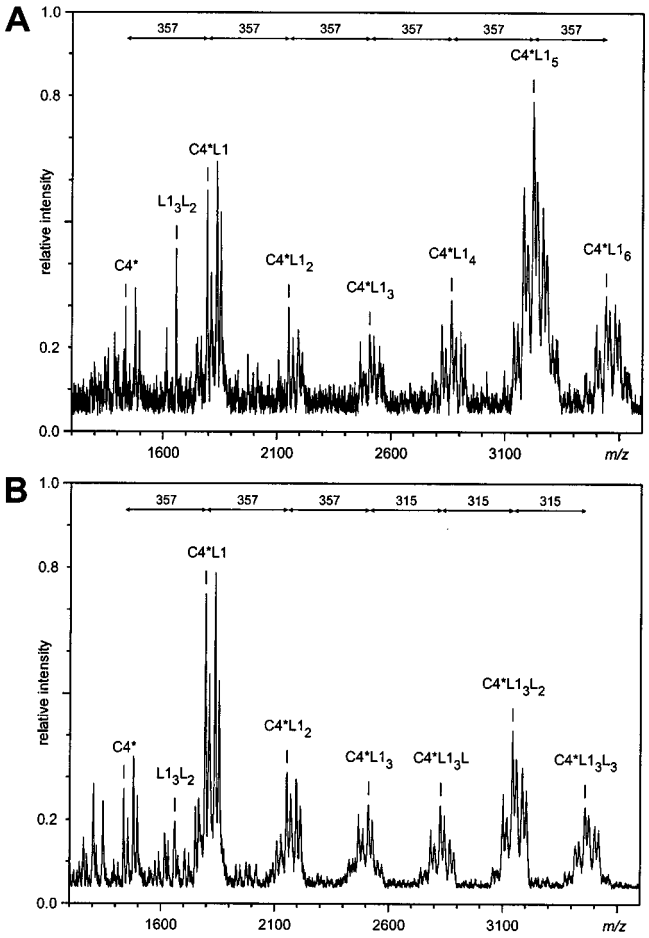

FIGURE 10: Parts of negative ion MALDI-TOF mass spectra of the short-chain OPS isolated by gel-permeation chromatography from acid-degraded LPS of the *L. pneumophila* Corby wild-type strain (A) and Corby mutant strain (B). Spectra were recorded in the linear configuration using 2,5-dihydroxybenzoic acid as the matrix: C4*, core heptasaccharide with four *O*-acetyl groups and modified Kdo[I]; L, legionaminic acid; and L1, 8-*O*-acetyllegionaminic acid.

DISCUSSION

Data on the biosynthesis of *L. pneumophila* LPS, including the polylegionaminic acid OPS, are scarce. They are limited to an observation of similarities in the biosynthesis pathway of legionaminic acid and neuraminic acid (5,7-diamino-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-non-2-ulosonic and 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acids, respectively) (*17*) and identification of genes that are homologous to those of bacterial *O*-acetyl (*18*) and methyl transferases (*17*). The *lag-1* gene that participates in 8-*O*-acetylation of polylegionaminic acid has been cloned, and it was demonstrated that complementation of a *lag-1*-negative mutant of strain Philadelphia 1 with *lag-1* completely restored the *O*-acetylation pattern of the OPS that was affected by mutation (*18*).

In the work presented here, we demonstrated that the *lag-1* gene could be expressed in a Sg 1 strain which does not naturally possess the gene (non-Pontiac group strains). However, gene expression or the *O*-acetyl transferase activity in the transformant strain is low and cannot provide such a high degree of 8-*O*-acetylation as in natural *lag-1* owners (Pontiac group strains). Another difference is related to the substrate specificity of *O*-acetyl transferase. While in strains of the Pontiac group legionaminic acid is 8-*O*-acetylated in all LPS species, in the transformant strain from the non-Pontiac group the *lag-1*-dependent 8-*O*-acetylation of legionaminic acid was limited to the long-O-chain LPS.

Detailed structural analysis of the isolated and fractionated OPS showed a highly complex *O*-acetylation pattern. Independent of the presence and activity of the *lag-1* gene, it was found that in the short-O-chain LPS the first three legionaminic acid residues next to the core are 8-*O*-acetylated. This feature was observed in all 12 Sg 1 strains that were studied and seems to be a conserved character of Sg 1 strains, including those that do not possess *lag-1*. Therefore, another *O*-acetyl transferase-encoding gene different from *lag-1* must exist, which is responsible for specific 8-*O*-acetylation of the first three legionaminic acid residues in the short-O-chain LPS.

On the other hand, in the long-O-chain LPS, 8-*O*-acetylation is strictly *lag-1*-dependent, but whether *lag-1* is present or not, the 5-*N*-acetimidoyl group of a single legionaminic acid residue proximal to the core is *N*-methylated (*22*). The *N*-methylated legionaminic acid derivatives can be further 8-*O*-acetylated by *lag-1*-encoded *O*-acetyl transferase, whereas *lag-1*-independent 8-*O*-acetylation occurs exclusively in the short-O-chain LPS that is devoid of *N*-methylated legionaminic acid. Therefore, both *N*-methylation and *lag-1*-independent 8-*O*-acetylation of legionaminic acid occur in the LPS species having a certain OPS chain length.

Previous studies (*8*) and this reinvestigation of the Sg 1 LPS core structure showed that the outer core region is highly *O*-acetylated and may contain up to five *O*-acetyl groups (Figure 11). The core *O*-acetylation pattern seems to be another common feature of Sg 1 strains. No mutant with affected *O*-acetylation of either the core or the first three legionaminic acid residues located next to the core in the short-O-chain LPS has ever been generated, and it remains to be elucidated how many genes are involved in this kind of *O*-acetylation. It has to be emphasized that not even in the OLDA phase variant (strain 811, Table 1), which has a considerably altered LPS structure, was the described *O*-acetylation pattern affected, thus, confirming the conserved nature of this structural feature. In *Neisseria gonorrhoeae* wild-type strain 1291, part of the LPS molecules carry a single *O*-acetyl group at a terminal 2-acetamido-2-deoxy-D-glucose residue in a side chain of the core oligosaccharide (*37*). After mutagenesis of this strain, which generated five pyocin-resistant strains, the carbohydrate composition of the core oligosaccharide was drastically altered but the position and the proportion of *O*-acetylation remained conserved (*37*).

O-Acetylation in bacterial glycopolymers is known to play an important (patho-)biological role. A loss of 3-*O*-acetylation at 2-acetamido-2-deoxy-D-mannuronic acid in the trisaccharide repeating unit of *Staphylococcus aureus* type 5 capsular polysaccharide significantly reduced the resistance of the bacterium to opsonophagocytic killing and the ability to colonize the kidneys in a mouse model (*38*). Virulence could be restored by complementing the mutant strain with the gene responsible for *O*-acetylation (*38*). 6-*O*-Acetylation at *N*-acetylmuramic acid residues of peptidoglycan from various human pathogens (*N. gonorrhoeae*, *Proteus mirabilis*, and *S. aureus*) was demonstrated to be responsible for

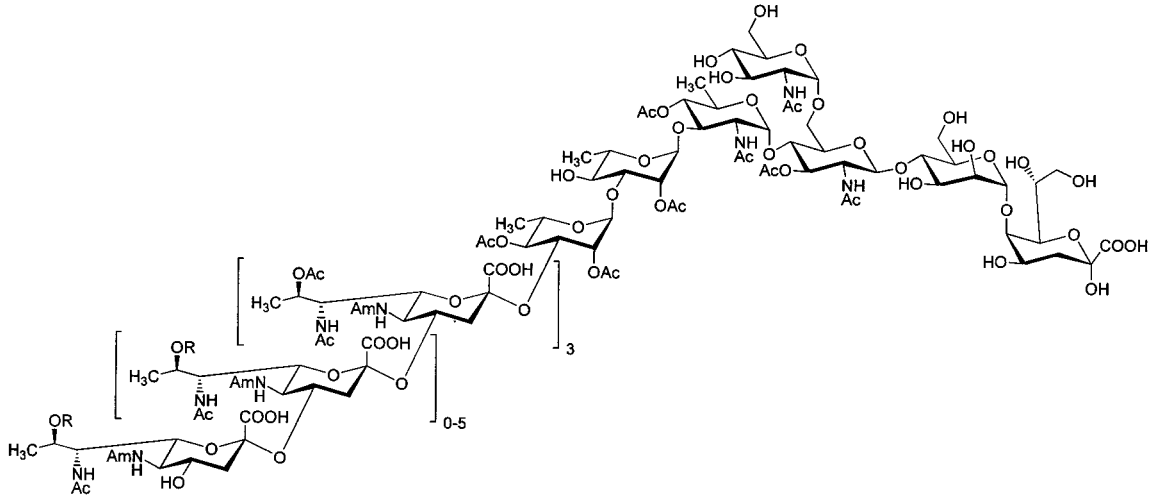

FIGURE 11: Structure of the short-chain OPS of *L. pneumophila* Sg 1 LPS with the defined positions of the *O*-acetyl groups. The first three legionaminic acid residues next to the core are 8-*O*-acetylated in all the Sg 1 strains that were studied; in the following, upstream legionaminic acid residues R = H or Ac in *lag-1*-negative or *lag-1*-positive strain, respectively. In some OPS species, there are fewer than four legionaminic acid residues in the total, each of which is 8-*O*-acetylated (see Figure 5B). The anomeric configuration of the ketosidic linkage of the legionaminic acid residue attached to the core may be different: AmN, acetimidoylamino group.

resistance of peptidoglycan to hydrolytic degradation by hen egg white lysozyme and most muramidases (*39*). A biosynthetic modification of bacterial alginates, an important virulence factor of some *Pseudomonas* species (*40*), by 2- and/or 3-(di-)*O*-acetylation at D-mannuronic acid has important implications for the alginate properties (*41*, *42*). *O*-Acetylation at position 3 in an α-(1→4)-linked homopolymer of 2-acetamido-2-deoxy-D-galacturonic acid forming the capsular polysaccharide (Vi antigen) of *Salmonella typhi* and *Citrobacter freundii* was reported to affect the binding properties of the carboxyl groups (*43*). *O*-Acetylation in LPS OPS is considered an important virulence factor as well, as creating antigenic variation enhances the survival of the bacteria because the host has to mount a specific immune response to each different serotype (*44*). An increased level of OPS *O*-acetylation serves as a tool for switching from predominantly hydrophilic to predominantly hydrophobic molecular forms of the LPS during the symbiotic bacteroid development of *Rhizobium leguminosarum* (*45*).

In contrast to *O*-acetylation in OPS (*46*), *O*-acetylation in the LPS core is rather uncommon (*47*). Apart from *N. gonorrheae* (*37*) mentioned above, *O*-acetylation was observed in the core of *Neisseria meningitidis* serotypes L2 (*48*), L4, and L5 (*49*, *50*), which have a single *O*-acetyl group in 30−60% of the LPS molecules. In *Haemophilus influenzae* RM.118-26, *O*-acetylation occurs in the outer core region of 60% of the LPS molecules and was suggested to be associated with phase variation (*51*), which is frequent in *Haemophilus* and *Neisseria* (*52*). Multiple *O*-acetylations were observed in the LPS core of *H. influenzae* type b; a major LPS species is *O*-acetylated at an L-*glycero*-D-*manno*-heptose residue, and minor species are additionally *O*-acetylated at a D-glucose residue and an unknown residue (*53*).

Therefore, *O*-acetylation in the LPS core seems to be a common feature of respiratory tract pathogens such as *N. meningitidis*, *H. influenzae*, and *L. pneumophila* but also for other mucosal pathogens such as *N. gonorrhoeae* and *Vibrio cholerae* (*54*, *55*). Most of these strains do not express OPS, which is usually described as being a hydrophilic LPS region, suggesting that a hydrophobic LPS provides a significant advantage in the host environment.

Together with the proximal legionaminic acid residues that are *O*-acetylated in the short-chain OPS, *N*-methylated, or both *N*-methylated and *O*-acetylated in the long-chain OPS, the outer core forms a highly hydrophobic region of the *L. pneumophila* Sg 1 LPS. In contrast to *Neisseria* and *H. influenzae*, *L. pneumophila* does express an OPS, but being a homopolymer of legionaminic acid, a sugar enriched in deoxy groups and *N*-acyl substituents, the OPS is rather hydrophobic. The hydrophobicity of the OPS and, hence, the whole *L. pneumophila* LPS may be further increased by *lag-1*-dependent 8-*O*-acetylation of polylegionaminic acid. The hydrophobicity of the LPS may play an important biological role, e.g., in the spread of *L. pneumophila* via aerosols and in the interaction of the bacterial surface with host cells, including amoebae and macrophages (*7*, *56*).

The *L. pneumophila* OPS is not only hydrophobic but also amphiphilic, having a zwitterionic character at neutral pH rendered by the negatively charged carboxyl groups and the positively charged acetimidoylamino groups. The first legionaminic acid residue linked to the di-*O*-acetylated Rha^{II} residue of the outer core occurs in a different chemical environment compared to the other residues and may play a particular biological role, e.g., in mAb recognition (see below). Interestingly, in *Pseudomonas fluorescens*, which also expresses a partially 8-*O*-acetylated polylegionaminic acid as OPS, the first legionaminic acid residue is linked to a 2-acetamido-3-*O*-acetyl-2,6-dideoxy-L-galactose→2-acetamido-2,6-dideoxy-D-glucose disaccharide in the outer core region (*11*), which is as highly hydrophobic as the outer core of *L. pneumophila* Sg 1.

As the epitope associated with *lag-1*-dependent 8-*O*-acetylation is frequently lost (*18*−*20*), it does not seem to be of stringent necessity. Another explanation could be the phage origin of the gene which is based on a high degree of homology between Lag-1 and Oac (*57*), an *O*-acetyl transferase from the bacteriophage SF6, which is in part responsible for serotype conversion by OPS modification of *Shigella flexneri* (*44*). Lysogenic conversion in *Salmonella*

enterica sv. Typhimurium by specific bacteriophages A3 and A4 has been reported to cause the OPS *O*-acetylation, which altered the binding behavior of the bacteriophages and prevented subsequent infection (*58*). Although no bacteriophage has yet been described for *L. pneumophila*, apart from a 30 kb instable genetic element of presumably phage origin being responsible for phase variation (*21*), it is conceivable that the *lag-1* gene is part of a cryptic or remnant bacteriophage genome integrated into the *L. pneumophila* chromosome.

The *O*-acetyl groups significantly contribute to the recognition of *L. pneumophila* LPS by monoclonal antibodies. Thus, binding of mAb 3/1 correlates with the major 8-*O*-acetylation of *L. pneumophila* Sg 1 strains (*15*) and is used to differentiate between Pontiac and non-Pontiac group strains (*5*, *14*). Furthermore, mAb 3/1 bound in Western blot analysis to LPS of only those Sg 1 strains that possess a functional *lag-1* gene. These data suggested that mAb 3/1 recognizes an epitope that is induced by *lag-1*-dependent 8-*O*-acetylation of polylegionaminic acid. In accordance with the *O*-acetylation pattern, binding of mAb 3/1 may be limited to LPS species with a certain OPS chain length, like in the OLDA strain transformed with *lag-1*, in which mAb 3/1 recognized only long-O-chain LPS. Several studies showed that the majority of strains isolated from patients react with mAb 2 and mAb 3/1 and that most strains isolated from environmental sources are not reactive with these antibodies (*5*, *13*, *14*, *59*−*61*). Therefore, the epitope recognized by these antibodies has been termed "virulence-associated" (*15*), but the molecular basis for this phenomenon could not been clarified by intracellular growth experiments (*62*). To date, it is concluded that cells expressing the epitope survive better in aerosols (*56*), which is supposed to be supported by the high hydrophobicity of the LPS (*7*).

The *O*-acetyl groups are evidently involved in the epitope of another LPS-specific monoclonal antibody, mAb LPS-1, since its binding ability in Western blot analysis was lost after mild alkaline de-*O*-acylation of the LPS (data not shown). A competition ELISA showed that mAb LPS-1 bound to the isolated core oligosaccharide from OLDA strain RC1 (*16*). SPR analyses with immobilized mAb LPS-1 showed that the affinity of the purified core heptasaccharides is dependent on the degree of their *O*-acetylation. The affinity is 2 orders of magnitude higher if the core heptasaccharide is substituted with a short-chain OPS. When the core heptasaccharide is substituted with a long-chain OPS that contains an *N*-methylated legionaminic acid derivative close to the core, the affinity is intermediate, indicating that the *N*-methyl groups interfere with the binding of mAb LPS-1. In Western blot analysis with LPS from OLDA strain RC1 fractionated prior by OPS chain length, binding of mAb LPS-1 strictly correlated with the absence of *N*-methylated legionaminic acid derivatives (*22*). Therefore, the full mAb LPS-1 epitope includes the *O*-acetylated outer region of the core with one or more legionaminic acid residues attached, which in turn may be (or need to be) 8-*O*-acetylated but not *N*-methylated.

In conclusion, the abundant *O*-acetyl groups in the *L. pneumophila* LPS contribute to the hydrophobicity of the cell surface and play an important role in the immunospecificity of *L. pneumophila* Sg 1 strains, in particular, in binding of mAb 3/1 and mAb LPS-1. Various LPS species and LPS-derived products with a defined *O*-acetylation and *N*-methylation pattern may be useful for characterization of other Sg 1 LPS-specific monoclonal antibodies. *O*-Acetylation of the Sg 1 LPS seems to be a multistep process involving a number of genes and interplaying somehow with *N*-methylation of legionaminic acid. Further studies are necessary to elucidate the biosynthesis of the core and polylegionaminic acid O-chain of the LPS in general and on *lag-1*-independent *O*-acetylation in particular.

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