Lipopolysaccharide and Aldoheptose Biosynthesis in Transketolase Mutants of *Salmonella typhimurium*

(sedoheptulose-7-phosphate synthesis/bile-salt sensitivity)

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Communicated by B. L. Horecker, May 17, 1971

ABSTRACT Genetic and biochemical evidence that sedoheptulose-7-phosphate is an obligatory precursor of the L-glycero-D-mannoheptose residues of the lipopolysaccharide of *Salmonella* was obtained by isolation and characterization of transketolase-negative mutants of *Salmonella typhimurium*. These mutants, which are defective in synthesis of sedoheptulose-7-phosphate, were found to produce an incomplete heptose-deficient lipopolysaccharide, and were also sensitive to bile salts, a characteristic property of heptose-deficient mutants. Phenotypic repair of the defect in lipopolysaccharide synthesis was obtained by addition of exogenous sedoheptulose-7-phosphate to growing cultures of the mutant strains. Characterization of revertants isolated either as transketolase-positive or heptose-positive provided further evidence that the heptose deficiency resulted from mutation at the transketolase locus. On the basis of these findings a possible pathway for conversion of sedoheptulose-7-phosphate to L-glycero-D-mannoheptose is proposed.

L-Glycero-D-mannoheptose is a characteristic component of the lipopolysaccharides of *Salmonella* and related Gram-negative bacteria, and is a specific and major constituent of the innermost backbone region of the polysaccharide (Fig. 1). The pathway of biosynthesis of this sugar is unknown, although it has been assumed to be derived from sedoheptulose-7-phosphate (1, 2). To test this hypothesis we needed a mutant incapable of making sedoheptulose-7-P.

While several reactions leading to formation of sedoheptulose-7-P are known, all depend on the presence of transketolase (TK):

Abbreviations: TK, transketolase; TA, transaldolase; Ald, aldolase; SDPase and FDPase, sedoheptulose diphosphatase and fructose diphosphatase; aro, aromatic; pent, pentose; hep, L-glycero-D-mannoheptose; KDO, 3-deoxy-D-mannoctulosonate.

In addition to reactions 1 and 2, catalyzed by transketolase, sedoheptulose-7-P can be derived from erythrose-4-P by reactions involving either transaldolase (reaction 4) or aldolase (reactions 5 plus 6); however, the only known sources of erythrose-4-P for these alternate pathways are reactions 2 and 3, which are themselves catalyzed by transketolase. Transketolase-negative mutants should therefore be deficient in synthesis of sedoheptulose-7-P, and, if this sugar is an obligatory precursor of L-glycero-D-mannoheptose, should produce an incomplete, heptose-deficient lipopolysaccharide. We now report that this is indeed the case, and that the defect in lipopolysaccharide synthesis can be corrected *in vivo* by addition of exogenous sedoheptulose-7-P to the culture medium.

Isolation of transketolase-negative mutants was first described by Josephson and Fraenkel (3) in *Escherichia coli*. These workers developed a method of selection based on the fact that transketolase is essential both for catabolism of pentoses and for synthesis of erythrose-4-P, an obligatory intermediate in the aromatic pathway. Transketolase mutants were successfully obtained after penicillin enrichment for the predicted pleiotropically pentose-negative, aromatic-requiring phenotype. For this investigation we have isolated transketolase mutants of *Salmonella typhimurium* by a method which combined this technique with an additional phage-

FIG. 1. Structure of the lipopolysaccharide of *S. typhimurium*. Noncarbohydrate substituents (phosphate, ethanolamine) are omitted. The point of termination of the incomplete polysaccharide of G-30 is as indicated.

TABLE 1. *Growth properties of mutants and revertants*

Strain	Medium				
	Minimal glucose	Minimal glucose + Aros ^a	Minimal pentoses	Minimal pentoses + Aros ^a	Bile salts ^c
G-30	+	+	+	+	+
TE-16	(±)	+	—	—	—
TE-21	(±)	+	—	—	—
TE-161 (bile ^c revertant)	+	+	—	—	+
TE-162 (aro ⁺ revertant)	+	+	—	—	+
TE-163 (pent ⁺ revertant)	+	+	+	+	+
TE-211 (bile ^c revertant)	+	+	—	—	+
TE-212 (aro ⁺ revertant)	+	+	—	—	+
TE-212 (pent ⁺ revertant)	+	+	+	+	+

The strains were grown to stationary phase in L broth and streaked on the plates specified. After 48 hr at 37°C the plates were scored for growth (+) or no growth (—). Where observed, faint growth was scored as (±).

^a Aromatic supplement.

^b L-Arabinose plus D-xylose.

MacConkey-glucose plates.

TABLE 2. *Transketolase activity of mutants and revertants*

Strain	Transketolase activity				
	Assay method				
	1	1x	2a	2ax	2b
G-30	96	194	284	567	132
TE-16	2	5	0-4	0-4	0
TE-21	1	3	0	8	0
TE-161 (bile ^c revertant)	11	—	—	—	—
TE-162 (aro ⁺ revertant)	13	31	23	32	—
TE-163 (pent ⁺ revertant)	98	189	263	421	—
TE-212 (aro ⁺ revertant)	7	—	—	—	—
TE-213 (pent ⁺ revertant)	84	168	293	363	—

Activity is reported in nanomoles per min per mg protein. Cell extracts were prepared by sonication in 50 mM Tris-HCl (pH 7.8)–10 mM MgCl₂–2 mM dithiothreitol. The soluble fraction obtained by centrifugation at 160,000 × *g* for 90 min was used as enzyme. The conditions of assays 1, 2a, and 2b were those of Josephson and Fraenkel (3). Assay 1 measured the formation of glyceraldehyde-3-P from ribose-5-P; assay 2a measured the formation of glyceraldehyde-3-P in the presence of ribose-5-P and erythrose-4-P; and assay 2b measured the formation of fructose-6-P under the conditions of assay 2a. The conditions in assay 1x were the same as assay 1 except that xylulose-5-P (0.25 mM) was also added. The conditions in assay 2ax were the same as those in assay 2a except that xylulose-5-P (0.25 mM) was present instead of ribose-5-P. Thiamine pyrophosphate (10 mM) had no effect on the TK activity of the parent or the mutants. The assays were carried out with a Gilford model 2400 recording spectrophotometer.

selection step designed to enrich specifically for mutants containing heptose-deficient lipopolysaccharide.

MATERIALS AND METHODS

S. typhimurium G-30 has been described (4). *E. coli* BJ-502 (TK[−]) and its parent, K-10, were kindly supplied by D. G. Fraenkel (Dept. of Bacteriology and Immunology, Harvard Medical School). Cells were grown at 37°C to late-exponential phase in proteose peptone beef extract broth (PPBE) (5) for isolation of lipopolysaccharide, and in minimal medium M9 (6) containing glucose (0.4%), thiamine·HCl (1 μg/ml), and an aromatic supplement for enzyme assays. The aromatic supplement was that described by Josephson and Fraenkel (3), except that 2,3-dihydroxybenzoic acid (1 mM) was also included.

Lipopolysaccharides were isolated by the method of Galanos *et al.* (7), and contained less than 1% protein and nucleic acid. Sugar compositions were determined by gas-liquid chromatography after total acid hydrolysis (1 N HCl, 5 hr, 100°C) and conversion to the alditol acetates. L-Arabinitol was added as internal standard. Columns were packed with 3% ECNSS-M Gas-Chrom Q, 100–120 mesh; analyses were carried out isothermally at 190°C.

All reagents were commercial products except volemitol, which was kindly supplied from the collection of Dr. F. Smith, Dept. of Biochemistry, University of Minnesota.

RESULTS

Isolation of transketolase-negative mutants of *S. typhimurium*

The procedure was based on the prediction that TK[−] mutants would have a heptose-deficient (hep[−]) lipopolysaccharide as well as the pentose[−] aro[−] phenotype established by Josephson and Fraenkel (3). Penicillin enrichment for the pentose[−] aro[−] character was therefore followed by a phage-selection step known to yield hep[−] mutants with high efficiency. Phage C21 was employed for this purpose. This phage adsorbs only to mutants that are blocked at an early stage in synthesis of the outer core region of the lipopolysaccharide, but retain the heptose residues of the backbone structure (8). Mutants lacking UDP-galactose-4-epimerase are of this type (Fig. 1). Selection for C21 resistance in such organisms yields predominantly derivatives with the hep[−] phenotype (8; M. J. Osborn, unpublished observations). Accordingly, a UDP-galactose-epimerase-negative, C21^s derivative of *S. typhimurium* LT-2, strain G-30, (Fig. 1) was employed as parent. Mutagenesis with ethyl methane sulfonate, and penicillin selection in the presence of L-arabinose, D-xylose, and an aromatic supplement was carried out essentially as described by Josephson and Fraenkel (3). Immediately after mutagenesis the cells were divided into five subcultures, which were carried through all subsequent steps in parallel in order to facilitate isolation of mutants arising from independent mutational events. The survivors of penicillin treatment were exposed to phage C21 as follows. For each subculture, 10 glucose-minimal plates containing the aromatic supplement were spread first with 2 × 10⁸ phages and then with 5 × 10⁴ bacteria. Each plate yielded approximately 100 C21-resistant colonies after 36–48 hr of incubation. These were screened for aromatic requirement and pentose-nonutilization by replication to minimal-glucose plates and minimal plates containing a mixture of L-arabinose, D-xylose, and the aromatic supplement. Of approximately 5000 colonies screened, two (TE-16

and TE-21), which arose from different subcultures, had the expected phenotype and were found in addition to be sensitive to bile salts, a characteristic property of heptose-deficient mutants. The growth properties are summarized in Table 1.

The TK activity of the parent and the mutants was determined by five different assays (Table 2). Both mutants are slightly leaky, TE-16 showing 0–2% and TE-21 0–4% of the parental TK activity, depending on the assay used. Since assays 1, 2a, and 2b depend on generation of xylulose-5-P from ribose-5-P by endogenous pentose phosphate epimerase and isomerase, the presence of these enzymes in the mutant extracts was confirmed by addition of purified transketolase (Sigma) to the incubation mixtures. Activity was restored to the expected levels in all cases.

Sugar composition of mutant lipopolysaccharides

The backbone region of the lipopolysaccharides of *Salmonella* and *E. coli* is composed of heptosyl-heptosyl-KDO (3-deoxy-D-mannooctulosonate) units, which are linked to the lipid portion of the polymer through additional residues of KDO (9, 10) (Fig. 1). The lipopolysaccharide of mutants blocked in biosynthesis of the heptose would be expected to lack this and all sugars distal to it in the polysaccharide chain, but to retain normal contents of KDO. The heptose content of the lipopolysaccharides isolated from the TK[−] mutants was markedly lower than that of the parent, G-30 (Table 3); the molar ratio of heptose to KDO was approximately 30% that of the parent in TE-16, and 45% in TE-21. In addition to the backbone constituents, the polysaccharide of G-30 contains glucose units linked to the terminal heptose residues (Fig. 1). As expected, the lipopolysaccharides of TE-16 and TE-21 showed a reduction in glucose content parallel to that of heptose. Table 3 also shows the results obtained with lipopolysaccharide isolated from strain BJ-502, a TK[−] mutant of *E. coli*, and strain K-10, its parent (3); the decrease in the heptose and glucose content was very similar to that observed for the TK[−] mutants of *S. typhimurium*.

Gas-liquid chromatography confirmed that the residual heptose present in the TK[−] mutants of both *S. typhimurium* and *E. coli* was exclusively the L-glycero-D-manno isomer. Analysis of the alditol acetates showed a single peak with a retention time (2.24–2.26 relative to glucose) identical to that of perseitol, the alditol of L-glycero-D-mannoheptose. No peak was observed with the relative retention time (1.95) of volemitol, the alditol of D-glycero-D-mannoheptose.

Isolation and characterization of revertants

To test whether the pentose negativity, aromatic requirement, and hep[−] character were due to a single mutation, we isolated revertants of TE-16 and TE-21 for each of the three characters. Since sensitivity to bile salts is closely correlated with the hep[−] phenotype (8), reversion to bile-salts resistance (bile⁺) provided a convenient means of selection for hep⁺ revertants. Accordingly, approximately 3×10^8 mutant bacteria were spread on plates containing bile salts (MacConkey glucose) for isolation of bile⁺, hep⁺ revertants, on glucose-minimal plates for aromatic-independent revertants (aro⁺), and on minimal plates containing L-arabinose, D-xylulose, and the aromatic supplement for pentose-positive revertants (pent⁺). One drop of ethyl methane sulfonate was placed in the center of each plate and revertant colonies were picked after 36–48 hr of growth. Spontaneous revertants were also isolated and gave similar results.

TABLE 3. Sugar composition of lipopolysaccharides

Strain	Weight per cent			Molar ratio		
	KDO	Hep	Glc	KDO	Hep	Glc
<i>S. typhimurium</i>						
G-30	9.7 (9.7)	9.7	5.0	1.0	1.14	0.65
	10.3	9.6	n.d.	1.0	1.08	n.d.
TE-16	13.5 (15.2)	4.6	2.4	1.0	0.38	0.23
	21.0	5.3	n.d.	1.0	0.29	n.d.
TE-21	16.1 (13.8)	7.8	4.0	1.0	0.54	0.32
	18.6	5.8	n.d.	1.0	0.36	n.d.
TE-162 (aro ⁺ revertant)	12.8 (13.0)	12.2	n.d.	1.0	1.08	n.d.
TE-163 (pent ⁺ revertant)	10.7 (9.5)	11.2	n.d.	1.0	1.18	n.d.
TE-213 (pent ⁺ revertant)	12.6 (9.2)	11.0	n.d.	1.0	0.99	n.d.
<i>E. coli</i>						
K-10	11.0 (10.1)	12.2	9.4	1.0	1.27	1.18
BJ-502	16.3 (12.2)	5.6	4.6	1.0	0.39	0.37

KDO was determined by the thiobarbituric acid method (20); values obtained by the semicarbazide procedure (21) are given in parentheses. Heptose was determined by the cysteine-sulfuric acid reaction as previously described (20). Crystalline L-glycero-D-mannoheptose-1-P (2) was used as standard; 1 μ mol gave a value of $\Delta A_{505-545} = 3.0$. Glucose was measured by gas-liquid chromatography.

n.d., not determined.

The phenotypic characteristics of the revertants are shown in Table 1. Eight revertants isolated as bile⁺ were uniformly also aro⁺ and conversely, 10 aro⁺ revertants examined were all bile⁺. However, none of the 18 revertants isolated as bile⁺ or aro⁺ were able to utilize the mixture of pentoses as sole carbon source, and the transketolase activity was found to be restored only to the extent of 10–15% that of the wild type (Table 2). On the other hand, seven revertants selected as pent⁺ showed full restoration of both the aro⁺ and bile⁺ phenotypes and essentially normal levels of TK activity (Table 2). It is not surprising that partial restoration of TK activity would suffice for biosynthesis of aromatic compounds and heptose, while full activity would be required to support pentose catabolism at rates adequate for growth.

The sugar composition of the lipopolysaccharides of representative partial and full revertants is included in Table 3. In all cases the heptose content was restored to the parental level.

Effect of exogenous sedoheptulose-7-P on lipopolysaccharide synthesis in vivo

The parental strain, G-30, which lacks UDP-galactose-4-epimerase (4), is unable to synthesize UDP-galactose in the absence of exogenous galactose, and is therefore conditionally defective in lipopolysaccharide synthesis. In galactose-free media a galactose-deficient lipopolysaccharide is formed which lacks all portions of the polysaccharide distal to the first glucosyl residue of the core (4, 9) (Fig. 1). However, synthesis of the nucleotide sugar can be initiated by addition of exogenous galactose, and the added galactose is then incorporated exclusively into the O-antigen chains and core region of lipopolysaccharide (4, 11). Since transfer of galactose into lipopolysaccharide depends on the integrity of the

FIG. 2. Incorporation of $[^{14}\text{C}]$ galactose into lipopolysaccharide. Cultures were grown to a density of approximately 5×10^8 cells per ml in PPBE medium at 37°C with vigorous aeration. Tris-HCl (pH 7.8) and $[^{14}\text{C}]$ galactose (7×10^6 cpm/ μmol) were then added to final concentrations of 10 mM and 0.25 mM, respectively; where indicated, sedoheptulose-7-P (Na^+) was also added to a concentration of 0.25 mM. For the experiment in the presence of chloramphenicol (CAP), nonradioactive galactose (0.25 mM) was added at cell density of 3×10^8 /ml. Growth was continued to a density of 5×10^8 /ml, the culture was centrifuged at 25°C , and the cells were resuspended to the same density in fresh medium containing 50 $\mu\text{g}/\text{ml}$ chloramphenicol. The culture was incubated for 5 min at 37°C with shaking before addition of Tris-HCl, $[^{14}\text{C}]$ galactose, and sedoheptulose-7-P as above. After addition of $[^{14}\text{C}]$ galactose, growth was continued for 60 min and aliquots were removed at the times indicated for measurement of incorporation of ^{14}C into lipopolysaccharide. The assay procedure was designed to distinguish lipopolysaccharide from galactose-containing polyisoprenol-P-linked intermediates of O-antigen synthesis which are synthesized independently of lipopolysaccharide, and accumulate under conditions in which synthesis of the internal core portion of lipopolysaccharide is incomplete (e.g., in hep^- strains) (18). The procedure is based on the lability of the glycosyl-pyrophosphoryl-polyisoprenol linkage of O-antigen intermediates to mild alkaline hydrolysis (19). Aliquots of the cultures (1 ml) were added to 1 ml of 2 N NH_4OH containing 10 mM MgCl_2 , incubated at room temperature for 90 min, and filtered through a Millipore filter (0.45 μm). The filters were washed three times with cold 5% trichloroacetic acid, dried, and counted in toluene-Liquifluor (New England Nuclear) in a Beckman LS-100 scintillation counter. Lipopolysaccharide is quantitatively adsorbed to the membrane filter under these conditions, while the products of alkaline hydrolysis of O-antigen intermediates pass through the filter.

heptose-containing backbone, mutants deficient in heptose biosynthesis should lack acceptor sites for galactose incorporation (Fig. 1). The ability of growing cultures of the TK^- derivatives to incorporate $[^{14}\text{C}]$ galactose into lipopolysaccharide was indeed greatly reduced (Fig. 2). The growth rates of the TK^- mutants were similar to that of G-30 in the enriched medium employed for these experiments.

That availability of sedoheptulose-7-P was the limiting factor in incorporation of $[^{14}\text{C}]$ galactose into lipopolysaccharide in the TK^- mutants was shown by addition of exogenous sedoheptulose-7-P to the culture medium (Fig. 2). Addition of sedoheptulose-7-P resulted in a striking increase in incorporation of $[^{14}\text{C}]$ galactose into lipopolysaccharide in both TE-16 and TE-21, the rate and extent of incorporation

TABLE 4. Specificity of sedoheptulose-7-P in restoring $[^{14}\text{C}]$ -galactose incorporation into lipopolysaccharide

Additions to medium	$[^{14}\text{C}]$ Galactose incorporation	
	nmol/ml culture/60 min	Per cent of control
None	3.63	100
Sedoheptulose-7-P (0.25 mM)	27.20	749
Glucose-6-P (0.25 mM)	2.51	69
Ribose-5-P (0.25 mM)	2.86	79
Erythrose (2.5 mM)	4.93	136
Erythrose-4-P (0.25 mM)	6.27	173
Sedoheptulose (0.25 mM)	7.88	217

Strain TE-16 was employed. Experimental conditions and assay procedures were as described in the legend to Fig. 2. Replacement of sedoheptulose-7-P by other compounds was at the concentrations indicated. Free sedoheptulose was prepared by treatment of sedoheptulose-7-P with *E. coli* alkaline phosphatase.

ultimately approaching those observed in G-30. The effect was specific both for the TK^- mutants and for sedoheptulose-7-P. Incorporation of galactose in G-30 was not significantly increased by supplementary sedoheptulose-7-P (Fig. 2), and other sugars and sugar phosphates had little effect in the TK^- mutants (Table 4). Glucose-6-P, ribose-5-P, and erythrose were completely ineffective, while partial restoration was observed with erythrose-4-P and free sedoheptulose. The effect of erythrose-4-P could be explained by transaldolase-catalyzed conversion to sedoheptulose-7-P (reaction 4), if the existence of a permease system capable of transporting the tetrose-P is assumed.

Rothfield and Pearlman-Kothencz (12) have previously shown that incorporation of galactose into lipopolysaccharide continues in strain G-30 in the presence of chloramphenicol. However, the effect of sedoheptulose-7-P on $[^{14}\text{C}]$ galactose in the TK^- mutants was abolished by prior addition of this inhibitor (Fig. 2). Since galactose permease and the enzymes of the galactose pathway were induced before addition of chloramphenicol, the inhibition could not be attributed to an effect on galactose metabolism or lipopolysaccharide synthesis *per se*. The results suggest that induction either of a sedoheptulose-7-P permease system or of enzymes related to aldohexose biosynthesis is necessary. The former appears to be the more likely possibility. The nature and specificity of the presumed transport system is not yet known, but inducible permeases for other sugar and polyol phosphates have been described (13-16).

DISCUSSION

The successful isolation of TK^- mutants points to the existence of a single transketolase enzyme in *S. typhimurium* that catalyzes several reactions (see *Introduction*). Josephson and Fraenkel have earlier shown that this is also the case in *E. coli* (3). In both organisms the TK^- mutants so far isolated have been significantly leaky with respect to both enzyme activity and phenotypic properties. This is reflected in detectable growth in the absence of the aromatic supplement, and, as shown in this investigation, in the L-glycero-D-mannoheptose content of the lipopolysaccharides. However, the amount of residual heptose in the lipopolysaccharide is ap-

parently insufficient in the *Salmonella* mutants to confer resistance to bile salts, or sensitivity to phage C21.

Although we have not yet mapped the TK mutation in *S. typhimurium*, the properties of the revertants indicated that a single mutation was responsible for pentose negativity, aromatic requirement, and heptose deficiency. Further strong evidence for this hypothesis was provided by the specific stimulatory effect of exogenous sedoheptulose-7-P on the incorporation of [¹⁴C]galactose into lipopolysaccharide in the mutants. If sedoheptulose-7-P were unrelated to biosynthesis of lipopolysaccharide heptose, and the heptose deficiency in these mutants were due to an independent mutation either in heptose biosynthesis or heptosyl transferase enzymes, phenotypic repair of the lesion by sedoheptulose-7-P would seem extremely unlikely.*

We thus conclude that sedoheptulose-7-P is the precursor of L-glycero-D-mannoheptose. We postulate the following pathway for biosynthesis of the aldoheptose, where NTP and NDP denote nucleoside triphosphate and diphosphate:

Ginsburg *et al.* (17) found GDP-D-glycero-D-mannoheptose in baker's yeast and postulated a similar pathway for its biosynthesis, starting also from sedoheptulose-7-P. By analogy with the epimerization of UDP-galactose to UDP-glucose we

postulate the epimerization at the C-6 position to occur at the nucleotide sugar level; alternative pathways involving epimerization at an earlier step are, of course, possible. Investigations of the intermediate steps in conversion of sedoheptulose-7-P to L-glycero-D-mannoheptose and the mechanism of incorporation of this sugar into lipopolysaccharide are in progress.

This research was supported by grants from the USPHS (AI-08650) and NSF (GB-8040). L. E. is the recipient of an Arthritis Foundation Postdoctoral Fellowship.

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* Incorporation of added sedoheptulose-7-P specifically into lipopolysaccharide heptose by growing cultures has been confirmed by use of highly purified [¹⁴C]sedoheptulose-7-P (over 99% pure).