Eur. J. Biochem. 76, 41–49 (1977)

# Isolation of Mutants Conditionally Blocked in the Biosynthesis of the 3-Deoxy-D-*manno*-octulosonic-acid–Lipid-A Part of Lipopolysaccharides Derived from *Salmonella typhimurium*

Volker LEHMANN, Eckhard RUPPRECHT, and Mary Jane OSBORN

Max-Planck-Institut für Immunbiologie, Freiburg, and Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut

(Received February 11, 1977)

A procedure is described for the selection of conditional 3-deoxy-D-*manno*-octulosonic-acid–Lipid A mutants which depends on temperature sensitivity for both synthesis of complete lipopolysaccharide and for growth. Using this procedure new types of mutants were isolated which cease growth and accumulate lipid A precursors following a shift to nonpermissive temperatures. All precursor molecules differ in their charge as judged by DEAE-cellulose chromatography. While they all contain glucosamine, phosphate and 3-hydroxymyristic acid, they lack detectable 3-deoxy-D-*manno*-octulosonic acid (dOclA) as well as the nonhydroxylated fatty acids of the complete lipid A structure.

Three mutants proved to be conditionally defective in dOclA metabolism, whereas one seems to be blocked at a relatively early step in lipid A synthesis. The phenotypes of all these mutants appear to be due to single mutations by reversion analysis and by characterization of the temperature-resistant revertants.

Studies of these mutants may shed light on the essential role of the complete dOclA–lipid A part of lipopolysaccharides in membrane function.

The lipopolysaccharides of *Salmonella* and related gram-negative bacteria are major components of the outer membrane [1]. They are made up of a complex polysaccharide which is linked through a 3-deoxy-D-*manno*-octulosonic acid (dOclA) trisaccharide to a unique lipid, designated lipid A (Fig. 1). Mutants with lesions in various steps of the polysaccharide synthesis, including the transfer of the first heptose residue, are easily obtained by conventional phage selection procedure [2]. They are all viable and display normal growth and physiology. The failure to detect mutants in the dOclA–lipid A region using the usual phage selection technique led to the assumption that the complete dOclA–lipid A region is indispensible for membrane functioning.

The successful isolation of a *Salmonella* mutant dependent on D-arabinose 5-phosphate for growth and synthesis of 3-deoxy-D-*manno*-octulosonic acid [3] clearly demonstrated the essential nature of this part of lipopolysaccharides. Since it did not seem feasible to use a similar supplementation procedure to obtain mutants in the lipid A part, a new and more general strategy was developed.

*Abbreviations.* dOclA, 3-deoxy-D-*manno*-octulosonic acid; dOclA-8-*P*, 3-deoxy-D-*manno*-octulosonate 8-phosphate.

In this paper we report a selection procedure of mutants which are conditionally defective (temperature-sensitive) in the dOclA–lipid A part and which accumulate incomplete lipid A molecules at restrictive temperatures.

## MATERIALS AND METHODS

### *Bacteria and Bacteriophages*

*Salmonella typhimurium* SL761 was supplied by Dr M. J. Osborn (University of Connecticut Health Center, Farmington, U.S.A.) and has the following genotype: $F^-$, *ilv*$^-$, *galE*$^-$, *pro*$^-$, *str*$^R$. It is a derivative of strain SL686 ($F^-$, *galE*$^-$, *pro*$^-$) which was obtained from Dr G. Schmidt at the Max-Planck Institute. Phage 9NA, specific for smooth strains of *Salmonella typhimurium* was obtained by Dr M. J. Osborn.

The rough-specific phages Br10, FP3, C21 and 6SR were provided by Dr G. Schmidt.

### *Media*

Cultures were grown at 25–30 °C with vigorous aeration in protease/peptone/beef extract medium

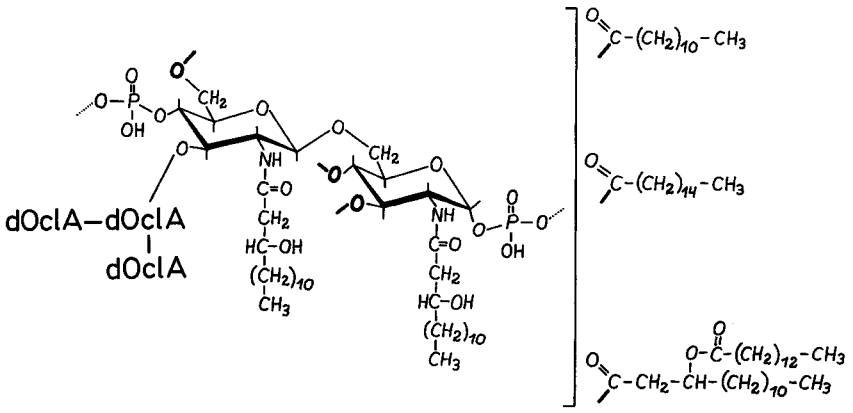


Fig. 1. *Structure of* Salmonella *lipid A substituted by a 3-deoxy-*D-manno-*octulosonic acid trisaccharide.* The three acyl residues are linked in an unknown manner to hydroxyl groups of lipid A [21]

(medium A) [4] or in Merck standard medium (medium B). Cultures after phage selection were plated out on medium A agar or Loeb agar [5].

*Screening of Mutants by Autoradiography*

The master plates with colonies that have reached 0.5 mm in diameter were covered with sterile filter paper discs (Whatman no. 42, 8-cm diameter) which, when taken off, yielded replicas of the colony pattern. The cells remaining on the master plates were allowed to grow at 25 °C until the colonies were visible again. The filters were transfered to prewarmed Loeb plates which contained 1 μCi of [$^{14}$C]galactose (2 Ci/mol) per 5 ml agar. After 45 min at 42 °C [$^{14}$C]-galactose incorporation was stopped by transferring the replica print to a petri dish containing 2 ml of 0.2 g/ml trichloroacetic acid solution. The solution also included 10 mM D-galactose as carrier to prevent nonspecific binding of [$^{14}$C]galactose to the paper. After 15 min unreacted [$^{14}$C]galactose was washed away with four portions (50 ml each) of cold trichloroacetic acid (0.2 g/ml). This was carried out exactly as described by Raetz [18] using a Büchner funnel by gentle suction with the colonies facing upward. The washed filter papers were then dried and pressed onto X-ray films and exposed for 7 days. When compared with the original plates 30 mutant colonies did not show black spots on the film. The mutants thus identified were purified from the original master plate.

*Labeling of Cultures*

*[$^{14}$C]Galactose Incorporation into Lipopolysaccharide.* Cultures (10 ml) were grown at 25 °C in medium B under vigorous aeration. At a density of $3 \times 10^8$ bacteria per ml [$^{14}$C]galactose (spec. act. = 2 Ci/mol) was added to a final concentration of 0.2 mM. After incubation for a further 30 min at 25 °C the temperature of the cultures was raised to 42 °C. Aliquots of 0.5 ml were removed from each culture at the indicated times and assayed for incorporation of [$^{14}$C]galactose into lipopolysaccharide [23].

*Labeling with* N-*Acetyl-[1-$^3$H]glucosamine.* 100-ml cultures (medium B) were grown at 25 °C to a density of about $3 \times 10^8$ cells/ml and then shifted to 42 °C. 10 min after the shift 50 μCi *N*-acetyl-[1-$^3$H]glucosamine (spec. act. 4000 Ci/mol) were added.

*Radioactive Material and Counting Procedure*

*N*-Acetyl-[$^3$H]glucosamine was obtained from Amersham Buchler (Braunschweig-Wenden). [$^{14}$C]-Galactose was purchased from New England Nuclear. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter, model 2450, using 3 g in a mixture of 250 ml Triton X-114, 50 ml water and 700 ml xylene as scintillation fluid [6].

*Chemicals*

D-Arabinose 5-phosphate was synthesized from D-glucosamine 6-phosphate according to the method of Volk [7]. All other reagents were commercial products.

*Analytical Procedures*

Protein was determined according to the method of Lowry [8] with bovine serum albumin as standard. 3-Deoxy-D-*manno*-octulosonic acid was measured by the thiobarbituric acid method [9], glucosamine according to Strominger *et al.* [10], organic phosphate according to Lowry *et al.* [11].

Phosphorylethanolamine was detected as follows: 0.5 mg precursor material were hydrolyzed in 0.1 ml 0.05 M HCl at 100 °C. After 60 min at 100 °C the hydrolysate was chilled and mixed with 0.2 ml chloroform and then centrifuged at room temperature. The

water phase was subjected to paper electrophoresis, either directly or after alkaline phosphatase treatment. The electrophoresis was carried out in pyridine/acetic acid/water (100/40/860, v/v/v, pH 5.3) [12]. Fatty acids were determined by gas-liquid chromatography after acid hydrolysis (4 M HCl, 5 h, 100 °C) and conversion into their methyl esters. The analysis was carried out at 170 °C in a Perkin-Elmer F-20 gas chromatograph, fitted with a flame ionisation detector, on a column containing 2.5% Castorwax on chromosorb G (80–100 mesh).

### *Enzyme Assays*

dOclA-8-*P* synthetase was assayed as described by Rick and Osborn [3], D-ribulose-5-*P* isomerase according to Volk [13] and CMP-dOclA synthetase according to Ghalambor and Heath [14] using an enzyme preparation enriched by protamine sulfate.

## RESULTS

### *Isolation of Conditionally Defective 3-Deoxy-D-manno-octulosonic-acid–Lipid-A Mutants of* Salmonella typhimurium

The enrichment of the desired mutants was based on the assumption that the synthesis of the complete dOclA–lipid A part of lipopolysaccharides is essential for continued growth. Therefore a method was devised which selects for temperature sensitivity both for synthesis of complete dOclA–lipid A and for growth. The procedure is based essentially on two properties of UDP-galactose epimerase mutants which were used as parent strains.

First, the lipopolysaccharide structure and the phage sensitivity is determined by the presence or absence of galactose in the medium. In the absence of exogenous galactose these mutants synthesize incomplete lipopolysaccharides which lack the O-antigens as well as the distal part of the core (Fig. 2). They are therefore resistant to phages 9NA and P22 which require the O-antigen as receptor. The addition of exogenous galactose, however, initiates the synthesis of complete lipopolysaccharide, whereby the cells become sensitive to these O-specific phages.

Second, galactose is not incorporated into previously existing incomplete core lipopolysaccharide synthesized prior to the addition of the sugar. Incorporation of galactose is therefore completely dependent on the *de novo* synthesis of lipopolysaccharide [15]. Mutants which are temperature-sensitive in the biosynthesis of the complete dOclA–lipid A, should therefore lack acceptor sites for galactose incorporation at the nonpermissive temperature and remain resistant to phages 9NA and P22. Provided the mutations are conditional lethal, a shift to the higher temperature should also lead to growth inhibition.

In a first approach (Fig. 3), the strain lacking UDP-galactose epimerase (strain SL761) was muta-

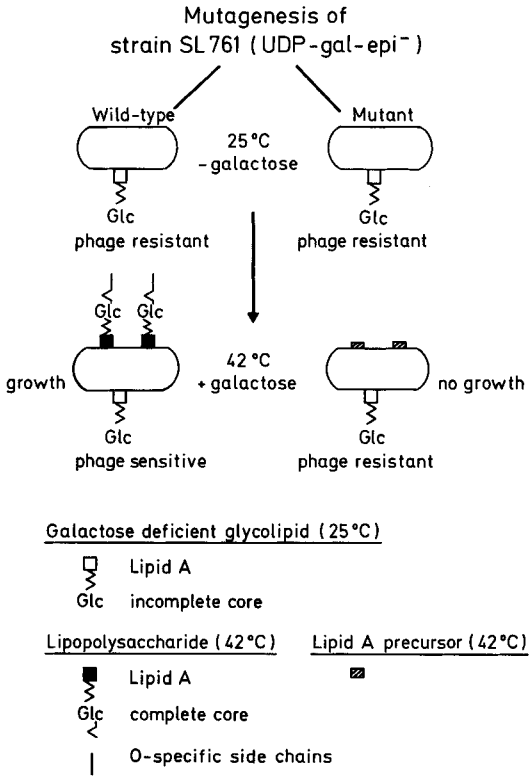


Fig. 3. *Summary of the experimental design for selection of temperature-sensitive dOclA–lipid A mutants*

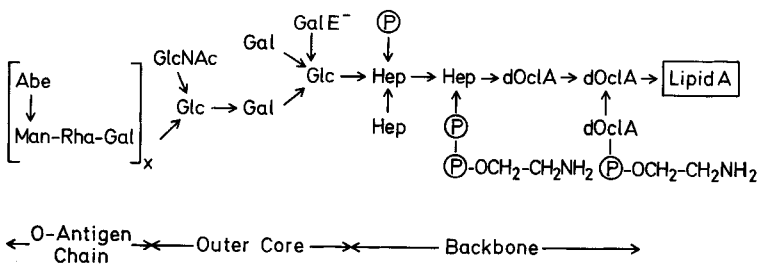


Fig. 2. *Structure of* Salmonella *lipopolysaccharide [22].* The biosynthetic step blocked in *galE*⁻ mutants is indicated by an arrow. The abbreviations are as follows: dOclA, 3-deoxy-D-*manno*-octulosonic acid; Hep, L-*glycero*-D-*manno*-heptose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Rha, L-rhamnose; Man, D-mannose; Abe, abequose

genized with ethylmethane sulfonate [16]. Immediately after mutagenesis the cells were divided into six subcultures, which were carried through all subsequent steps in parallel to obtain mutants arising from independent mutational events. Cells were then grown overnight at 25 °C (permissive temperature) to express the phenotype. 0.1 ml of the overnight culture were diluted in 10 ml medium A and grown to a density of about $3 \times 10^8$ bacteria per ml at 25 °C. The culture was then shifted to 42 °C and, after 15 min, galactose and glucose were added to a final concentration of 1 mM each. This glucose was necessary to prevent galactose toxicity [17]. After incubation for an additional 45 min, the culture was exposed to phages as follows: 0.1 ml of a phage suspension containing phage 9NA (multiplicity of infection = 100) was added to 0.1 ml culture and the phages permitted to adsorb for 15 min at 42 °C. The killing was greater than 99%. Survivors were washed with medium A, plated out and incubated at 25 °C. The colonies were replicated and the replica plates incubated at 42 °C. Approximately 0.1% of the survivors were unable to grow at 42 °C. They were picked and screened for galactose incorporation. Of 30 temperature-sensitive isolates examined, one (mutant Ts1) stopped the incorporation immediately after the shift (Fig. 5). Genetic evidence (unpublished results) and biochemical evidence indicate that the mutant defect is due to an altered dOclA-8-*P* synthetase which catalyses the following reaction: D-arabinose-5-*P* + phospho*enol*pyruvate → dOclA-8-*P* + $P_i$ [14]. Characteristics of this mutant are presented below. During the course of characterisation of this mutant it was shown that the UDP-galactose epimerase mutation, superimposed by a second mutation in dOclA-8-*P* synthesis, causes an increased toxicity towards galactose. The conditions used for the selection permitted normal growth for the parent strain, but often produced marked lysis of the mutant.

Based on this information the selection procedure was repeated with the following modifications. Strain SL686, the parent of SL761, was mutagenized and grown as described above. At the time of the shift the temperature-sensitive dOclA-8-*P* synthetase mutant (Ts1) obtained after the first enrichment was added to the mutagenized culture of strain SL686 as internal standard at a ratio 1 : 1000000. This was done to ensure that lysis of dOclA-negative mutants and eventually also of lipid A mutants does not occur under the conditions of the selection. Mutant Ts1 is a derivative of strain SL761 (*ilv*$^-$, *str*$^R$) and can easily be distinguished from strain SL686 (*ilv*$^+$, *str*$^S$) by two markers. The phage selection included a mixture of phages (Table 1) to kill the smooth parental strain as well as undesirable rough mutants. Of 15000 surviving colonies (grown at 25 °C), 120 were shown to be temperature-sensitive in growth. They were picked and screened for potential dOclA−lipid A mutants using an autoradiographic method. It represents an adaptation of the procedure of Raetz [18] originally applied by Olivera and Bonhoeffer [19]. The method includes a replica plating procedure (see Methods) in which colonies growing on membrane filters were tested for their ability to incorporate exogenous, radioactive galactose into lipopolysaccharides. 30 mutants were unable to incorporate [$^{14}$C]galactose (at 42 °C) and were further investigated. Five of them exhibited an isoleucine/valine requirement and were resistant to streptomycin. They were assumed to be identical reisolates of strain Ts1 which were added to the mutagenized culture of SL686 as internal control. Considering a 500-fold enrichment during the phage selection step and a yield of temperature-sensitive mutants of about 1%, the above number is in good agreement with the input ratio of the internal control, indicating that lysis of Ts1 did not occur.

Table 1. *Composition of a mixed phage suspension which was used to select for conditional dOclA−lipid A mutants by killing off the parent strain and trivial rough mutants*

| Phage | Multiplicity | Chemotype of target cells |
|---|---|---|
| FP3 | 30 | Re, $Rd_1$, $Rd_2$ |
| Br10 | 30 | Rb, Ra |
| 6SR | 40 | Ra |
| 9NA | 100 | smooth |

Of the remaining 25 strains, 17 showed the desired phage pattern. In galactose-free medium these mutants are sensitive to phage C21. They are also sensitive to phage 9NA when galactose is added to the medium at the permissive temperature. As expected, however, they are resistant to O-specific phages under conditions employed for their selection. All mutants could be classified into three groups. The properties of three representative mutants, designated Ts2−Ts4 respectively, together with Ts1 obtained by the first enrichment procedure, are given below.

Since strain SL761 (parent of Ts1) and strain SL686 (parent of Ts2−Ts4) behaved identically in the experiments performed, only data obtained for strain SL761 are shown.

### *Isolation of Revertant Strains*

In order to establish whether single mutations were responsible for the observed pleiotropic effects on the lipopolysaccharide synthesis and growth, spontaneous revertants of Ts1−Ts4 were isolated which possessed restored ability to grow at 42 °C. About $10^6 - 10^7$ cells were plated on Loeb plates and exposed to 42 °C. Revertant colonies were picked after 24 h of incubation. The spontaneous reversion frequencies to temperature resistance ranged, depending

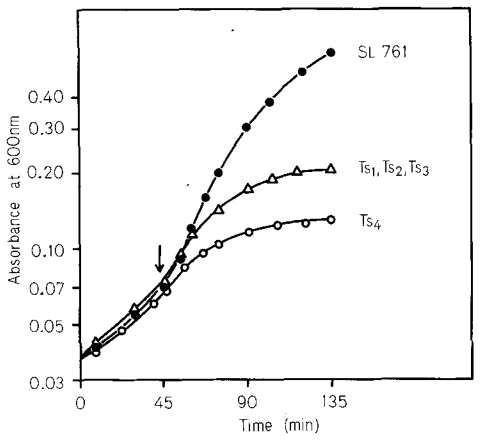


Fig. 4. *Effect of temperature shift from 25 °C to 42 °C on the growth of the parent and mutant strains*

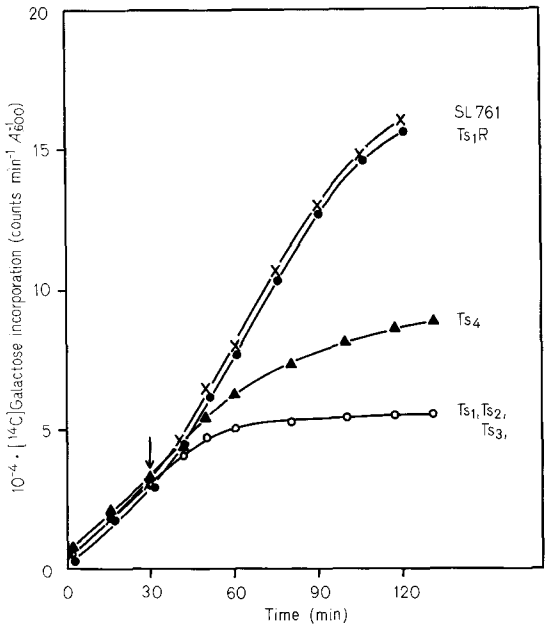


Fig. 5. *[$^{14}$C]Galactose incorporation into lipopolysaccharide from cultures of parent, mutants and one revertant from mutant Ts1*

on the mutant, from $10^{-5}$ to $10^{-7}$. More properties of the revertant strains are presented below.

### *Effect of Temperature on Growth and Viability of Strains Ts1 – Ts4 (Fig. 4)*

These strains and the parent strain SL761 grow exponentially in Merck standard medium at 25 °C with a doubling time of about 45 min. When log-phase cultures are shifted to 42 °C growth continues in mutants Ts1 – Ts3 for one generation at a normal rate but stops after about one and a half generations. Strain Ts4, however, stops growth after one generation following a temperature shift to 42 °C.

At 42 °C the viable counts paralleled essentially the increase in turbidity. No significant loss in viability within 3 h was observed when the cells were incubated in Merck standard medium at 42 °C and then plated out at 30 °C.

### *Effect of Temperature Shift on Lipopolysaccharide Synthesis (Fig. 5)*

To measure the effect of the temperature shift on the synthesis of lipopolysaccharide, the incorporation of [$^{14}$C]galactose was used as specific measure for the *de novo* synthesis of lipopolysaccharides. When the parent, revertant and mutant strains were cultured at 25 °C, incorporation of [$^{14}$C]galactose in all cases was almost identical. After raising the temperature to 42 °C the incorporation of [$^{14}$C]-galactose continued at an approximately linear rate in the parent strains for about 90 min. While the incorporation at this temperature continued at a decreased rate in Ts4, it is heavily reduced in mutants Ts1 – Ts3. The severe reduction of [$^{14}$C]galactose incorporation in mutants Ts1 – Ts3 at the nonpermissive temperature prior to detectable effects on growth rate indicated that the block in lipopolysaccharide synthesis in those mutants is the cause, and not the effect, of the inhibition of growth.

In all revertants (as shown in Fig. 5 for one revertant from mutant Ts1), assayed for [$^{14}$C]galactose incorporation, lipopolysaccharide synthesis was restored at 42 °C.

### *Isolation of Lipid A Precursor*

Evidence that mutants Ts1 – Ts4 are producing lipid A precursor was obtained as follows (Fig. 6). Mutants and parent cells were labeled with *N*-acetyl-[$^{3}$H]glucosamine at 42 °C and extracted with chloroform/methanol (2/1) before treatment with phenol/chloroform/petroleum ether according to the method of Galanos *et al.* [20]. As shown in Table 2, after precipitation of the parent lipopolysaccharide and removal of chloroform/petroleum ether only minor amounts of $^{3}$H remained in the phenol supernatant. In the mutants, however, the lipopolysaccharide could account for only small fractions of the total radioactivity. The major amount of $^{3}$H radioactivity was still present in the phenol supernatant after precipitation of lipopolysaccharide. In order to show that the radioactive material remaining in the phenol supernatant is different from lipopolysaccharide, paper chromatography in 70% phenol was carried out on the total phenol extracts derived from mutant and parent cells which had been labeled with *N*-acetyl-[$^{3}$H]glucosamine. Using extracts from the parent strain, the total radioactivity stayed near the origin

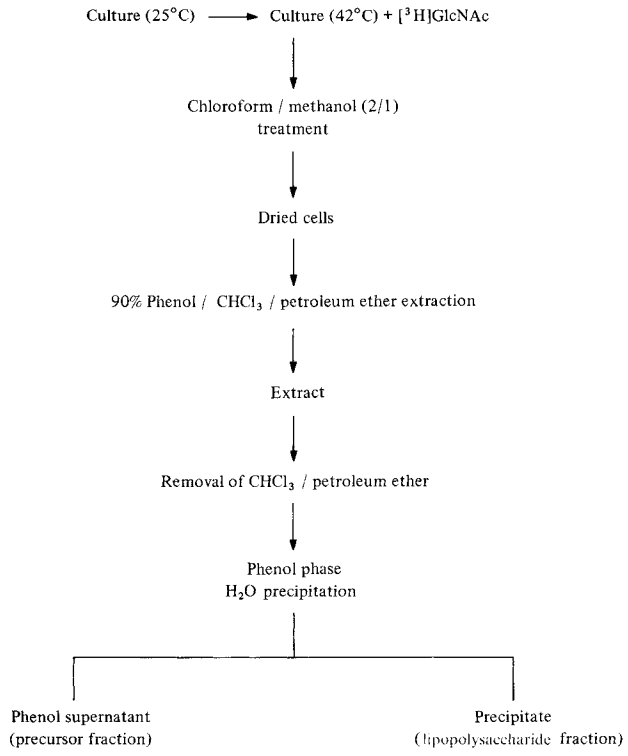


Fig. 6. *Scheme of isolation of lipid A precursor from mutants Ts1 – Ts4*

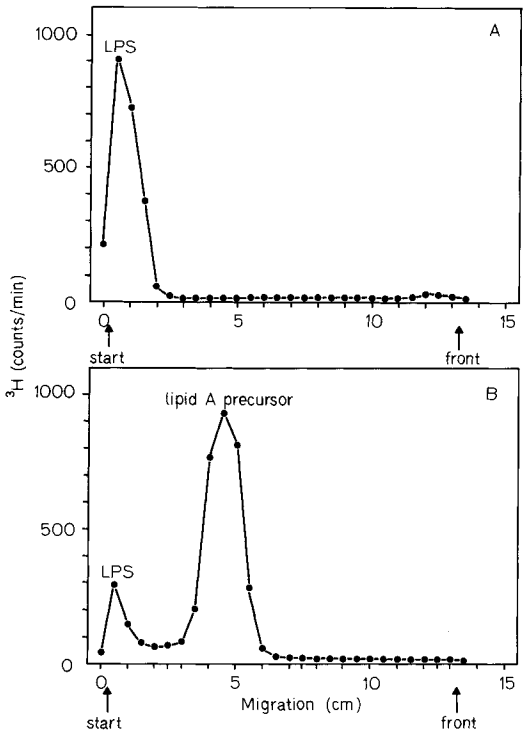


Fig. 7. *Paper chromatography of lipopolysaccharide and lipid A precursor obtained by phenol/chloroform/petroleum ether extraction from cells labeled with* N-*acetyl-[1-³H]glucosamine.* (A) Parent strain, (B) mutant Ts1. Solvent system: 70% phenol. LPS = lipopolysaccharide

Table 2. *Distribution of ³H derived from* N-*acetyl-[1-³H]glucosamine in various fractions from parent strain SL761, mutants Ts1 – Ts4 and revertant Ts4R*
Cells were extracted twice with chloroform/methanol (2/1) (10 ml) followed by phenol/chloroform/petroleum ether according to Galanos [20] (see Fig. 6)

| Fraction | $10^{-6} \times {}^3H$ in | | | | | |
|---|---|---|---|---|---|---|
| | SL761 | Ts1 | Ts2 | Ts3 | Ts4 | Ts4R |
| | counts/min | | | | | |
| Dried cells | 22.0 | 15.0 | 14.5 | 16.0 | 8.5 | 21.0 |
| Extract | 9.0 | 4.5 | 5.5 | 5.1 | 3.5 | 7.5 |
| Precursor fraction | 1.1[a] | 4.1 | 4.5 | 3.8 | 1.8 | 0.8[a] |
| Lipopoly-saccharide fraction | 7.0 | 0.3 | 0.5 | 0.9 | 1.3 | 6.0 |

[a] The radioactivity represents material which is different from lipid A precursors; when subjected to paper chromatography in 70% phenol it stays at the origin (Fig. 7).

(Fig. 7 A). In the mutants Ts1 – Ts4 the major amount was found as single peaks migrating faster than lipopolysaccharide with $R_F$ values (Fig. 7 B) ranging from 0.1 to 0.3. These faster-moving peaks specific for the mutants were still detectable when the lipopolysaccharides were precipitated prior to paper chromatography.

No, or only a trace, of lipid A precursor was produced when revertant cells were exposed to 42 °C. Revertants which synthesize both lipopolysaccharide as well as traces of precursor material at 42 °C possessed reduced ability to grow. Reversions in these strains thus appear to be caused by intragenic suppression rather than true back reversions.

### *Purification of Putative Lipid A Precursors by DEAE-cellulose Chromatography*

Further information on the nature of these potential lipid A precursor molecules was obtained by chromatography on DEAE-cellulose using a methanol/ammonium acetate gradient ranging from 0 to 0.8 M. The phenol supernatants derived from mutants Ts1 – Ts4 after precipitation of the respective lipopolysaccharides were directly applied to the column. Four different elution pattern were obtained (Fig. 8) which indicated the presence of at least four different putative lipid A precursors (fractions I to IV). They elute at ammonium acetate concentrations of 0, 0.08, 0.20 and 0.23 M respectively (see Table 3). In order to show that fractions I to IV are related to

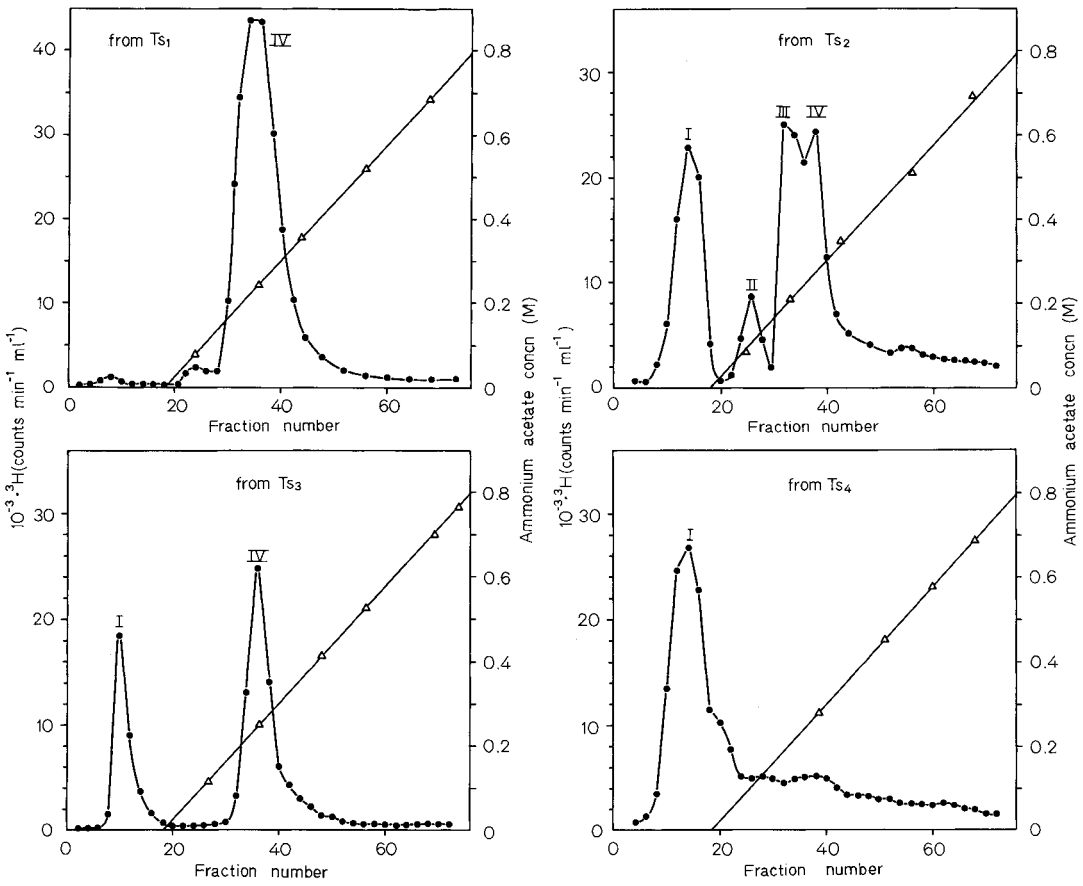


Fig. 8. *Purification of $^3$H-labeled precursor fractions from mutants Ts1–Ts4 by DEAE-cellulose chromatography.* Chromatography was performed with a DEAE-cellulose column (1 $cm^2 \times 8$ cm; Servacel type: DEAE 23 SS) at 25 °C using a linear gradient of ammonium acetate in methanol ranging from 0–0.8 M. Fractions of about 1 ml were collected and screened for radioactivity. In all cases the recovery of radioactivity was better than 70%

the lipid A structure the materials were freed from salts by dialysis and submitted to a chemical analysis.

### *Chemical Analysis of Precursor Fractions*

Analysis of the purified precursor fractions from the four mutants are summarized in Table 3. All components contain glucosamine, 3-hydroxymyristic acid and phosphate. They all lack 3-deoxy-D-*manno*-octulosonic acid as well as lauric and myristic acids which are constituents of the complete lipid A structure. When the precursor fractions were subjected to mild acid hydrolysis (0.05 M HCl, 100 °C, 60 min) phosphorylethanolamine was released from all preparations except fraction IV. The presence of 3-hydroxymyristic acid, glucosamine and phosphate is consistent with the view that fractions I–IV are precursor molecules of the complete lipid A structure. The charge difference between fractions I, II and III has yet to be explained but it may be due to a different substitution of glucosamine with phosphate. Experiments are currently in progress to clarify this point.

Table 3. *Composition of the lipid A precursor fractions obtained after DEAE-cellulose chromatography*
Precursor fraction I was derived from mutants Ts2–Ts4, fractions II and III from Ts2 and fraction IV from Ts1–Ts3

| Component | Precursor fraction | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Glucosamine | + | + | + | + |
| Phosphate | + | + | + | + |
| 3-Hydroxymyristic acid | +[a] | + | + | + |
| Lauric acid | – | – | – | – |
| Myristic acid | – | – | – | – |
| Palmitic acid | traces | traces | traces | traces |
| dOclA | – | – | – | – |
| Phosphorylethanolamine | + | + | + | – |
| | *n* | | | |
| Concentration of ammonium acetate at which precursor material is eluted from DEAE-cellulose column (Fig. 8) | 0 | 0.08 | 0.2 | 0.23 |

[a] Mutant Ts4 contains lower amounts of 3-hydroxymyristic acid as compared to mutants Ts1–Ts3.

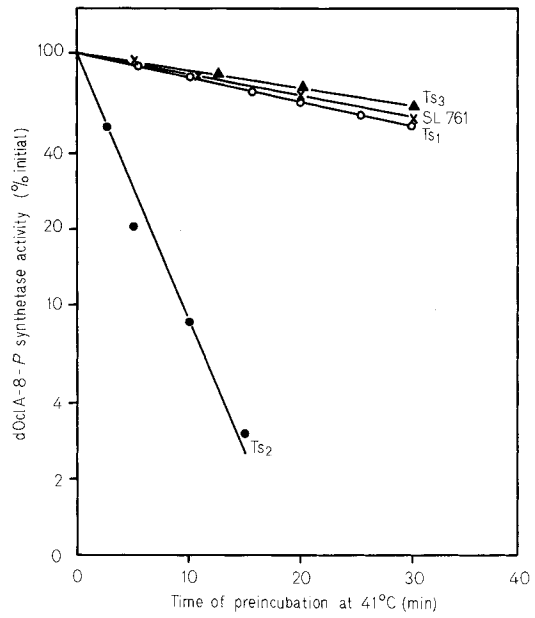


Fig. 9. *Thermolability of dOclA-8-P synthetase activity of parent strain SL761 and mutants Ts1 – Ts3.* Extracts were prepared by sonication and assayed for dOclA-8-*P* synthetase activity as described by Rick and Osborn [3]. The data are expressed relative to an unheated sample of the same extract (= 100%)

Table 4. *Enzymatic synthesis of 3-deoxy-D-*manno*-octulosonic acid from D-arabinose-5-P ($Na^+$ salt) and phosphoenolpyruvate (tricyclohexylammonium salt)*
Extracts from parent, mutants and revertants were prepared from cells grown in medium A at 25 °C and assayed at 25 °C or 42 °C

| Source of enzyme | dOclA formed in 10 min at | |
|---|---|---|
| | 25 °C | 42 °C |
| | nmol/mg protein | |
| SL761 | 61 | 356 |
| Ts1 | 9 | 61 |
| Ts2 | 50 | 51 |
| Ts3 | 45 | 60 |
| Ts4 | 62 | 371 |
| Ts1 $R_1$ | 56 | 341 |
| Ts2 $R_2$ | 51 | 301 |

Table 5. *Specific activities of the dOclA-8-P synthetase derived from parent strain SL761 and mutant Ts1*
Chloramphenicol (CA) was added to the culture after the shift to 42 °C in a concentration of 100 µg/ml. Extracts for dOclA-8-*P* synthetase activity were assayed at 25 °C

| Strain | Time of incubation of cells at 42 °C | Specific activity | |
|---|---|---|---|
| | | −CA | +CA |
| | min | nmol $min^{-1}$ $mg^{-1}$ (%) | |
| SL761 | 30 | 6.30 (100) | 6.10 |
| Ts1 | 0 | 0.94 ( 15) | 0.92 |
| | 5 | 0.90 ( 14.3) | 0.10 |
| | 15 | 0.68 ( 10.8) | 0.90 |
| | 30 | 0.47 ( 7.5) | 0.93 |

## *Properties of 3-Deoxy-*D-manno*-octulosonic acid-8-phosphate Synthetase in Mutants Ts1 – Ts4*

Although the mutants Ts1 – Ts4 synthesize different precursors at the elevated temperature (DEAE-cellulose chromatography), a study of the properties of the dOclA-8-*P* synthetase showed these mutants to be divided into only three classes.

*Class I Mutants (Ts1, Ts2).* The thermolability of the dOclA-8-*P* synthetase derived from mutants Ts1 – Ts4 was compared with the wild-type enzyme. The results are depicted in Fig. 9. Cell extracts obtained from parent and mutant cultures grown at 29 °C were incubated at 42 °C for various times before assaying at 29 °C. The only enzyme which displayed thermolability was derived from mutant Ts2. Ts1 exhibits no thermolability of the dOclA-8-*P* synthetase when tested *in vitro* (see also Table 4). It seems, however, to be impaired in the synthesis of active enzyme at the elevated temperature. Extracts from mutant Ts1 grown in medium A at 25 °C had only 15% of the parent enzyme activity. When shifted to 42 °C, the level of enzyme activity did not increase although growth continued for one and a half generations (Fig. 4). Thus, when extracts were prepared from mutant and parent cells incubated at 42 °C for 30 min (one generation), the mutant extracts possessed only 7.5% of the activity of that from the parent strain (Table 5). That this decrease of activity is not due to a temperature-induced inactivation of the enzyme was shown in a similar experiment, where chloramphenicol was added immediately after a shift to 42 °C (Table 5). Within 30 min at 42 °C no loss of activity was detectable. Two other enzymes involved in 3-deoxy-D-*manno*-octulosonic acid metabolism have been examined in mutant Ts1. Neither of these enzymes was significantly affected by heating at 42 °C.

*Class II Mutant (Ts3).* Although the dOclA-8-*P* synthetase is not thermo-labile (Fig. 9), dOclA synthesis appears to be affected by the mutation at the nonpermissive temperature (42 °C) (Table 4). A possible induction of the degradative 3-deoxy-D-*manno*-octulosonic acid aldolase was excluded (data not shown). Further studies are in progress to define the enzymatic defect.

*Class III Mutant (Ts4).* This mutant has a normal dOclA-8-*P* synthetase. The enzyme lesion has not yet been identified, but preliminary structural analysis of the lipid A precursor (see Table 3) together with the growth pattern (Fig. 4) suggest that the enzymatic block may occur before the introduction of the dOclA residues.

## DISCUSSION

It was hoped that the selection procedure described in this paper would yield mutants deficient in different essential steps of the dOclA–lipid A biosynthesis. Three classes of mutants were obtained which produce lipid A precursors under nonpermissive temperatures. Three mutants appear to be impaired in dOclA metabolism whereas one mutant (class III) seems to be defective in an early stage in lipid A synthesis, presumably in a step prior to dOclA incorporation. Other mutants were isolated using the same technique. They all have lesions in the dOclA part of lipopolysaccharides. Failure of this selection method to yield mutants defective at a very early stage in lipid A synthesis suggests that such mutants may not survive the selection procedure. It is also possible that many potentially lethal mutations affecting membrane-bound enzymes may be phenotypically silenced by the stabilizing environment of the membrane.

Of the three mutants unable to synthesize dOclA-8-*P*, two produced different precursor molecules at elevated temperatures. We have at present no information concerning the nature of the differences as well as the factors which generate structural diversity of precursor molecules. It is possible that this structural heterogeneity reflects a highly efficient mechanism by which the membrane can regulate the charge pattern on the cell surface. Experiments are in progress to determine how changes in the membrane of these mutants can effect the structure of the lipid A precursors.

All precursor molecules derived from mutants unable to synthesize dOclA possess a common feature. While they contain as constituents glucosamine, phosphate and 3-hydroxymyristic acid, they lack dOclA, myristic and lauric acids.

In a separate paper it will be shown that the lipid A precursor from mutant Ts1 contains the basic structure of the complete lipid A molecule and represents the true intermediate in the biosynthesis of the dOclA–lipid A part of lipopolysaccharides. The isolation of conditional lethal mutants, unable to synthesize complete dOclA–lipid A and unable to grow at restrictive temperatures, confirms the initial assumption of the essential nature of the complete dOclA–lipid A complex. Cessation of growth in these mutants could result from lack of sufficient dOclA–lipid A molecules to participate in some vital membrane function. On the other hand, accumulation of lipid A precursors may itself affect membrane function and prove lethal to the cell. In order to decide between these two possibilities more detailed studies of membrane function are required under conditions in which synthesis of complete lipid A is inoperative.

We are grateful to Miss Inge Minner for expert technical assistance. We like also to express our gratitude to Dr O. Lüderitz for helpful discussion and for reading this manuscript. A part of these investigation which were performed at the University of Connecticut was supported by grants of the *Deutsche Forschungsgemeinschaft* (V. L. and E. R.). Further investigations at the Max-Planck-Institut were supported by grants of the *Max-Planck-Gesellschaft* (V. L.).

## REFERENCES

1. Osborn, M. J., Gander, J. E., Parisi, E. & Carson, J. (1972) *J. Biol. Chem. 247*, 3962–3972.
2. Stocker, B. A. D. & Mäkelä, P. H. (1971) in *Bacterial Endotoxins*, vol. 4 of *Microbial Toxins* (Weinbaum, K., Kadis, S. & Ajl, A. J., eds) Academic Press, New York.
3. Rick, P. D. & Osborn, M. J. (1972) *Proc. Natl Acad. Sci. U.S.A. 69*, 3756–3760.
4. Rothfield, L. & Pearlman-Kothencz, M. (1969) *J. Mol. Biol. 44*, 477–492.
5. Loeb, T. & Zinder, N. D. (1961) *Proc. Natl Acad. Sci. U.S.A. 47*, 282–289.
6. Anderson, L. E. & McClure, W. O. (1973) *Anal. Chem. 51*, 173–175.
7. Volk, W. A. (1966) *Methods Enzymol. 9*, 38–39.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
9. Waravdekar, V. S. & Saslaw, L. D. (1959) *J. Biol. Chem. 234*, 1945–1950.
10. Strominger, J. L., Park, L. T. & Thompson, R. E. (1959) *J. Biol. Chem. 234*, 3263–3268.
11. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L. & Farr, A. L. (1954) *J. Biol. Chem. 207*, 1–17.
12. Kickhöfen, B. & Warth, R. (1968) *J. Chromatogr. 33*, 558–560.
13. Volk, W. A. (1966) *Methods Enzymol. 9*, 585–588.
14. Ghalambor, M. A. & Heath, E. C. (1966) *J. Biol. Chem. 241*, 3216–3221.
15. Osborn, M. J., Gander, J. E. & Parisi, E. (1972) *J. Biol. Chem. 247*, 3963–3972.
16. Josephson, B. L. & Fraenkel, D. G. (1969) *J. Bacteriol. 100*, 1289–1295.
17. Fukasawa, T. & Nikaido, H. (1961) *Biochim. Biophys. Acta, 48*, 470–483.
18. Raetz, C. R. H. (1975) *Proc. Natl Acad. Sci. U.S.A. 72*, 2274–2278.
19. Olivera, B. M. & Bonhoeffer, F. (1974) *Nature (Lond.) 250*, 513–514.
20. Galanos, C., Lüderitz, O. & Westphal, O. (1969) *Eur. J. Biochem. 9*, 245–249.
21. Rietschel, E. Th., Gottert, H., Lüderitz, O. & Westphal, O. (1972) *Eur. J. Biochem. 28*, 166–173.
22. Lüderitz, O., Westphal, O., Staub, A. M. & Nikaido, H. (1971) in *Bacterial Endotoxins*, vol. 4 of *Microbial Toxins* (Weinbaum, K., Kadis, S. & Ajl, A. J., eds) Academic Press, New York.
23. Eidels, L. & Osborn, J. J. (1971) *Proc. Natl Acad. Sci. U.S.A. 68*, 1673–1677.

V. Lehmann, Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg-Zähringen, Federal Republic of Germany

E. Rupprecht, Dr Karl Thomae GmbH, D-7950 Biberach/Riß, Federal Republic of Germany

M. J. Osborn, Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut, U.S.A. 06032