Characterization of a Novel Lipid A Containing D-Galacturonic Acid That Replaces Phosphate Residues

THE STRUCTURE OF THE LIPID A OF THE LIPOPOLYSACCHARIDE FROM THE HYPERThERMOPHILIC BACTERIUM AQUIFEX PYROPHILUS*

(Received for publication, December 1, 1999, and in revised form, January 11, 2000)

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According to the 16 S rRNA phylogenetic tree, the hyperthermophilic bacterium Aquifex pyrophilus represents the deepest and shortest branching species of the kingdom Bacteria. We show for the first time that an organism, which is phylogenetically ancient on the basis of its 16 S rRNA and that exists at extreme conditions, may contain lipopolysaccharide (LPS). The LPS was extracted from dried bacteria using a modified phenol/water method. SDS-polyacrylamide gel electrophoresis and silver stain displayed a ladder-like pattern, which is typical for smooth-form LPS (possessing an O-specific polysaccharide). The molecular masses of the LPS populations were determined by matrix-assisted laser-desorption ionization mass spectrometry. Lipid A was precipitated after mild acid hydrolysis of LPS. Its complete structure was determined by chemical analyses, combined-gas-liquid chromatography-mass spectrometry, matrix-assisted laser-desorption ionization mass spectrometry, and one- and two-dimensional NMR spectroscopy. The lipid A consists of a β-(1→6)-linked 2,3-diamino-2,3-dideoxy-α-D-glucopyranosyl residue, which is linked to the 3-hydroxy fatty acid residue, which is linked to an octadecanoic acid in the nonreducing end. This structure represents a novel type of lipid A.

Within the kingdom Bacteria, Aquifex pyrophilus (1) exhibits with 95 °C the highest growth temperature. It does not belong to any of the known phyla and represents the deepest branching species of the kingdom Bacteria in the 16 S rRNA-based universal phylogenetic tree (2, 3). Hyperthermophiles are represented among the deepest and shortest lineages of this tree and are discussed to be still rather primitive (4). A. pyrophilus was isolated from hot marine sediments (depth: 106 m) at the Kolbeinsey Ridge, Iceland and is growing microaerophilically under oxygen reduction at temperatures in the range of 67 to 95 °C. Cells are Gram-negative highly motile rods exhibiting a complex envelope consisting of murein, an outer membrane, and a surface protein layer (1). However, it was not investigated whether A. pyrophilus contains lipopolysaccharide (LPS) or LPS analogous structures.

LPS (5) are characteristic components of the cell wall of Gram-negative bacteria where they are located in the outer leaflet of the outer membrane. They contribute to the highly effective permeation barrier function of the outer membrane and, furthermore, participate in various physiological membrane functions essential for growth and survival of Gram-negative bacteria. LPS play also an important role in the interaction of the bacteria with suitable hosts. They are the endotoxins of Gram-negative bacteria and are responsible for a broad spectrum of biological activities. Chemically, LPS are composed of three regions, namely (a) the O-specific polysaccharide (6, 7), which is built up of a varying amount of repeating oligosaccharide units, (b) the core oligosaccharide (8), and (c) the lipid A (9, 10), which anchors the molecule in the membrane and was shown to represent the toxic principle of LPS (11). Endotoxic active lipid A, e.g. enterobacterial lipid A, is known to possess a rather conserved structure, which is characterized by a β-(1→6)-linked 2-amino-2-deoxy-4-glucopyranosyl (α-GlcN) disaccharide backbone with phosphate groups attached to O-1 and O-4′ that carries (R)-3-hydroxy fatty acids and (R)-3-aclyxyacyl residues at positions 2 and 3, and 2′ and 3′, respectively. LPS isolated from other bacterial species showed a greater variability of their lipid A including 2,3-diamino-2,3-dideoxy-4-glucopyranosyl (DAG) instead of GlcN and variations in the fatty acid and phosphate substitution pattern (9). LPS are discussed to be valuable (chemo-)taxonomic and also phylogenetic markers because of the compositions of their lipid A and inner core regions. This might also be true for the taxonomy of A. pyrophilus and related species. In this work we describe the isolation of the LPS from A. pyrophilus and the characterization of the structure of its lipid A moiety which is different from all previously known lipid A.

EXPERIMENTAL PROCEDURES

Bacterial Cultures and LPS Isolation—Batch cultures of A. pyrophilus were grown in 300-liter fermentors in modified SME medium under microaerophilic conditions at 85 °C as described (1). After centrifuga-

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‡ The abbreviations used are: LPS, lipopolysaccharide; DAG, 2,3-diamino-2,3-dideoxy-4-glucopyranosyl; Kdo, 3-deoxy-4-manno-oct-2-ulopyranosonic acid; GLC, gas-liquid chromatography; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; GaLA, galacturonosyl; GaLPa, galactopyranosyl; GlcN, 2-amino-2-deoxy-glucopyranosyl; TLC, thin-layer chromatography.
tion, the sedimented bacteria (yield: 166 g) were washed successively with ethanol, acetone (twice), and ether and dried (27 g). For the extraction of LPS, the phenol/chloroform/light petroleum (2:5:8 (v:v:v)) method (12) was tried. For better yields and purity, it was necessary to extract the dried bacteria using hot phenol/water supplemented with 2% sodium-2-nauroylsarcosine (w/v) (13): yield: 450 mg LPS, 1.7% dry bacterial mass.

**Isolation of Lipid A**—Free lipid A was obtained by treatment of LPS (100 mg) with HCl (0.1 N, 100 °C, 90 min) and ultracentrifugation for 4 h at 100,000 × g (yield: 27 mg, 27% LPS) (14).

**De-O-acylation of Lipid A**—Lipid A was de-O-acylated by mild hydrolysis (as described (15)).

**General and Analytical Methods**—Analysis for neutral sugars, total GlcN, reducing GlcN, 3-deoxy-D-manno-oct-2-ulosonaric acid (Kdo), and phosphate were performed as described (16). The method for the analysis of neutral sugars was equally employed for the detection of uronic acids and amino sugars with the following variations: for the determination of uronic acids, samples were methanolyzed (0.5 N methanolic HCl, 85 °C, 45 min) and then carboxyl-reduced (NaB\(_2\)H\(_4\), in anhydric H\(_2\)SO\(_4\), and compounds corresponding to each detected band were scraped out of the plate and extracted from silica gel with CHCl\(_3\) : acetic acid (gentisic acid, DHB, Aldrich) in methanol and aliquots of 0.5 μl were deposited on a metallic sample. The mass spectra shown are the average of at least 50 single analyses. Mass scale calibration was performed internally with similar compounds of known chemical structure. Details of the applied methods, especially of the preparation of TLC-separated spots and on laser-induced fragmentation, are given in Refs. 13 and 27.

**NMR Spectroscopy**—NMR spectra were recorded of a solution of 5 mg in 0.5 ml Me\(_2\)SO-\(d_6\), with a Bruker DRX 600 (operating frequencies 600 MHz for \(^1\)H, 150.9 MHz for \(^{13}\)C, and 243 MHz for \(^{31}\)P) or DRX 360 (operating frequency 90.6 MHz for \(^{13}\)C) spectrometers at 298 °C using Bruker standard software. The \(^1\)H resonances were measured relative to the methyl group signal of Me\(_2\)SO (2.5 ppm). The assignment of the proton chemical shifts was achieved by correlation spectroscopy, total correlation spectroscopy, and double-quantum-filtered correlated spectroscopy experiments. The assignment of carbon chemical shifts was achieved by \(^1\)H,\(^{13}\)C heteronuclear multiple quantum coherence experiments and, for galactopyranuronic acid (GalNA), by comparison to published \(^13\)C NMR data (28). \(^13\)C resonances were determined relative to the methyl group signal of Me\(_2\)SO (40.0 ppm). Nuclear Overhauser effect contacts were identified using nuclear Overhauser effect spectroscopy experiments.

**RESULTS**

**Isolation and Characterization of LPS**—Extraction of dried bacterial cells using the phenol/chloroform/light petroleum method gave LPS in low yield together with a coextracted phospholipid. Therefore, the modified hot phenol/water method was applied. The LPS was isolated from the water phase after ultracentrifugation (450 mg, 1.7% of dry bacterial mass) and was found to be free of other lipids, proteins, DNA, and RNA. On SDS-polyacrylamide gel electrophoresis of the LPS preparation, a ladder-like pattern indicating the presence of S-form LPS with different sizes of the O-specific polysaccharide moieties was identified (Fig. 1). The negative ion MALDI-TOF mass spectrum of the native LPS revealed the molecular masses of the LPS populations (Fig. 2, M\(_1\), M\(_2\), . . . . .). Each repeating unit was found to possess an average mass of 708 Da, which indicated that it is composed of two heptose and two hexose residues. Furthermore, the spectrum comprises prominent peaks originating from laser-induced cleavage of the labile linkage between lipid A and Kdo I of the core oligosaccharide (29, 30). Thus, the fragment ion peak at m/z 1916 could be identified as the main free lipid A component (see below).

Quantitative sugar analysis of the isolated LPS revealed that it consists of GlcN, DAG, Kdo, galacturonic acid (GalA), Man, Glc, and α-glycerol-3-manno-heptose (Table 1). The LPS does not contain phosphate. Fatty acid analysis revealed the

![Fig. 1. SDS-polyacrylamide gel electrophoresis analysis of LPS isolated from A. pyrophilus. LPS of: lane 1, Salmonella enterica sv. Minnesota R595 (chemotype Re); lane 2, S. enterica sv. Minnesota R7 (Rd); lane 3, S. enterica sv. Minnesota R5 (Rc); lane 4, S. enterica sv. Minnesota R345 (Rb); lane 5, S. enterica sv. Minnesota R60 (Ra); lane 6, S. enterica sv. Abortus Equi (S-Form); lane 7, A. pyrophilus LPS extracted with phenol/chloroform/light petroleum; lane 8, A. pyrophilus LPS extracted by the modified phenol/water method; lane 9, A. pyrophilus whole cell lysate.](http://www.jbc.org/)

**Fig. 1. SDS-polyacrylamide gel electrophoresis analysis of LPS isolated from A. pyrophilus**.
presence of 3-hydroxytetradecanoic acid (14:0(3-OH)) and 3-hydroxyhexadecanoic acid (16:0(3-OH)) in amide linkage as well as octadecanoic acid (18:0) in ester linkage (Table II).

Compositional Analysis of Lipid A—Lipid A was obtained on mild hydrolysis of the LPS in a yield of 27% LPS. Compositional analyses of the lipid A revealed the presence of DAG and GalA. No phosphate or additional sugars were detected. A colorimetric assay proved the presence of uronic acids in lipid A. Mild methanolysis followed by reduction of the carboxyethyl ester using NaB2H4 and the preparation of alditol acetates, identified on GLC and GLC-MS with comparison to authentic standard derivatives GalA. GLC analyses of the acetylated (R)- and (S)-butyl glycosides proved that DAG and GalA are d-configured.

Analysis of Fatty Acid Substitution—Fatty acid analysis of lipid A identified 14:0(3-OH) and 16:0(3-OH) in amide linkage as well as 18:0 in ester linkage in a molar ratio of 2:2:1, respectively. As revealed by MALDI-MS (see below, Fig. 4), each DAG residue possesses one 14:0(3-OH) and 16:0(3-OH) in amide linkage. The absolute configurations of 14:0(3-OH) and 16:0(3-OH) were determined to be R. The ester-bound octadecanoic acid is exclusively linked to 14:0(3-OH) resulting in 14:0(3-OH)-18:0.

Analysis of Lipid A—The negative ion MALDI-TOF mass spectrum of the isolated lipid A fraction (Fig. 3) indicated a heterogeneous preparation comprising four quasimolecular ions, (M - H)− at m/z 1916.5, 1741.4, 1651.2, and 1474.9. The first ion corresponds to a species consisting of two DAG, two 14:0(3-OH), two 16:0(3-OH), one 18:0, and two GalA (calculated mass, 1917.6 Da). The ions at m/z 1741.4 and m/z 1651.2 correspond to species lacking one uronic acid (Δm/z 176) or lacking the octadecanoic acid (Δm/z 266) and or lacking both (Δm/z 442), respectively. No masses representing phosphate containing oligosaccharides were detected. Minor fractions with Δm/z 28 originate from species containing one shorter acyl residue. To unequivocally analyze the distribution of the fatty acids, the lipid A was separated using TLC, and the fraction containing pentaacyl lipid A was eluted and analyzed using MALDI-TOF-MS. In Fig. 4A, the negative MALDI-TOF mass spectrum is shown, which reveals that only minor amounts of tetraacylated lipid A are present in this fraction. The positive ion MALDI-TOF mass spectrum (Fig. 4B) reveals two abundant oxonium ions at m/z 1084.8 and 908.9, which are generated after laser-induced cleavage of the nonreducing DAG I (31, 32). They correspond to (DAG II + GalA + 14:0(3-OH) + 16:0(3-OH) + 18:0)− and (DAG II + 14:0(3-OH) + 16:0(3-OH) + 18:0)−, respectively. The loss of GalA in the second product is thought to originate from fragmentation. The presence of the oxonium ion at m/z 1084.8 (possessing the octadecanoic acid) indicates that the 18:0 is located on the nonreducing DAG II. The identified small amounts of an oxonium ion at m/z 818.8 for (DAG II + GalA + 14:0(3-OH) + 16:0(3-OH))− (without the 18:0) originate from the tetraacylated lipid A fraction.

| Component | nmol/mg of LPS |
|-----------|---------------|
| GlcN      | 770           |
| GlcNred   | 0             |
| DAG       | 480           |
| Kdo       | 700           |
| GalA      | 1180          |
| Man       | 1690          |
| Glc       | 870           |
| L,D-Hep   | 630           |

a GlcNred, reducing glucosamine; L,D-Hep, L-glycero-D-manno-heptose.

| Component | nmol/mg of LPS |
|-----------|---------------|
|          | (R)-14:0-18:0 | (R)-13:0-16:0 |
| Total fatty acid content | 280 | 450 | 470 | 1200 |
| Ester-bound fatty acids | 230 | 0 | 0 |
| Amide-bound fatty acids | 50 | 420 | 500 | 1200 |

* Sum of ester- and amide-bound fatty acids.
De-O-acylation of Lipid A and Characterization of the Product—

Lipid A was treated with hydrazine under mild conditions, and the product was analyzed by MALDI-MS, which identified an ion at \(m/z\) 1650.9 that represents a homogeneous compound completely missing the ester-bound fatty acid.

Methylation analysis of de-O-acylated lipid A employed the carboxyl and carbonyl reduction using NaBD₄, converting GalA to galactose. Analysis using GLC-MS revealed only 1,5-di-O-\(\text{acetyl}-2,3,4,6\)-tetra-O-methyl-[1-\(\text{H}_{6},6'-\text{H}_{2}\)]-galactitol proving that both GalA residues are pyranoses and exclusively present as terminal sugars.

NMR Spectroscopy of De-O-acylated Lipid A—De-O-acylated lipid A was well soluble in dimethyl sulfoxide (Me₂SO), thus, it was possible to analyze it in Me₂SO-\(d_{6}\) by NMR spectroscopy. The \(^1\)H and \(^{13}\)C chemical shift assignments (Table III) are based on one-dimensional \(^1\)H and \(^{13}\)C NMR-spectra and two-dimensional double quantum filter correlated spectroscopy (Fig. 5), total correlation spectroscopy (not shown), and heteronuclear multiple quantum coherence experiments (Fig. 6). The chemical shifts assigned for the GalpA residues are comparable to those published in Ref. 28.

In the \(^1\)H NMR spectrum, four signals between 7.4 and 7.9 ppm were attributed to the four NH protons. Four other signals were identified in the anomeric region, of which three were attributed to H-1 of \(\alpha\)-linked hexoses (residues A, B, and D; for labeling see Fig. 8) and one to H-1 of a \(\beta\)-linked (C) hexoses, as characterized by the chemical shifts and \(J_{H-1,H-2}\) coupling constants (A, 4.91 ppm (2.6 Hz); B, 4.80 ppm (3.5 Hz); D, 5.07 ppm (2.2 Hz); C, 4.39 ppm (8.4 Hz)). At 4.34 ppm and 4.10 ppm, the chemical shifts of H-5 of the two uronic acids were identified. The signals of the remaining ring protons are in the region 3.29–4.05 ppm. The chemical shifts of the fatty acids are between 0.6 and 2.3 ppm. The signals of their \(\alpha\)-CH₂ are between 2 and 2.2 ppm, and those of the other CH₂-groups between 1 and 1.5 ppm. The signals of the CH₃ protons are between 0.7 and 0.9 ppm. In agreement with the results of other experiments the substance was found to be composed of four sugars, substituted with fatty acids.

The \(^{13}\)C NMR spectrum was assigned by a heteronuclear multiple quantum coherence experiment (Fig. 6, Table III). Six carboxyl resonances (of the two uronic acids and the four amide-bound 3-hydroxy fatty acids) are present between 170 and 173 ppm (spectrum not shown). In the anomeric region, four signals were identified between 92 and 103 ppm. Of the ring sugar signals in the region 50–78 ppm, those four between 50 and 55 ppm are assigned to the C-2 and C-3 atoms of the two DAG residues. The intensive signals of the fatty acids are in the region 13–33 ppm, with that of the CH₃ groups at 13 ppm. The results confirm the presence of two DAG and two uronic acid residues. The complete assignment of chemical shifts and the determination of vicinal \(^1\)H,\(^1\)H-coupling constants confirmed that both uronic acids are \(4\text{C}_{1}\) \(\alpha\)-\(\text{d-galacturonic acid residues. The coupling constants for GalpA A and GalpA D are } J_{H-1,H-2}\)
Structure of A. pyrophilus Lipid A

TABLE III

| Atom         | Chemical shift (coupling constants) in residue |
|--------------|---------------------------------------------|
| C-1         | 91.74 (92.25) 102.55 99.29                  |
| C-2         | 67.2–68.0 51.63 53.47 68.41                  |
| C-3         | 67.2–68.0 50.77 54.37 69.30                  |
| C-4         | 70.44 67.53 74.00 69.76                     |
| C-5         | 70.93 71.39 77.09 71.41                     |
| C-6         | 170–173 68.40 60.79 170–173                 |

* NH group at C-2.
* NH group at C-3.
* Nonresolved.

The sequence of the monosaccharides was established by nuclear Overhauser effect spectroscopy experiments (Fig. 7). Interresidual Nuclear Overhauser effect contacts were identified between protons C1 and B6a, b. Together with the downfield shift of the carbon B6 (68.4 ppm) it proved the (1–4)-linkage between DAG residues B and C. The (1–1)-linkage between GalA and DAG B was identified by a Nuclear Overhauser effect contact between protons A1 and B1, and the (1–4)-linkage between GalA D and DAG C by a Nuclear Overhauser effect contact between proton D1 and C4, together with the downfield shift of carbon C4 (74.0 ppm). Thus, the monosaccharide sequence D → C → B → A (Fig. 8) is unambiguously proven. A $^{31}$P NMR spectrum gave no phosphate signals, which is in agreement with all other analyses. Taken together, our data establish the structure of the lipid A moiety of LPS from A. pyrophilus as shown in Fig. 8.

**DISCUSSION**

For the first time, LPS was isolated from a hyperthermophilic bacterium, namely from *A. pyrophilus*. The LPS was shown to be of the S-form, as indicated by varying numbers of repeating units in the O-specific polysaccharide resulting in a ladder-like banding pattern in SDS-polyacrylamide gel electrophoresis. The molecular masses of LPS populations were identified by MALDI-MS. The average mass of one repeating unit represented a mixture of compounds. Thus, for the final structure of the lipid A moiety. The isolated lipid A was found to be a homogeneous compound. The complete structure of the carbohydrate backbone was characterized as $\alpha$-D-GalA-(1–4)$\beta$-D-DAG-(1–4)-$\alpha$-D-DAG-(1–4)$\alpha$-D-GalpA. This is the first lipid A backbone that comprises two GalA residues. The complete structure, as revealed by MALDI-MS, is composed of this carbohydrate backbone, which is substituted by four (R)-3-hydroxy fatty acids in amide linkage and possesses a 14:0(3-OH) acyloxyacyl group at the nonreducing end. Each DAG residue carries one 14:0(3-OH) and one 16:0(3-OH); however, it was not possible to determine the exact position (N-2 or N-3) of each fatty acid. Smaller lipid A moieties were also present in the preparation, which lack either 18:0 or one GalA acid residue or both. Because only one lipid A fragmentation peak at m/z 1916 was found in the native LPS (see Fig. 3), it must be concluded that the observed heterogeneity in the free lipid A preparation does not originate from intrinsic biological heterogeneity but from cleavages during the chemical isolation procedure (33).

The DAG disaccharide is not substituted by phosphate but carries two D-GalA residues instead. DAG has been identified in lipid A from *Bordetella pertussis* and *Legionella pneumovirus*. Differences in the fatty acyl substitutions of the DAG disaccharide in lipid A from *A. pyrophilus* and *L. pneumovirus* suggest different functions in the lipooligosaccharide. The DAG disaccharide is not substituted by phosphate but carries two D-GalA residues instead. DAG has been identified in lipid A from *Bordetella pertussis* and *Legionella pneumovirus*. Differences in the fatty acyl substitutions of the DAG disaccharide in lipid A from *A. pyrophilus* and *L. pneumovirus* suggest different functions in the lipooligosaccharide.
Structure of A. pyrophilus Lipid A

Figure 6. Heteronuclear multiple quantum coherence spectrum of de-O-acylated lipid A. The spectrum was recorded at 600 MHz and 47 °C. The letters refer to the carbohydrate residues as shown in Fig. 8, and the arabic numerals refer to the protons in the respective residues.

Figure 7. Nuclear Overhauser effect spectroscopy spectrum of de-O-acylated lipid A. The spectrum was recorded at 600 MHz and 47 °C. The letters refer to the carbohydrate residues as shown in Fig. 8, and the arabic numerals refer to the protons in the respective residues.

In the case of lipid A from B. pertussis and L. pneumophila, both positions O-1 and O-4' are substituted by monophosphate groups. In the few cases of phosphateless lipid A, no DAG has been identified. The structure of lipid A of R. leguminosarum is unique. Its carbohydrate backbone comprises β-D-GlcPN-(1→6)-2-amino-2-deoxy-gluconic acid that is substituted at O-1 and O-4' by D-Gal that is acylated at N-2, O-3, N-2' and O-3' by four 14:0(3-OH) residues (34). This precursor is then processed differently than in E. coli (35–38), forming the lipid A-core region. During that process, the phosphate residues at O-1 and O-4' are removed by 1- and 4'-phosphatases followed in later steps by the transfer of α-9-bisphosphorylated (2→4)-linked Kdo disaccharide at O-6' of the lipid A backbone. It is possible that the gene encoding this transferase possesses no or only some sequence similarity to lpxL and lpxM, because lpxL displays already distant sequence similarities to lpxL (40). This may be the reason why at present no “lpxS” gene has been identified in Aquifex. Despite this, we hypothesize that in LPS biosynthesis of A. pyrophilus the same precursor as in E. coli is first furnished and then processed by specific enzymes to a structure comprising the unique lipid A. Notably, this process includes the replacement of phosphate groups by α-GalP residues at O-1 and O-4' of the lipid A backbone.

Lipid A is considered a valuable chemotaxonomic and phylogenetic marker. From comparative analyses of presently known lipid A structures, the lipid A of A. pyrophilus is unique and, thus, reflects the separated position of this species in the phylogenetic tree, which is based on 16 S rRNA homology studies (2). In agreement with this, amino acid sequence alignments of KdsA (Kdo transferase) and WaaC and WaaF (heptosyl transferases) result in trees possessing Aquifex as a separate branch. Interestingly, Thermotoga maritima (41), the second deepest branching Bacterium according to the 16 S rRNA tree, does not possess the genes for LPS biosynthesis (42).
(GenBank™ accession number AE000512). This finding is confirmed by our own finding\(^2\) that no LPS can be isolated from this species.

Acknowledgments—We thank H.-P. Cordes and H. Lüthje for recording the NMR and MALDI-TOF spectra, respectively, R. Engel and H. Moll for help with GC-MS of fatty acids and sugars, V. Susott for help with the NMR and MALDI-TOF spectra, respectively, R. Engel and H. Moll for help with the NMR data, and W. Brabetz for his interest and for discussion.

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\(^2\) B. M. Ploetz, B. Lindner, K. O. Stetter, and O. Holst, unpublished data.
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J. Biol. Chem. 2000, 275:11222-11228.
doi: 10.1074/jbc.275.15.11222

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