SHORT COMMUNICATIONS

Occurrence of 2,3-Diamino-2,3-dideoxy-D-glucose in Lipid A from Lipopolysaccharide of *Pseudomonas diminuta*

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INTRODUCTION

It is generally considered that the lipopolysaccharides of most Gram-negative bacteria share a common architecture, whereby an O-specific, polymeric side-chain is attached to the hydrophobic lipid A via a core oligosaccharide, of which the inner region is typically rich in ionizing functions (Galanos *et al.*, 1977; Wilkinson, 1977). Whereas the side-chain is highly variable in composition and structure, the lipid A and inner core regions of lipopolysaccharides representing different genera are usually similar. The inner core region is usually distinguished by the presence of an aldohexose (typically L-glycero-D-manno-hexose) and 3-deoxy-D-manno-octulosonic acid (KDO), while lipid A consists of a phosphorylated, 1,6'- β -linked disaccharide of D-glucosamine carrying O- and N-fatty acyl substituents. Deviations from this monosaccharide constitution and differences in fatty acid composition can have taxonomic significance.

Although the phosphorylated disaccharide of glucosamine forms the backbone of lipid A in most lipopolysaccharides that have been studied (Rietschel *et al.*, 1977), additional variations can arise through the attachment of other residues. Elaborations of this sort have been demonstrated for lipid A fractions from *Salmonella* species (Mühlradt *et al.*, 1977), *Chromobacterium violaceum* (Hase & Rietschel, 1977) and *Rhodospirillum tenue* (Tharathan *et al.*, 1978), and seem likely to be widespread. The absence from lipid A of glucosamine represents a more fundamental difference, currently recognized only for *Pseudomonas diminuta* (Wilkinson *et al.*, 1973), *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris* (Roppel *et al.*, 1975), *Thermus* strains (Pask-Hughes & Williams, 1978) and *Caulobacter crescentus* (Agabian & Unger, 1978). In lipid A from the two *Rhodopseudomonas* species, glucosamine is replaced by 2,3-diamino-2,3-dideoxy-D-glucose (Roppel *et al.*, 1975; Keilich *et al.*, 1976). The present study shows that the same diaminoheptose also occurs in lipid A from *P. diminuta* and probably in that from *Pseudomonas vesicularis*.

METHODS

Organisms and growth conditions. *Pseudomonas diminuta* NCTC 8545 was grown in nutrient broth (Oxoid no. 2) at 30 °C for 24 h in a 20 l fermenter with aeration at 20 l min⁻¹. *Pseudomonas vesicularis* NCTC 10900 was grown on nutrient agar (Oxoid) at 30 °C for 48 h. After harvesting and washing, the bacteria from *P. vesicularis* were freeze-dried; those from *P. diminuta* were used to prepare walls (Wilkinson *et al.*, 1973).

Preparation of lipopolysaccharide and lipid A. For *P. diminuta*, both products were obtained from defatted, isolated walls as described previously (Wilkinson *et al.*, 1973). For *P. vesicularis*, the defatted whole cells (7.5 g) were stirred with 45% (w/v) phenol at 68 °C for 15 min. The aqueous phase obtained after cooling and centrifugation was dialysed at 4 °C for 3 d against deionized water, concentrated by rotary evaporation and clarified by centrifugation (108 000 g, 1 h). Freeze-drying of the supernatant solution gave the crude lipopolysaccharide (560 mg).

Release of the diaminoheptose. Samples of lipid A or lipopolysaccharide were hydrolysed under N₂ with 6.1 M-HCl at 105 °C for 4 h. The hydrolysates were dried repeatedly *in vacuo* over P₂O₅ and KOH. The diaminoheptose from *P. diminuta* was isolated from a hydrolysate by adsorption on Dowex 50 resin (H⁺ form), followed by stepwise elution with HCl (0 to 2 M; Roppel *et al.*, 1975).

Derivatives of the diaminohehexose. The amino sugar from *P. diminuta* was converted into the di-*N*-acetyl derivative and into the peracetylated alditol (with and without a deuterium label at C-1) as described by Roppel *et al.* (1975). The corresponding derivatives were also prepared from a reference sample of 2,3-diamino-2,3-dideoxy-D-glucose dihydrochloride (Sefochem-Fine Chemicals, Emek Hayarden, Israel).

Oxidation of the diacetamidohexose. Samples (about 0.5 mg each) of the product from *P. diminuta* and the reference compound with the *gluco* configuration were oxidized with 50 mM-NaIO₄ (0.5 ml) for 18 h at 4 °C then for 24 h at 20 °C. To the reaction mixtures were added 0.05 M-I₂ in 0.1 M-KI (0.5 ml) and 0.1 M-NaOH (0.25 ml). After 10 min at 20 °C, further 0.1 M-NaOH (0.15 ml) was added, and after 35 min the reactions were terminated by the addition of 6.1 M-HCl (one drop). After removal of I₂ by repeated extraction with CHCl₃, the solutions were dried and the residues were hydrolysed with 6.1 M-HCl at 105 °C for 4 h. Neutral amino acids in the products were isolated by preparative paper electrophoresis at pH 5.3 and were subjected to chromatographic comparisons with reference DL-2,3-diaminosuccinic acid (ICN Pharmaceuticals, Cleveland, Ohio, U.S.A.).

Paper chromatography and electrophoresis. Chromatography and high-voltage electrophoresis were carried out with Whatman no. 1 paper. Solvent systems used were: A, ethyl acetate/pyridine/water/acetic acid (5:5:3:1, by vol.); B, propan-1-ol/18 M-ammonia/water (6:3:1, by vol.); C, methanol/water/10 M-HCl/pyridine (32:7:1:4, by vol.). Buffer systems used were: D, pyridine/acetic acid/water (5:2:43, by vol.) pH 5.3; E, pyridine/acetic acid/formic acid/water (2:3:20:180, by vol.) pH 2.8. Separations were carried out for about 1 h at 50 to 70 V cm⁻¹. Detection reagents used were ninhydrin, alkaline silver nitrate (Trevelyan *et al.*, 1950), aniline hydrogen oxalate (Charalampous & Mueller, 1953), the Ehrlich reagent (Partridge, 1948) and the acid/molybdate reagent for phosphates (Hanes & Isherwood, 1949).

Other analytical methods. The Elson-Morgan reaction was carried out as described by Rondle & Morgan (1955). Ion-exchange chromatography was carried out with an amino acid analyser (Locarte, London EC3). Aminoalditol acetates were examined by using a Pye 104 gas chromatograph with packed columns containing the following stationary phases: I, 2% (w/w) OV-17 at 200 °C; II, 3% (w/w) OV-225 at 210 °C. Flow rates (N₂) were 30 ml min⁻¹ in both cases. Combined gas-liquid chromatography/mass spectrometry was carried out with a Pye 104 chromatograph and column I coupled to an LKB spectrometer, model 2091. Optical rotations were determined with a Bendix polarimeter, model 143A. Measurements of circular dichroism were made with a Cary spectropolarimeter, model 61.

RESULTS AND DISCUSSION

Previous studies (Wilkinson *et al.*, 1973) have shown that lipid A from *P. diminuta* contains phosphorus (1.7%), nitrogen (2.9%) and a range of fatty acids, the major ones being 3-hydroxydodecanoic acid, 3-hydroxytetradecanoic acid and tetradecanoic acid. The infra-red spectrum of the lipid revealed a predominance of amide over ester functions. These data, and the subsequent studies of *Rhodopseudomonas* lipids (Roppel *et al.*, 1975), suggested that *P. diminuta* might also contain a diamino sugar. Acid hydrolysates of lipid A were therefore examined for such a sugar by paper electrophoresis. A major cationic product ($m_{\text{Gl:N}}$ 1.2 at pH 5.3, 1.6 at pH 2.8) was detected, which gave an orange-brown colour with ninhydrin, reduced alkaline silver nitrate (but only gave a weak yellow-brown colour on prolonged heating with aniline hydrogen oxalate) and gave a bright yellow colour with the Ehrlich reagent. After isolation, this major product gave a strong Elson-Morgan reaction with A_{max} 520 nm (the reaction products from glucosamine had A_{max} 540 nm). A minor product in the lipid A hydrolysate (almost neutral at pH 5.3) gave the same reactions with ninhydrin and the Ehrlich reagent, but also reacted with the Hanes-Isherwood reagent, indicating that it was a phosphate ester of the cationic amino product. Further studies showed that the electrophoretic properties and reactions of the latter were identical with those of reference 2,3-diamino-2,3-dideoxy-D-glucose. Identical behaviour was also observed on paper chromatography of the respective *N*-acetyl derivatives with solvent system A, and on gas-liquid chromatography of the peracetylated aminoalditols with columns I and II. The use of column I permits the differentiation of 2,3-diamino-2,3-dideoxyglucose from the isomers with the *allo* and *galacto* configurations (Roppel *et al.*, 1975).

The mass spectra of the aminoalditol acetates from the *P. diminuta* compound and 2,3-diamino-2,3-dideoxy-D-glucose were also identical and in accord with the published spectrum for the reference compound (Roppel *et al.*, 1975). The primary fragment at m/e 215

(and secondary fragments derived from it), which are diagnostic for the derivative of a 2,3-diamino-2,3-dideoxyhexose, showed the expected shift of one mass unit in the derivative carrying a deuterium label at C-1. The *threo* disposition of the amino groups was demonstrated by oxidative degradation of the di-*N*-acetyl derivatives of the amino sugars and chromatographic comparisons with DL-2,3-diaminosuccinic acid. The properties of the reaction products were identical and closely similar to those of the reference compound (which apparently contained minor impurities). On paper chromatography with solvent system C, two elongated spots with low mobilities and giving a blue colour with ninhydrin were observed. With solvent system B, two round purple spots linked by a yellow-brown streak were obtained. On autoanalysis with continuous elution in standard citrate buffer pH 3.25 (Moore *et al.*, 1958), a major peak appeared after 58 min and minor peaks after 44 min and 72 min. The reason for the complex chromatographic behaviour of 2,3-diaminosuccinic acid is not clear.

Polarimetric measurements were made to decide the D/L configuration of the 2,3-diamino-2,3-dideoxyglucose from *P. diminuta* and to confirm its identity. In aqueous solution, the di-*N*-acetyl derivative was clearly laevorotatory as expected for the D-isomer, for which $[\alpha]_D$ at equilibrium is -46° (Baer & Neilson, 1967). However, the amount of material available was insufficient for the specific rotation to be determined. The circular dichroism spectrum of the solution showed the expected bands: an intense, negative band with $\lambda_{\max}$ 196 nm, and a weak, positive band with $\lambda_{\max}$ 222 nm. These data were identical with those for the reference compound and closely similar to literature values (Keilich *et al.*, 1976).

Pseudomonas diminuta and *P. vesicularis* are recognized as closely related species (Ballard *et al.*, 1968), and it was therefore of interest to look for 2,3-diamino-2,3-dideoxyglucose in a lipopolysaccharide from *P. vesicularis*. A brief examination of the crude extract from strain NCTC 10900 revealed the presence of a major component with the expected chromatographic and electrophoretic properties, but little or no glucosamine.

Although *P. diminuta* and *P. vesicularis* are still encompassed by a broad definition of the genus *Pseudomonas*, the most appropriate location for these organisms is uncertain. They possess several distinctive morphological and nutritional properties (Ballard *et al.*, 1968; Palleroni & Doudoroff, 1972) and can be set apart from other subgroups within the genus on the basis of ribosomal RNA homologies (Palleroni *et al.*, 1973) and by the absence of the host factor for coliphage Q β RNA replication (DuBow & Ryan, 1977). Studies of the cellular fatty acids of *P. diminuta* and *P. vesicularis* (Kaltenbach *et al.*, 1975) confirm the close relationship between the species, while the EDTA-resistance of *P. diminuta* (Wilkinson, 1967) and its unique range of glycolipids (Wilkinson, 1969; Wilkinson & Bell, 1971) further distinguish the organism from the mainstream pseudomonads. The results of the present study underline these points and unexpectedly suggest a phylogenetic link between the two species and *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris*.

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REFERENCES

- AGABIAN, N. & UNGER, B. (1978). *Caulobacter crescentus* cell envelope: effect of growth conditions on murein and outer membrane protein composition. *Journal of Bacteriology* **133**, 987–994.
- BAER, H. H. & NEILSON, T. (1967). A new synthesis of 2,3-diamino-2,3-dideoxy-D-glucose. *Journal of Organic Chemistry* **32**, 1068–1072.
- BALLARD, R. W., DOUDOROFF, M., STANIER, R. Y. & MANDEL, M. (1968). Taxonomy of the aerobic pseudomonads: *Pseudomonas diminuta* and *P. vesiculare*. *Journal of General Microbiology* **53**, 349–361.
- CHARALAMPOUS, F. C. & MUELLER, G. C. (1953). Synthesis of erythrulose phosphate by a soluble enzyme from rat liver. *Journal of Biological Chemistry* **201**, 161–173.
- DUBOW, M. S. & RYAN, T. (1977). Host factor for coliphage Q β RNA replication as an aid in elucidating phylogenetic relationships: the genus *Pseudomonas*. *Journal of General Microbiology* **102**, 263–268.
- GALANOS, C., LÜDERITZ, O., RIETSCHEL, E. T. & WESTPHAL, O. (1977). Newer aspects of the chemistry and biology of bacterial lipopolysaccharides, with special reference to their lipid A component. In *International Review of Biochemistry. Biochemistry of Lipids II*, vol. 14, pp. 239–335. Edited by T. W. Goodwin. Baltimore: University Park Press.
- HANES, C. S. & ISHERWOOD, F. A. (1949). Separation of the phosphoric esters on the filter paper chromatogram. *Nature, London* **164**, 1107–1112.
- HASE, S. & RIETSCHEL, E. T. (1977). The chemical structure of the lipid A component of lipopolysaccharides from *Chromobacterium violaceum* NCTC 9694. *European Journal of Biochemistry* **75**, 23–34.
- KALTENBACH, C. M., MOSS, C. W. & WEAVER, R. E. (1975). Cultural and biochemical characteristics and fatty acid composition of *Pseudomonas diminuta* and *Pseudomonas vesiculare*. *Journal of Clinical Microbiology* **1**, 339–344.
- KEILICH, G., ROPPEL, J. & MAYER, H. (1976). Characterization of a diaminoheptose (2,3-diamino-2,3-dideoxy-D-glucose) from *Rhodopseudomonas viridis* lipopolysaccharides by circular dichroism. *Carbohydrate Research* **51**, 129–134.
- MOORE, S., SPACKMAN, D. H. & STEIN, W. H. (1958). Chromatography of amino acids on sulfonated polystyrene resins. An improved system. *Analytical Chemistry* **30**, 1185–1190.
- MÜHLRADT, P. F., WRAY, V. & LEHMANN, V. (1977). A ³¹P-nuclear-magnetic-resonance study of the phosphate groups in lipopolysaccharide and lipid A from *Salmonella*. *European Journal of Biochemistry* **81**, 193–203.
- PALLERONI, N. J. & DOUDOROFF, M. (1972). Some properties and taxonomic subdivisions of the genus *Pseudomonas*. *Annual Review of Phytopathology* **10**, 73–100.
- PALLERONI, N. J., KUNISAWA, R., CONTOPOULOU, R. & DOUDOROFF, M. (1973). Nucleic acid homologies in the genus *Pseudomonas*. *International Journal of Systematic Bacteriology* **23**, 333–339.
- PASK-HUGHES, R. A. & WILLIAMS, R. A. D. (1978). Cell envelope components of strains belonging to the genus *Thermus*. *Journal of General Microbiology* **107**, 65–72.
- PARTRIDGE, S. M. (1948). Filter paper partition chromatography of sugars. *Biochemical Journal* **42**, 238–250.
- RIETSCHEL, E. T., HASE, S., KING, M.-T., REDMOND, J. & LEHMANN, V. (1977). Chemical structure of lipid A. In *Microbiology-1977*, pp. 262–268. Edited by D. Schlessinger. Washington: American Society for Microbiology.
- RONDLE, C. J. M. & MORGAN, W. T. J. (1955). The determination of glucosamine and galactosamine. *Biochemical Journal* **61**, 586–589.
- ROPPEL, J., MAYER, H. & WECKESSER, J. (1975). Identification of a 2,3-diamino-2,3-dideoxyhexose in the lipid A component of lipopolysaccharides of *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris*. *Carbohydrate Research* **40**, 31–40.
- THARANATHAN, R. N., WECKESSER, J. & MAYER, H. (1978). Structural studies on the D-arabinose-containing lipid A from *Rhodospirillum tenue*. *European Journal of Biochemistry* **84**, 385–394.
- TREVELYAN, W. E., PROCTER, D. P. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, London* **166**, 444–445.
- WILKINSON, S. G. (1967). The sensitivity of pseudomonads to ethylenediaminetetra-acetic acid. *Journal of General Microbiology* **47**, 67–76.
- WILKINSON, S. G. (1969). Lipids of *Pseudomonas diminuta*. *Biochimica et biophysica acta* **187**, 492–500.
- WILKINSON, S. G. (1977). Composition and structure of bacterial lipopolysaccharides. In *Surface Carbohydrates of the Prokaryotic Cell*, pp. 97–175. Edited by I. W. Sutherland. London: Academic Press.
- WILKINSON, S. G. & BELL, M. E. (1971). The phosphoglucolipid from *Pseudomonas diminuta*. *Biochimica et biophysica acta* **248**, 293–299.
- WILKINSON, S. G., GALBRAITH, L. & LIGHTFOOT, G. A. (1973). Cell walls, lipids, and lipopolysaccharides of *Pseudomonas* species. *European Journal of Biochemistry* **33**, 158–174.