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Isolation of a Mutant of *Salmonella typhimurium* Dependent on D-Arabinose-5-phosphate for Growth and Synthesis of 3-Deoxy-D-mannoctulosonate (Ketodeoxyoctonate)

(lipopolysaccharide biosynthesis/ketodeoxyoctonate-8-phosphate synthetase/D-arabinose-5-phosphate dependence)

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ABSTRACT **A new type of auxotrophic mutant of *Salmonella typhimurium* has been isolated that is defective in the synthesis of the 3-deoxy-D-mannooctulosonate (ketodeoxyoctonate) region of the lipopolysaccharide and requires D-arabinose-5-phosphate for growth. Genetic and biochemical evidence indicated that the mutant defect was due to an altered ketodeoxyoctonate-8-phosphate synthetase with an apparent K_m for D-arabinose-5-phosphate 35-fold higher than that of the parental enzyme. As a result of this enzymatic lesion, the mutant strain was dependent on exogenous D-arabinose-5-phosphate both for growth and for synthesis of a complete lipopolysaccharide.**

The lipopolysaccharides of *Salmonella* and related Gram-negative bacteria are major components of the outer membrane of the cell envelope (1, 2), and are composed of complex polysaccharide chains covalently attached to a unique lipid containing glucosamine, Lipid A (Fig. 1). The mechanisms of biosynthesis of the outer core and *O*-antigen regions of the polysaccharide are now well established (3), but little is yet known about biosynthesis of Lipid A and the innermost region of the polysaccharide containing ketodeoxyoctonate (KDO). Experimental approach to this problem has been hampered by the continued absence of known mutants in this region of the polymer. Mutants blocked at various steps in biosynthesis of the polysaccharide, including incorporation of the first heptose residue (Fig. 1*A*), are readily obtained by phage-selection procedures (4). Such mutants are viable, but produce incomplete lipopolysaccharides lacking all parts of the polymer distal to the site of the biosynthetic lesion. Failure of conventional selection techniques to yield mutants defective in synthesis of the KDO or Lipid A region of the polymer suggested that such mutations are lethal and that the normal $(KDO)_3$–Lipid A structure (Fig. 1*B*) is essential for maintenance of the structural or functional integrity of the cell.

Mutants conditionally defective in biosynthesis of specific sugar constituents of the polysaccharide have been a major tool in previous biosynthetic studies (3), and we therefore sought to isolate analogous conditional mutants in synthesis of KDO. The pathway of biosynthesis of KDO involves a series of three reactions, catalyzed, respectively, by D-ribulose-5-P isomerase, KDO-8-P synthetase, and KDO-8-P phosphatase (5, 6):

$$\text{D-ribulose-5-P} \rightleftharpoons \text{D-arabinose-5-P} \quad [1]$$

$$\text{D-arabinose-5-P} + \text{phosphoenolpyruvate} \rightarrow \text{KDO-8-P} + P_i \quad [2]$$

$$\text{KDO-8-P} \rightarrow \text{KDO} + P_i \quad [3]$$

Finally, free KDO is converted to the nucleotide–sugar, CMP-KDO (7), the donor of KDO residues in lipopolysaccharide biosynthesis (8). We report here the isolation and properties of a D-arabinose-5-phosphate-dependent *Salmonella typhimurium* with an altered KDO-8-P synthetase.

MATERIALS AND METHODS

S. typhimurium PRX was derived from SA722 (9) and had the following genotype: HfrK10, ade$^-$thi$^-$, *galE*$^-$, phosphotransferase system I-negative (pts-I$^-$), hexose-phosphate transport (*uhp*) constitutive. The UDP-galactose-4-epimerase mutation (*gal*E) was introduced by transduction with phage P22 from strain G30 (10), and the constitutive *uhp* character was obtained as described by Dietz and Heppel (11). Cultures were grown at 37° with vigorous aeration in proteose peptone–beef extract medium (PPBE) (12) containing 0.05 volume of a 10 times-concentrated solution of M9 salts (13). D-Arabinose-5-P was present at a concentration of 0.2 mM unless indicated otherwise.

Lipopolysaccharide was purified according to Romeo *et al.* (14). D-Arabinose-5-phosphate and D-[^{3}H]arabinose-5-phosphate were synthesized from D-glucosamine-6-phosphate and D-[^{3}H]glucosamine-6-phosphate (15). The preparations were free of detectable contamination, as determined by high-voltage paper electrophoresis (pyridine–acetic acid–H_2O, 1:10:89, pH 3.5) and by paper chromatography (butanol–95% ethanol–H_2O, 52:32:16) after dephosphorylation with *Escherichia coli* alkaline phosphatase (Worthington Biochemical Co.). In addition, the preparations contained less than 3% keto-sugar as determined by the cysteine-carbazole method (16). All other reagents were commercial products.

RESULTS

Isolation of a mutant conditionally defective in synthesis of KDO-8-P

The selection was based on the assumption that mutation in the pathway of biosynthesis of KDO would result in a phenotype requiring KDO, in which growth as well as synthesis of a normal lipopolysaccharide would be dependent on an exogenous source of KDO. Unfortunately, KDO itself was taken up poorly by our strains of *S. typhimurium*, and efforts

Abbreviation; KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-octulosonate).

to isolate derivatives with improved transport of KDO were unsuccessful. The finding that the obligatory precursor of KDO, D-arabinose-5-P, is a substrate (though not an inducer) of the hexosephosphate permease (P. D. Rick and M. J. Osborn, unpublished observations) suggested the use of this precursor as supplement. D-Arabinose-5-P is not used as carbon source for *S. typhimurium*, and preliminary studies indicated that it is used essentially exclusively for synthesis of KDO. After growth of the parental strain, PRX, for two generations in the presence of 0.05 mM D-[5-^{3}H]arabinose-5-P, 10% of the radioactivity was incorporated into acid-precipitable material, and this was recovered quantitatively in the purified lipopolysaccharide. After removal of Lipid A by mild acid hydrolysis (17), over 50% of the total ^{3}H was found in free KDO, and the remainder was associated with anionic components electrophoretically similar to polysaccharide fractions containing reducing-terminal KDO.

The parent strain, PRX, was UDP-galactose epimerase-negative and hexosephosphate-transport constitutive. Cells were mutagenized with ethylmethane sulfonate (18) and grown overnight in PPBE medium containing 0.1 mM D-arabinose-5-P. Phage selection was then used to enrich for the desired conditional KDO-negative phenotype. The procedure was based on the properties of UDP-galactose epimerase mutants. In the absence of exogenous galactose, such mutants produce incomplete lipopolysaccharides lacking *O*-antigen chains as well as the distal portion of the core (Fig. 1*A*), and are resistant to phages such as 9NA that require *O*-antigen for adsorption (19). Addition of galactose to the medium initiates synthesis of complete lipopolysaccharide, and the cells rapidly become sensitive to *O*-specific phage. However, superimposition of a second mutation in KDO synthesis should prevent galactose-dependent formation of complete lipopolysaccharide, and, in the absence of D-arabinose-5-P, the double mutant should remain resistant to phage 9NA. Accordingly, the culture was grown for 3.5 generations in the presence of 0.1 mM D-arabinose-5-P and 1 mM D-fucose (to induce the galactose operon). Chloramphenicol (100 μg/ml) was then added, and the cells were harvested and depleted of internal D-arabinose-5-P by washing with 0.9% NaCl containing 100 μg of chloramphenical per ml and 0.5 mM fructose-6-P. Fructose-6-P is also a substrate for hexose-phosphate permease, and exchanges rapidly with the intracellular pool of D-arabinose-5-P. Chloramphenicol was added throughout the period of starvation for D-arabinose-5-P, since it was not known whether residual growth under nonpermissive conditions would result in suicide of the desired mutant. In fact, this does not occur (see below). Synthesis of lipopolysaccharide *de novo* continues in the presence of chloramphenicol for prolonged periods of time (20). The washed cells were suspended in fresh medium containing chloramphenicol but lacking D-arabinose-5-P and incubated 30 min at 37° to permit expression of the KDO-negative phenotype. Galactose (1 mM) was then added and incubation was continued for 60 min. At this time phage 9NA was added (multiplicity = 30) and permitted to adsorb for 15 min. Killing was greater than 90%. Survivors were washed to remove free phage, plated on PPBE agar containing 0.1 mM D-arabinose-5-P, and replicated to plates lacking this supplement. Of 6000 colonies screened, three were dependent on D-arabinose-5-P for growth. One of these, PRX2, was found to have an altered KDO-8-P synthetase, and is described here. The other two mutants, which were appreciably leaky in their requirement for D-arabinose-5-P, have not yet been investigated further.

Spontaneous revertants of strain PRX2 were also isolated in order to establish whether the observed effects on lipopolysaccharide synthesis and growth were due to a single mutation. About 1.5×10^6 cells were plated on PPBE agar in the absence of exogenous D-arabinose-5-P, and revertant colonies were picked after 36–48 hr of incubation. The spontaneous reversion frequency was about 1×10^{-6}. The characteristics of one of the revertant organisms, PRX2R, are presented.

Effect of D-arabinose-5-P on growth and viability of strain PRX2

The effect of D-arabinose-5-P starvation on the growth of PRX2 is shown in Fig. 2. The generation time of the mutant was 40 min in PPBE-salts medium supplemented with 0.2 mM D-arabinose-5-P, and was similar to that of the parent. After removal of D-arabinose-5-P, growth continued at the normal rate for about one generation, and then rapidly declined to a final doubling time of about 5 hr. The viable count paralleled the increase in turbidity, and no loss in viability was observed over a period of 5 hr in the absence of D-arabinose-5-P. In separate experiments, growth resumed with little lag when D-arabinose-5-P was added again to cultures after 5 hr.

The requirement for D-arabinose-5-P was highly specific. Other pentose- and hexose-phosphates, D- and L-arabinose, and various common metabolites were inactive. KDO was also ineffective in supporting growth, presumably because of poor uptake coupled with the rapid degradation of the compound by an induced KDO aldolase (21).

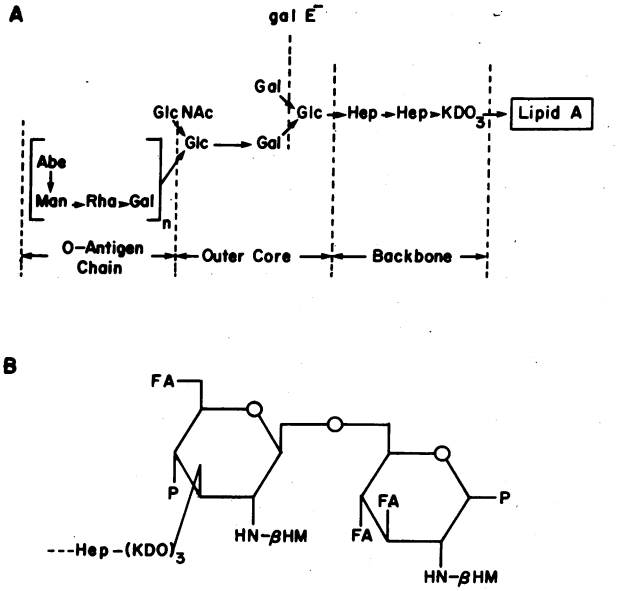

FIG. 1. (*A*) Structure of the lipopolysaccharide of *S. typhimurium* (3). Noncarbohydrate substituents (phosphate, ethanolamine) are omitted. The point of termination of the incomplete lipopolysaccharide of *galE*⁻ mutants is as indicated. (*B*) Structure of Lipid A. (26, 27). The abbreviations are as follows: KDO, 3-deoxy-D-mannoctulosonate; FA, fatty-acid ester (palmitate, myristate, laurate); βHM, β-hydroxymyristate: P, phosphate; Hep, L-glycero-D-mannoheptose; Abe, abequose; Man, D-mannose; Rha, L-rhamnose: Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Glc, D-glucose.

Effect of exogenous D-arabinose-5-P on lipopolysaccharide synthesis *in vivo*

Incorporation of [^{14}C]galactose was used as index of the effect of D-arabinose-5-P deprival on lipopolysaccharide synthesis *in vivo*. Strains PRX and PRX2 lack UDP-galactose-4-epimerase, and added [^{14}C]galactose is incorporated exclusively into the core and *O*-antigen of lipopolysaccharide (10, 22). It should be emphasized that galactose is not incorporated into previously existing, incomplete core lipopolysaccharide formed before addition of the sugar (23), and incorporation is therefore a specific index of *de novo* synthesis of polysaccharide chains after the addition of galactose. Since incorporation of galactose into lipopolysaccharide requires synthesis of the KDO–trisaccharide region of the molecule (Fig. 1), mutants defective in KDO synthesis should lack acceptor sites for the incorporation of galactose.

In order to divorce direct effects of D-arabinose-5-P deprival on lipopolysaccharide synthesis from possible secondary effects due to changes in growth rate, incorporation of [^{14}C]-galactose was measured in the presence of chloramphenicol (Fig. 3). Cultures of the parent, mutant, and revertant strains were grown in the presence of D-arabinose-5-P, washed, and incubated in fresh medium lacking D-arabinose-5-P for 30 min, at which time the growth rate of the mutant was still normal (see Fig. 2). Chloramphenicol was then added, and after 5 min, the cultures were divided into three parts. [^{14}C]Galactose was added either alone or together with D-arabinose-5-P or KDO. Under these conditions, incorporation of galactose into lipopolysaccharide continued at approximately linear rates for at least 2 hr. As predicted, the ability of the mutant, PRX2, to incorporate [^{14}C]galactose into lipopolysaccharide was severely reduced after a 30-min period of starvation for D-arabinose-5-P, and was completely restored by addition of this sugar (Fig. 3*B*). KDO was also effective in restoring incorporation of [^{14}C]galactose, presumably because chloramphenicol prevented induction of the degradative KDO aldolase. The rate of [^{14}C]galactose incorporation into lipopolysaccharide by parent (Fig. 3*A*) and revertant (Fig. 3*C*) strains

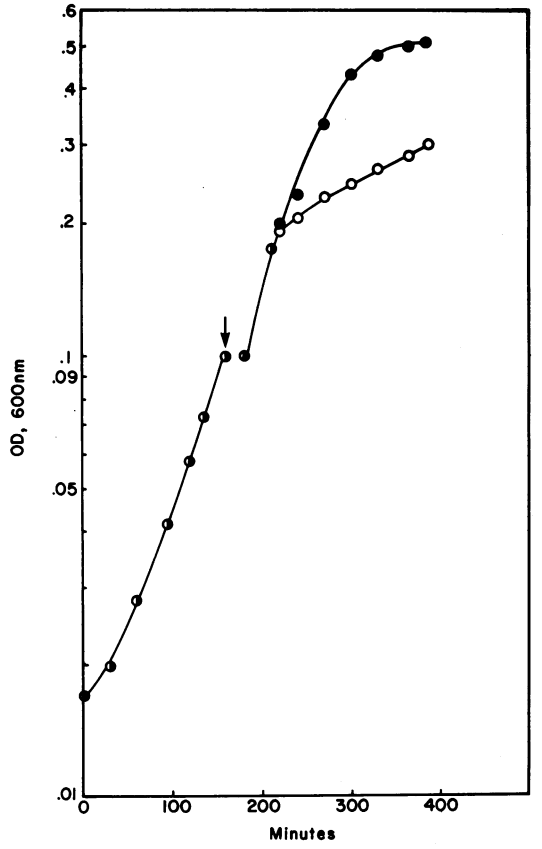

FIG. 2. Effects of D-arabinose-5-P starvation on the growth of *S. typhimurium*, strain PRX2. A culture was grown in PPBE-salts medium (see *Methods*) containing D-arabinose-5-P (0.2 mM). The culture was grown at 37° with vigorous aeration to a density of about 3 × 10^8 bacteria per ml (*arrow*). The cells were then isolated by centrifugation at 30° and washed with PPBE-salts medium in the absence of D-arabinose-5-P. The culture was then divided in half, and the resulting cultures were suspended in fresh medium in the presence and absence of D-arabinose-5-P (0.2 mM). The density of each culture was adjusted to 3.0 × 10^8 bacteria per ml, and the cultures were incubated at 37° with vigorous aeration. Growth was monitored turbidimetrically at 600 nm with a Coleman Jr. II spectrophotometer. With D-arabinose-5-P, ●——●; without D-arabinose-5-P, ○——○.

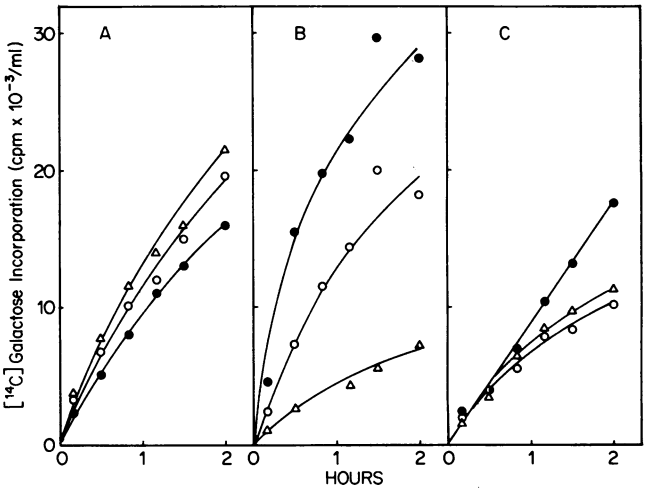

FIG. 3. [^{14}C]Galactose incorporation into lipopolysaccharide. Cultures of the parent (*A*), mutant (*B*), and revertant (*C*) strains were grown at 37° with vigorous aeration in PPBE-salts medium containing D-arabinose-5-P (0.2 mM) and D-fucose (1 mM). At a density of about 3 × 10^8 bacteria per ml the cells were harvested by centrifugation at 30° and washed with PPBE-salts medium in the absence of D-arabinose-5-P. The washed cells were resuspended in fresh medium without D-arabinose-5-P and adjusted to a density of 3 × 10^8 bacteria per ml. The cultures were then incubated at 37° with vigorous aeration. Growth of the mutant in the absence of D-arabinose-5-P continued at a normal rate for one generation (40 min). When the cultures reached a density of 5.4 × 10^8 bacteria per ml (30 min), chloramphenicol was added to a final concentration of 100 μg/ml and the cultures were further incubated for 5 min. To 7 ml of each culture was then added [^{14}C]galactose (3 Ci/mol) to a final concentration of 0.25 mM plus the following: D-arabinose-5-P (0.2 mM), ●——●; KDO (6 mM), ○——○; no additions, ▲——▲. Aliquots of 1 ml were removed from each culture at the indicated times and assayed for incorporation of [^{14}C]galactose into lipopolysaccharide (4).

TABLE 1. *Enzymatic synthesis of KDO from* D-*ribulose-5-P*

Source of enzyme	KDO formed (μmol/30 min per mg of protein)
PRX	0.344
PRX2	<0.001

The synthesis of KDO from D-ribulose-5-P was assayed in extracts of the parent and mutant strain as follows. Cell extracts were prepared by sonication in 100 mM glycylglycine buffer (pH 8.0)–1 mM EDTA. The soluble fraction obtained by centrifugation at 98,000 × *g* for 90 min was used as enzyme. Reaction mixtures contained the following: 250 mM glycylglycine (pH 8.0), 4.7 mM D-ribulose-5-P (Na^+), 6.25 mM phosphoenolpyruvate (tricyclohexylammonium salt), and enzyme to a final volume of 0.4 ml. Reaction mixtures were incubated at 37° for 30 min, and reactions were stopped by addition of 0.1 ml of the reaction mixture to 0.15 ml of cold 10% trichloroacetic acid. The resulting precipitate was removed by centrifugation, and 0.1 ml of the supernatant solution was assayed for KDO by the thiobarbituric acid assay (17). Heat-inactivated enzyme was used for a blank. Protein was determined by the method of Lowry *et al.* (24).

were similar and were not markedly influenced by addition of D-arabinose-5-P or KDO. Indeed, the incorporation in the parent was somewhat inhibited by D-arabinose-5-P.

Identification of the enzymatic defect in strain PRX2

We had assumed that selection for the D-arabinose-5-P-dependent phenotype would preferentially yield mutants in D-ribulose-5-P isomerase, the enzyme responsible for synthesis of the required sugar (reaction 1). Indeed, formation of KDO from D-ribulose-5-P could not be detected in crude extracts of PRX2 (Table 1). This reaction, which requires the combined activity of the isomerase and KDO-8-P synthetase (reactions 1 + 2), was readily demonstrated in extracts of the parent, PRX. However, in direct assays of D-ribulose-5-P isomerase (Table 2), the activity of the mutant was identical to that of the parent. The ability of the mutant to synthesize D-arabinose-5-P thus appeared to be unimpaired.

Further analysis showed that PRX2 contains an altered KDO-8-P synthetase with an extremely high K_m for D-arabinose-5-P. At a concentration of D-arabinose-5-P of 1 mM, which was essentially saturating for the parental enzyme, extracts of PRX2 showed less than 10% of the wild-type KDO-8-P synthetase activity (Table 2). The activity of the wild type was not inhibited by addition of mutant extract. Apparent K_m values for D-arabinose-5-P were determined from Lineweaver–Burk plots (Fig. 4) and are summarized in Table 2. The value obtained for the mutant, 6 mM, is about 35-fold higher than that observed in the parent (0.17 mM). The apparent V_{max} of the synthetase was not markedly affected by the mutation (Table 2).

Strain PRX2R was isolated as a spontaneous revertant that no longer required D-arabinose-5-P for growth, and incorporation of [^{14}C]galactose into lipopolysaccharide was also independent of added D-arabinose-5-P in this strain (Fig. 3*C*). The growth rate of PRX2R and PRX were found to be the same in the presence or absence of exogenous D-arabinose-5-P. The activity of KDO-8-P synthetase, at a D-arabinose-5-P concentration of 1 mM, was restored to about 55% that of the wild type (Table 2). Interestingly, however, kinetic analysis (Fig. 4*C*, Table 2) yielded an apparent K_m for D-arabinose-5-P

TABLE 2. *Enzyme activities and kinetic parameters*

Enzyme	Strain	Specific activity (nmol/min per mg)	Apparent K_m for D-arabinose-5-P (mM)	Apparent V_{max} (nmol/min per mg)
KDO-8-P synthetase	PRX	51.0*	0.17	51
	PRX2	4.5*	6.0	33
	PRX2R	27.5*	6.0	218
D-Ribulose-5-P isomerase	PRX	52.8	—	—
	PRX2	50.1	—	-

KDO-8-P synthetase was assayed as described in the legend to Fig. 4. Cell extracts for the assay of D-ribulose-5-P isomerase were prepared in the same manner and the activity was determined as described by Volk (25).

* Specific activity at a concentration of 1.0 mM D-arabinose-5-P.

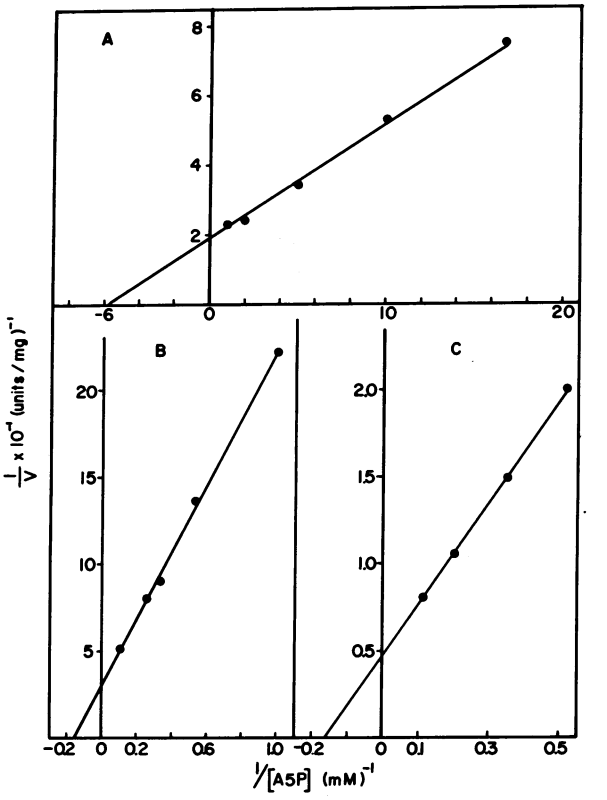

FIG. 4. Double reciprocal plots for KDO-8-P synthetase with respect to D-arabinose-5-P (A5P). The activity of the KDO-8-P synthetase of the parent (*A*), mutant (*B*), and revertant (*C*) strains was determined as follows. Cell extracts were prepared by sonication in 100 mM Tris·HCl (pH 7.5)–1 mM EDTA. The soluble fraction obtained by centrifugation at 98,000 × *g* for 90 min was used as enzyme. Reaction mixtures contained the following: 100 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, 50 mM phosphoenolpyruvate (tricyclohexylammonium salt), D-arabinose-5-P (Na^+), enzyme, and H_2O to a final volume of 0.5 ml. Reaction mixtures were incubated at 37° for 10 min. Formation of KDO was measured as described in the legend to Table 1. One unit of activity is defined as the formation of 1 μmol of product per min.

identical to that of the mutant synthetase of PRX2. The independence of PRX2R from D-arabinose-5-P appears rather to be due to a marked elevation in the apparent V_{max} of the synthetase reaction. This was increased 4-fold over that of wild-type PRX extracts and 7-fold over that of PRX2 extracts. It is not yet known whether the higher apparent V_{max} in PRX2R results from an increase in catalytic activity of the enzyme or from an increase in its amount. Genetic control of the activities of enzymes specifically related to lipopolysaccharide synthesis has not been described.

DISCUSSION

We describe here the isolation of a new class of rough mutant of *S. typhimurium*, defective in the synthesis of the KDO-region of the lipopolysaccharide. The mutation results in dependence on exogenous D-arabinose-5-P for growth as well as for synthesis of a normal lipopolysaccharide. Evidence is presented that the mutant phenotype is due to the synthesis of an altered KDO-8-P synthetase, with an apparent K_m for D-arabinose-5-P 35-fold higher than that of the parent strain. Although the ability of the mutant to synthesize D-arabinose-5-P appeared to be unimpaired, the normal rate of synthesis of this pentose phosphate is apparently insufficient to compensate for the mutant defect. Thus, in the absence of a high intracellular concentration of D-arabinose-5-P, the synthesis of KDO-8-P in the mutant strain is effectively blocked. The constitutive hexose-P permease of the mutant strain allows transport of D-arabinose-5-P into the cell to an intracellular concentration of about 11.5 mM (P. D. Rick, unpublished results). Thus, addition of D-arabinose-5-P to cultures of the mutant results in phenotypic repair of the lesion. Added D-arabinose-5-P is not an effective carbon source, and appears to be used solely for synthesis of KDO.

Although we have not yet mapped the KDO-8-P synthetase mutation in *S. typhimurium*, the properties of the revertant indicate that the pleiotropic effects on lipopolysaccharide synthesis and growth are due to a mutation in the KDO-8-P synthetase. These data suggest strongly that the structural integrity of the KDO–Lipid A region of the lipopolysaccharide is essential for growth. Early observations of Heath *et al.* (8) suggest the possibility that KDO is incorporated at an early stage in Lipid A synthesis, before introduction of *O*-fatty acyl residues (see Fig. 1*B*). In this case, mutants unable to incorporate KDO should produce an incomplete, underacylated Lipid A.

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