Biosynthesis of the Core Part of the Lipopolysaccharide of *Pseudomonas aeruginosa*

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The incorporation of rhamnose and glucose into the core part of the lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* was studied using enzyme preparations from strain PAC1R and LPS-defective mutants derived from it. Crude membrane preparations from the LPS-defective mutant PAC556 transferred rhamnose from dTDP-L-[¹⁴C]rhamnose to material insoluble in trichloroacetic acid. The preparations contained both transferase enzyme and acceptor, the former being destroyed by heating. Between 60 and 70% of the radioactive rhamnose transferred to the membranes was extractable by aqueous phenol and non-diffusible. The material extracted did not move in any of the chromatography solvents tested and contained rhamnose as the sole radioactive component. Soluble dTDP-L-rhamnose-LPS rhamnosyltransferase was obtained from the parent strain PAC1R by ammonium sulphate precipitation of a 105000 g supernatant fraction from broken bacteria. It was most active at pH 8 with 5 mM-MgCl₂ and required heat-treated membranes of PAC556 as acceptor. This mutant, whose LPS lacks both O-antigenic side-chains and rhamnose in the core, was shown to lack either the epimerase or the NADP-dependent oxidoreductase used to synthesize dTDP-rhamnose. After preincubation with soluble transferase and UDPglucose, heated membranes of mutant strains PAC611, PAC612 and PAC605 could also act as acceptors for rhamnose. These mutants all lacked some or all of the glucose as well as the rhamnose from the core of their LPS and the experiments thus provided confirmation that rhamnose was the terminal hexose of the core in *P. aeruginosa* PAC1.

INTRODUCTION

The series of reactions leading to the biosynthesis of the core region of the lipopolysaccharide (LPS) have been well studied in the *Enterobacteriaceae*, particularly in *Salmonella typhimurium*. Transferase enzymes act sequentially to transfer the required sugar from its nucleotide derivative to the growing LPS core in the inner membrane (for review, see Osborn & Rothfield, 1971). Progress in these studies depended initially on the isolation of mutants unable to complete the core structure and which could provide sources of incomplete core to which the sugars were transferred. Until recently, the lack of LPS-defective mutants in *Pseudomonas aeruginosa* prevented similar studies in this species. Mutants of *P. aeruginosa* PAC1R which lack the O-antigenic side-chains and parts of the core have now been isolated (Koval & Meadow, 1977; Meadow & Wells, 1978) and we have used preparations from them to study core biosynthesis in *P. aeruginosa*. It was hoped that the experiments would also provide some evidence for the proposed sequence of sugars and mutant lesions in the LPS core of this organism. Evidence for the sequence, based on the partial structure proposed for *P. aeruginosa* NCTC 10999 (Drewry *et al.*, 1975), comes mainly from analysis of polysaccharide fractions from defective mutants (Koval & Meadow, 1977) and from the pyocin-sensitivity patterns of these mutants (Meadow & Wells, 1978).

Complete chemical evidence for this structure is not yet available. A preliminary account of some of this work has been published (Asonganyi & Meadow, 1978).

METHODS

Pseudomonas aeruginosa strains. The parent strains PAC1 (NCIB 10848) and PAC1R and the LPS-defective mutants PAC556, PAC605, PAC610 and PAC611 have been described previously (Koval & Meadow, 1977; Meadow *et al.*, 1978; Meadow & Wells, 1978). Strain PAC612 was isolated by D. A. and R. A. Rodin from PAC609 (Meadow & Wells, 1978) as a mutant resistant to the pyocin from *P. aeruginosa* IS4. Analysis of its partially degraded LPS and examination of its response to the R-type pyocins by the methods previously described (Meadow & Wells, 1978) suggested that its LPS contained no O-antigenic side-chains and lacked rhamnose and all but one glucose moiety in the LPS core. It thus resembled PAC611 in LPS structure.

All cultures were maintained on slopes of nutrient agar (Oxoid no. 2) at room temperature after overnight growth at 37 °C.

Growth conditions and preparation of bacterial fractions. All cultures were grown in 1 l minimal medium with 0.5% (w/v) trisodium citrate. 2H₂O as carbon source (Clarkson & Meadow, 1971) in 5 l conical flasks at 37 °C with shaking. They were harvested in mid-exponential phase (A_{670} 1.0) by centrifugation (10000 g, 10 min), washed with 50 mM-Tris/HCl, 7 mM-2-mercaptoethanol, pH 8 (TM buffer) and stored as a frozen pellet until required. To prepare fractions, bacteria from 1 l culture were thawed and resuspended in 20 ml TM buffer. They were broken at 83 MPa in a chilled French press and unbroken bacteria were removed by centrifugation at 5000 g for 30 min at 4 °C. A portion (1 ml) of the supernatant fluid (extract) was stored. The remainder was centrifuged again at 27 000 g for 20 min. The pellet so formed was resuspended in 2 ml TM buffer and used as the wall fraction. After removing 1 ml of the supernatant fluid (supernatant I), the rest was centrifuged at 105 000 g for 2 h, yielding supernatant II and a pellet. The latter was resuspended in 2 ml TM buffer and used as the membrane fraction. The protein from supernatant II was concentrated by adding ammonium sulphate to 80% saturation at 4 °C. After stirring for 20 min the resulting precipitate was collected by centrifugation at 10000 g for 15 min, dissolved in 2 ml TM buffer and dialysed overnight against 4 l of the same buffer. It was designated soluble transferase. The protein content of the fractions was determined by the Lowry method using bovine serum albumin as standard. 3-Deoxy-D-manno-octulosonic acid (KDO) was determined by the method of Ellwood (1970).

Enzyme assays. UDPglucose pyrophosphorylase (EC 2.7.7.9) and dTDPglucose pyrophosphorylase (EC 2.7.7.24) were measured in the supernatant fluid (100 000 g, 1 h) from extracts by the method of Pazur & Shuey (1961). dTDP-D-glucose 4,6-dehydratase (EC 4.2.1.46) was measured in similar extracts except that the bacteria had been resuspended in 50 mM-Tris/HCl, 10 mM-MgCl₂, 1 mM-EDTA, pH 8. When incubated in 0.1 M-NaOH for 20 min, dTDP-4-keto-6-deoxy sugars develop a secondary absorption maximum at 318 nm (Okazaki *et al.*, 1962) and the appearance of this absorption forms the basis of the assay used (Glaser & Kornfeld, 1966). In the presence of dTDP-4-ketorhamnose 3,5-epimerase (EC 5.1.3.13), the product of the dehydratase is converted to the rhamnose epimer which has the same absorption and hence would not be determined separately. The presence of the epimerase could be detected since it is one of the enzymes necessary for the NADP-dependent conversion of dTDPglucose to dTDP-rhamnose which was measured by the method of Glaser & Kornfeld (1961).

The activity of dTDP-L-rhamnose-LPS rhamnosyltransferase in bacterial fractions was determined in duplicate in tubes (10 × 750 mm). The assay mixture normally contained (in 200 to 230 µl) 50 mM-Tris/HCl, pH 8, 10 mM-MgCl₂, dTDP-L-[¹⁴C]rhamnose [20 µmol, sp.act. 0.5 µCi µmol⁻¹ (18.5 kBq µmol⁻¹)] and 200 µg bacterial protein. After incubation at 37 °C for 60 min, the reaction was stopped by adding 3 ml ice-cold 5% (w/v) trichloroacetic acid (TCA) and cooling at 0 °C for 10 min. The precipitate was collected on a Millipore filter (0.54 µm pore size) and washed thoroughly with ice-cold distilled water. The filter was then transferred to a scintillation vial for counting. In some experiments, material containing more than 200 µg protein was used and this prevented good separation by filtration. Such assay mixtures were centrifuged at 3000 g on a bench centrifuge and the resulting pellets were washed once with ice-cold distilled water. The pellets were finally washed into scintillation vials with 10 ml scintillation fluid. The two methods yielded similar results.

To assay the soluble transferase, heated membranes were required as acceptor. Each assay normally included 500 µg soluble transferase protein and 500 µg heated membrane protein in a total volume of 230 µl. Incubation was at room temperature for 60 min. All other conditions were as above.

In some experiments, membranes were preincubated with UDPglucose before assaying for dTDP-rhamnose transferase activity. Heated membranes (500 µg protein) were mixed with 20 nmol UDPglucose and soluble transferase (500 µg protein) in 10 mM-MgCl₂, 50 mM-Tris/HCl, pH 8, in a total volume of 230 µl. After incubation at 37 °C for 30 min, 3 ml ice cold 50 mM-Tris/HCl, pH 8, was added and the tubes were centri-

fused (3000 g, 10 min). The pellets were resuspended in 100 μ l of the same buffer, and dTDP-L-[14 C]-rhamnose (sp.act. 0.5 μ Ci μ mol $^{-1}$, 20 μ mol in 20 μ l water), MgCl $_2$ (100 mM in 25 μ l of the same buffer) and soluble transferase (100 μ l, 500 μ g protein) were added. Incubation and further treatment was as usual.

Radioactive substrates. [U- 14 C]Glucose 1-phosphate [sp.act. 150 μ Ci μ mol $^{-1}$ (5.5 MBq μ mol $^{-1}$)] was obtained from The Radiochemical Centre, Amersham. dTDP-L-[14 C]rhamnose was prepared enzymically from it in two stages by the methods of Kornfeld & Glaser (1961) and Glaser & Kornfeld (1966). The overall yield was about 15%. Radioactivity in samples was measured in 10 ml scintillation fluid [BBOT scintillator (Ciba, Duxford, Cambs.), 4 g; naphthalene, 80 g; 2-methoxyethanol, 400 ml; toluene, 600 ml] in a Beckman liquid scintillation counter LS22.

Characterization of TCA-insoluble product. Carrier membrane fraction (containing 1 mg protein) was added to the tube in which incubation had been completed before stopping the reaction with TCA. The precipitate was washed twice with 4 ml distilled water at 4 °C and then washed into a Universal container with another 5 ml water. A sample (0.1 ml) was removed for counting and the residue was neutralized with 10 mM-NaOH. It was warmed to 68 °C before adding 5 ml aqueous phenol (90%, w/v) also warmed to 68 °C. After 15 min incubation at this temperature with vigorous intermittent shaking, the suspension was cooled in ice and centrifuged at 3000 g for 10 min to separate the two layers. The upper aqueous layer was removed with a Pasteur pipette and the phenol layer was re-extracted with an equal volume of water. The aqueous layers were combined and, after removing a sample for counting, were dialysed overnight against distilled water at 4 °C. The radioactivity in a sample of the dialysed material was measured and the residue was applied to silica gel thin-layer plates and to Whatman 3MM chromatography paper. The plates and paper were developed with solvents used by previous workers to chromatograph LPS. They were, for the plates, isobutyric acid/NH $_4$ OH (sp.gr. 0.880)/water (59:4:39, by vol.) (Krag, 1978), chloroform/methanol/water (130:45:7, by vol.) and propan-2-ol/1 M-NH $_4$ OH (60:40, v/v) (Buttke & Ingram, 1975) and, for the paper, isobutyric acid/1 M-NH $_4$ OH (7:3, v/v) (Boman & Monner, 1975).

A portion (containing about 10 5 c.p.m.) of acid-precipitated product was washed twice with 4 ml distilled water and hydrolysed with 1 M-HCl (100 °C, 1 h). The resulting solution was evaporated to dryness, resuspended in 10 ml water and evaporated to dryness again. The residue was dissolved in 50 μ l water and applied to Whatman 3MM chromatography paper with rhamnose and glucose as markers. It was subjected to descending chromatography overnight using butan-1-ol/pyridine/water (6:4:3, by vol.) as solvent. When dry, the paper was cut into strips 20 mm wide and counted in scintillation fluid to detect radioactive components.

RESULTS

dTDP-L-rhamnose-LPS rhamnosyltransferase activity in fractions from P. aeruginosa

Mutant PAC556 has an incomplete LPS core which lacks rhamnose (Koval & Meadow, 1977). It was therefore fractionated as described in Methods and each fraction was tested for the ability to incorporate rhamnose from dTDP-L-[14 C]rhamnose into TCA-insoluble material. Some activity was detected in all fractions except supernatant II but the most active was the membrane fraction (Table 1). Membrane fractions from the parent strain PAC1R and from two other LPS-defective mutants were inactive, as were heat-treated membranes from PAC556 (Table 1). Membrane fractions from PAC556 thus appeared to be providing both transferase enzyme and an LPS acceptor which had a vacant rhamnose site. Membranes from PAC556 were therefore used in preliminary experiments to establish some of the properties of the transferase. Maximum activity was obtained at pH 8, virtually none being detected below pH 5 or above pH 10. Transferase activity was markedly stimulated by Mg $^{2+}$ with the highest activity at about 5 mM. Added Mn $^{2+}$ slightly stimulated activity but was inhibitory above 5 mM. Transferase activity was unchanged between 20 and 32 °C but decreased linearly and rapidly above 37 °C, falling to about 50% at 40 °C. The amount of rhamnose incorporated increased linearly with time, stopping after about 90 min incubation at 37 °C. It was directly proportional to the amount of membrane protein up to 4 mg ml $^{-1}$. Using 500 μ g membrane protein, 5 mM-MgCl $_2$ and 60 min incubation at 30 °C, the apparent K_m for dTDP-rhamnose was 50 μ M.

Heated membrane fractions from PAC556 were used as acceptors to test other bacterial fractions for transferase activity. Strain PAC1R was used to provide transferase since any membrane fragments present had been shown to lack acceptor activity (see Table 1).

Fig. 1. Incorporation of rhamnose from dTDP-L-[¹⁴C]rhamnose into TCA-insoluble material by soluble transferase from PAC1R with heated membranes from PAC556 as acceptor. (a) Effect of increasing amounts of soluble transferase using membranes containing 1 nmol KDO ml⁻¹. (b) Effect of increasing amounts of membranes using soluble transferase at 1.5 mg protein ml⁻¹.

Table 1. *dTDP-L-rhamnose transferase activity in various bacterial fractions*

Fractions were prepared and tested for their ability to transfer radioactivity from dTDP-L-[¹⁴C]-rhamnose into TCA-insoluble material as described in Methods. Each tube contained dTDP-L-[¹⁴C]rhamnose (20 μ mol, 0.5 μ Ci μ mol⁻¹), MgCl₂ (10 mM), Tris/HCl buffer (50 mM, pH 8.0) in 200 μ l. The reaction was started by adding the fraction to be tested (200 μ g protein, except for supernatant II which contained 100 μ g protein). The tubes were incubated for 1 h at 37 °C. dTDP-rhamnose transferase activities are expressed as nmol rhamnose incorporated (mg protein)⁻¹ h⁻¹.

Fraction	Bacterial source	Transferase activity
Extract	PAC556	3.40
Walls	PAC556	4.19
Supernatant I	PAC556	2.88
Supernatant II	PAC556	1.00
Membranes	PAC556	8.46
Membranes (heated 4 min at 100 °C)	PAC556	0.50
Membranes	PAC1R	0.30
Membranes	PAC605	0.50
Membranes	PAC610	0.45

Soluble transferase was isolated from supernatant II from PAC1R by ammonium sulphate precipitation as described in Methods. It was tested for dTDP-L-rhamnose transferase activity under the standard conditions described in Methods with heated membrane preparations from PAC1R, PAC605, PAC610, PAC612 as well as PAC556 as acceptors. Only when incubated with heated membranes from PAC556 was significant activity detected (see Table 3).

Properties of soluble dTDP-L-rhamnose transferase from PAC1R

In the presence of heated membranes from PAC556, the soluble transferase from PAC1R catalysed the incorporation of radioactivity from dTDP-L-[¹⁴C]rhamnose into TCA-insoluble material. The amount of incorporation depended both on the amount of transferase (Fig. 1a) and the amount of acceptor (Fig. 1b) present. Extraction of the heated membranes with chloroform/methanol (2:1, v/v) removed their acceptor activity, but attempts to reactivate them by adding back the extract under various conditions were unsuccessful.

The radioactivity incorporated into TCA-insoluble material was released by hydrolysis with 1 M-HCl as described in Methods and, after paper chromatography of the hydrolysate,

Table 2. Fractionation of radioactivity incorporated in TCA-insoluble material using heated membranes from PAC556, soluble transferase from PAC1R and dTDP-L-[¹⁴C]rhamnose

The conditions for transferase activity and the methods of fractionating the product are described in Methods.

Fraction no.	Treatment	Radioactivity detected (d.p.s.)	Percentage of total radioactivity
1.	TCA precipitate	11 870	100
2.	Extracted into aqueous layer after phenol treatment	8 300	71
3.	Remaining in pellet after phenol extraction	1 000	9
4.	Present after dialysis of fraction 2	7 290	62

Table 3. Effect of preincubation with UDPglucose on ability of heated membranes to act as acceptors for the dTDP_rhamnose transferase

The heated membranes were preincubated with UDPglucose and crude transferase from strain PAC1R and washed before assaying for dTDP_rhamnose transferase activity all as described in Methods. The transferase activities are expressed as nmol rhamnose incorporated (mg protein)⁻¹ h⁻¹.

Source of heated membranes	Core sugars*	Transferase activity	
		UDPglucose in preincubation	
		None	20 nmol
PAC1R	Rha ₂ Glc ₅ GalN	0.27	0.22
PAC556	Glc ₅ GalN	2.92	2.70
PAC610	Rha ₂ Glc ₄ GalN	0.29	0.22
PAC611	Glc GalN	0.22	3.08
PAC612	Glc GalN	0.31	2.92
PAC605	GalN	0.22	1.12

* Rha, Rhamnose; Glc, glucose; GalN, galactosamine.

was found in a single radioactive component with the same mobility as rhamnose. Phenol extraction of the TCA-insoluble radioactive product released 70% of the radioactivity into the aqueous layer. The material so extracted was mainly non-diffusible (Table 2) and did not move in any of the chromatography solvents listed in Methods.

The core LPS of *P. aeruginosa* PAC1R is thought to contain rhamnose as the terminal hexose with a branched glucose pentasaccharide linking it through galactosamine, heptose and KDO to the lipid A part of the molecule (Meadow & Wells, 1978). It seemed possible that some indication of the sequence of sugars and the lesions in the LPS-defective mutants might come from the effects of preincubation with UDPglucose on rhamnose incorporation using different heated membranes as acceptors. After preincubation with UDPglucose as described in Methods, heated membranes from PAC611 and PAC612 were as active as those from PAC556 while membranes from PAC605 showed some activity (Table 3). The results thus support the view that in some mutants it is necessary to transfer glucose to incomplete core in order to generate suitable rhamnose acceptor sites.

Synthesis of UDPglucose and dTDP_rhamnose by P. aeruginosa and its LPS-defective mutants

In order to synthesize LPS, the bacteria require both the specific transferase and the relevant nucleotide sugar. The lack of glucose or rhamnose in the LPS of a mutant could thus result from its inability to make the nucleotide sugar derivative. The enzymes required to synthesize UDPglucose and dTDP_rhamnose were therefore assayed in extracts of PAC1R and some of its LPS-defective mutants as described in Methods. There was no significant difference between any of the mutants and the parent strain in UDPglucose pyrophosphorylase, dTDPglucose pyrophosphorylase or in dTDPglucose 4,6-dehydratase

Table 4. *Activities of enzymes involved in the synthesis of UDPglucose and dTDP-rhamnose by strain PAC1R and its LPS-defective mutants*

Enzyme activities were assayed in extracts prepared and used as described in Methods. Pyrophosphorylase activities were measured as rates of production of nucleotide sugars and dehydratase activity as the rate of production of dTDP-4-keto-6-deoxy sugar; these activities are all expressed as $\mu\text{mol (mg protein)}^{-1} \text{ h}^{-1}$. dTDP-L-rhamnose synthase activity was measured by following the disappearance of NADPH in the presence of dTDPglucose and NAD (Glaser & Kornfeld, 1961) and so it requires the activities of dTDP-4-ketorhamnose 3,5-epimerase and dTDP-4-ketorhamnose reductase (EC 1.1.1.133) as well as the dehydratase; this activity is expressed as $\mu\text{mol NADPH oxidized (mg protein)}^{-1} \text{ min}^{-1}$.

Strain	UDPglucose pyrophosphorylase	dTDPglucose pyrophosphorylase	dTDPglucose 4,6-dehydratase	dTDP-L-rhamnose synthase
PAC1R	0.63	0.29	0.73	1.0
PAC556	0.80	0.27	0.66	0.08
PAC611	0.68	0.38	0.64	1.3
PAC605	0.68	0.44	0.80	0.9

activity (Table 4). However, the dTDP-L-rhamnose synthase activity of PAC556 was very much lower than that found in the parent strain and other mutants tested (Table 4). Therefore, the lack of rhamnose in this mutant probably arises from its inability to synthesize dTDP-L-rhamnose through defects in either the dTDP-4-ketorhamnose 3,5-epimerase or the NADP-dependent dTDP-4-ketorhamnose reductase. The methods used did not allow us to distinguish between them.

DISCUSSION

The rhamnose transferase system of *Pseudomonas aeruginosa* PAC1R described here appears very similar to the transferase involved in the biosynthesis of the core part of the LPS of *Salmonella typhimurium* (Osborn & Rothfield, 1971). The transferase was associated with the membrane fraction but was readily separated from it. The soluble transferase had an alkaline pH optimum and required for activity heated membrane fractions with suitable acceptor sites, the relevant nucleotide sugar derivative and divalent metal ions. It is possible that the *P. aeruginosa* system, like that of *S. typhimurium* (Rothfield & Takeshita, 1966), requires phospholipids for activity since their extraction from heated membrane fractions eliminated acceptor activity. However, proof of this would require reactivation of phospholipid-depleted membranes and so far our attempts to achieve this have not been successful.

The only native membranes active as acceptors for rhamnose were those derived from PAC556 in which the LPS core appears to lack only rhamnose. We have now shown that this defect results from its inability to synthesize dTDP-rhamnose. Soluble dTDP-L-rhamnose-LPS rhamnosyl transferase activity was detected in all the mutants tested including PAC556 (results not shown). Although the radioactive product has not been completely identified its properties are consistent with the assumption that it contained LPS core with rhamnose as the sole radioactive component.

The effect of preincubation with UDPglucose on the ability of membranes to accept rhamnose from dTDP-rhamnose provides useful confirmation for the proposed sequence of sugars in the LPS core. The structure of this core has still not been completely elucidated (see Wilkinson, 1977) but in *P. aeruginosa* NCTC 1999, a partial structure containing the sequence rhamnose-glucose-rest-of-core has been obtained (Drewry *et al.*, 1975). Analysis of fractions from LPS-defective mutants of PAC1 (Koval & Meadow, 1977) and their pyocin-sensitivity patterns (Meadow & Wells, 1978) are consistent with the same sequence. Membranes from three of the LPS-defective mutants - PAC611, PAC612 and PAC605 - were able to accept rhamnose only after preincubation with crude transferase and UDPglucose, suggesting that the terminal rhamnose of the core is added to a glucose moiety.

After such preincubation, membranes from PAC611 and PAC612 were as active as untreated membranes from PAC556 suggesting that the soluble transferase preparation contained all the enzymes required to incorporate the glucose residues into the core. It is perhaps surprising that, after preincubation with transferase and UDPglucose, membranes from PAC605 whose LPS is thought to differ from that of PAC611 and PAC612 by a single glucose unit (see Table 3) had only one-third the acceptor activity of PAC556 membranes. However, the amounts of membrane fraction added were estimated in terms of protein content only and may not be comparable in terms of available core units.

These experiments have shown that the core of *P. aeruginosa* LPS is synthesized by the sequential addition of glucose followed by rhamnose, both from their nucleotide derivatives, and that the defect in the LPS core of PAC556 derives from its inability to synthesize dTDP-L-rhamnose. Until we have some means of distinguishing between the different transferases presumably involved in adding the five glucose units to the core, it will be difficult to identify the defects in the other mutants.

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