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Relationship Between Cell Death and Altered Lipid A Synthesis in a Temperature-Sensitive Lethal Mutant of *Salmonella typhimurium* That Is Conditionally Defective in 3-Deoxy-D-Manno-Octulosonate-8-Phosphate Synthesis

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The relationship between the inability to synthesize a complete 3-deoxy-D-manno-octulosonate region of lipopolysaccharide and cell death was investigated in a temperature-sensitive lethal mutant of *Salmonella typhimurium*. The defect in lipopolysaccharide synthesis is due to a mutation in the structural gene for 3-deoxy-D-manno-octulosonate-8-phosphate synthetase (designated *kdsA*) and results in the synthesis of a temperature-sensitive enzyme. Expression of the *kdsA* lesion at elevated temperatures, at which the synthesis of 3-deoxy-D-manno-octulosonate is complete blocked, is required for expression of the temperature-sensitive lethal phenotype. However, the defect in lipopolysaccharide synthesis is not alone sufficient cause for the observed cell death. Genetic evidence is presented which indicates that the mutant possesses a second mutation, or possibly multiple mutations, whose lethal expression is dependent on the inability of the mutant to synthesize a fully acylated and 3-deoxy-D-manno-octulosonate-substituted lipid A portion of lipopolysaccharide at elevated temperatures.

The accompanying paper (15) described the isolation and characterization of a new mutant of *Salmonella typhimurium* conditionally defective in the synthesis of the 3-deoxy-D-manno-octulosonate (KDO) region of the lipopolysaccharide (15). Biochemical evidence established that the defect in lipopolysaccharide synthesis is due to a temperature-sensitive 3-deoxy-D-manno-octulosonate-8-phosphate (KDO-8-P) synthetase. The inability of the mutant to synthesize KDO-8-P under nonpermissive conditions results in the accumulation of an incomplete and underacylated lipid A. Characterization of the incomplete lipid A indicates that it appears to be identical in structure to the lipid A precursor accumulated under nonpermissive conditions by previously reported mutants of *S. typhimurium* conditionally defective in KDO-8-P synthesis (6, 12). However, the new mutant suffers a pronounced loss of viability at temperatures nonpermissive for the synthesis of KDO-8-P. In contrast, all previously characterized mutants temperature sensitive in KDO-8-P synthesis only undergo growth inhibition at nonpermissive conditions, and no loss in viability is observed even after extended periods of time at elevated temperatures (7, 14).

We present here evidence that the defect in KDO-8-P synthesis in the new mutant, *S. typhimurium* PR32, is required but not alone sufficient for expression of the temperature-sensitive lethal phenotype. Data are presented which indicate that the mutant strain possesses an additional unknown mutation, or possibly multiple defects, whose lethal expression is dependent on the inability to synthesize a fully acylated and KDO-substituted lipid A at elevated temperatures.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains relevant to the present study are listed in Table 1. Cultures were grown with vigorous aeration in proteose-peptone-beef extract (PPBE) medium (16) or M9 minimal medium (8) as indicated.

Chemicals and isotopically labeled compounds. D-[6-^{3}H]glucosamine (19 Ci/mmol), $^{32}P_i$, and Liquifluor were purchased from New England Nuclear Corp. D-Arabinose-5-phosphate was synthesized from D-glucosamine-6-phosphate (17). Biosolv BBS-3 was purchased from Beckman Instruments, Inc. All other chemicals were obtained from standard commercial sources.

Genetic methods. Transductions were carried out with phage P22HT4. Recipient strains were grown in PPBE broth to a density of 5×10^8 cells per ml and then concentrated 10-fold in the same medium. Recipient strains possessing a *galE* lesion were grown in media supplemented with 0.1 mM D-galactose to allow synthesis of phage receptors. Strain HD50 was grown in the presence of 15 μM D-galactose since higher concentrations of galactose resulted in galactose toxicity. Phage were added at a multiplicity of infection of

TABLE 1. Characteristics of *S. typhimurium* strains

Strain	Relevant properties	Source or Reference
SU453	*hisF1009 trpB2 metA22 xyl-1 strA* F⁻	SGSC[a]
PR122	*galE nag* derivative of SU453	(15)
PR32	*kdsA* derivative of PR122	(15)
PR32-TR5	Tn*10*-*kdsA*$^+$ derivative of PR32[b]	This work
PR32-TS5	Tn*10*-*kdsA* derivative of PR32[b]	This work
HD50	*kdsA galE fadE nag metA trpE hisF xyl ilvA pyrE malA strA*	M. J. Osborn
LT2	Wild type	M. J. Osborn
G30	*galE*	M. J. Osborn

[a] Salmonella Genetic Stock Centre; K. E. Sanderson, Department of Biology, University of Calgary, Calgary, Alberta T2N 1N4, Canada.

[b] The designation Tn*10*-*kdsA* indicates that the transposon Tn*10* is located near the *kdsA* locus at min 39 and was used for introduction of the relevant *kdsA* allele into the desired genetic backgrounds by cotransduction with selection for Tet^r.

0.8, and the suspensions were incubated at 30°C for 20 min. Samples of the suspension were then plated on PPBE agar medium under selective conditions. Temperature-resistant transductants were selected on PPBE plates after 24 h of incubation at 42°C. Tetracycline-resistant transductants were selected on PPBE plates containing tetracycline at a final concentration of 25 μg/ml. All PPBE plates used for transductions contained ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetracetic acid (10 mM) to prevent secondary phage adsorption. Transductants were purified and checked for recipient markers and P22 sensitivity.

Insertion of the transposable element Tn*10* carrying tetracycline resistance (Tet^r) into strain PR32 at a locus 90% contransducible with the *kdsA* locus was accomplished by using phage P22 *c*2ts29 12am*N*11 13*H*101 *int-3* Tn*10* by the general method of Davis et al. (3). Tetracycline-resistant transductants were screened for temperature-resistant growth at 42°C, purified, and checked for nonlysogeny. Linkage of Tn*10* to the *kdsA* locus was established by determining the frequencies of cotransduction of Tet^r with temperature-resistant growth at 42°C by employing the Tet^r transductants and strain PR32 as donors and recipient, respectively. One of the transductants, strain PR32-TR5, carried Tn*10* inserted in the chromosome at a locus 90% cotransducible with the wild-type *kdsA* allele. Strain PR32-TR5 grew at wild-type rates at 42°C and did not lose viability or accumulate detectable lipid A precursor at 42°C.

Strains PR32-TR5 and PR32 were used as donor and recipient, respectively, in order to isolate temperature-sensitive transductants of strain PR32 with Tn*10* closely linked to the $kdsA_{PR32}$ allele. Tet^r transductants of strain PR32 were obtained at a frequency of approximately 5×10^{-5}, and Tet^r was 10% cotransducible with temperature-sensitive growth at 42°C. One of the temperature-sensitive transductants, strain PR32-TS5, was employed as the donor for the transduction of the $kdsA_{PR32}$ allele into other genetic backgrounds.

The proximity of Tn*10* to the *kdsA* locus was confirmed by employing strain PR32-TR5 as donor in the phage P22-mediated transduction of Tet^r into the temperature-sensitive KDO-8-P synthetase mutant, *S. typhimurium* HD50. Tet^r transductants of strain HD50 were obtained at a frequency of approximately 10^{-5}, and Tet^r was 90% cotransducible with temperature-resistant growth. The corresponding Tet^r temperature-sensitive transductants of strain HD50 carried Tet^r at a locus 90% cotransducible with the $kdsA_{HD50}$ allele and were employed as donors for the phage P22-mediated transduction of this allele into other genetic backgrounds.

Determination of phospholipid compositions. Cultures were grown at 28°C in 30 ml of PPBE medium to an absorbancy at 660 nm of 0.3. The cultures were then divided into two 10-ml portions. One culture was maintained at 28°C, whereas the other culture was shifted to 42°C. Carrier-free $^{32}P_i$ (55 μCi) was added to each culture at the time of the shift, and the cultures were incubated with shaking for 3 h. Phospholipids were then extracted from the cells by the method of Bligh and Dyer (2) as modified by Ames (1). Briefly, the cells were harvested by centrifugation, and the cell pellets were suspended in 0.8 ml of phosphate-buffered saline (4), after which 2 ml of methanol and 1 ml of chloroform were added with thorough mixing. The resulting suspensions were allowed to stand at room temperature for 1 h, and then 1 ml of chloroform and 1 ml of phosphate-buffered saline were added with thorough mixing. The mixtures were next subjected to a brief low-speed centrifugation, and the labeled phospholipids present in the lower chloroform phase were separated by one-dimensional thin-layer chromatography on glass-backed LHP-K plates (Whatman, Inc.) with chloroform–methanol–acetic acid (65:25:10, vol/vol/vol) as the solvent. Individual phospholipid classes were visualized by autoradiography employing Kodak XR-5 medical X-ray film. Radioactive areas were scraped from the plate, and the amount of isotope in each phospholipid class was analyzed by liquid scintillation counting with toluene-Liquifluor (15).

Rates of total phospholipid synthesis. Cultures of PR32 were grown at 28°C to an absorbancy at 600 nm of 0.2 in M9 minimal medium supplemented with glucose (0.2%) and 50 μg of each required amino acid per ml. The cultures were then divided into two equal portions; one portion was maintained at 28°C, whereas the other portion was shifted to 42°C. Immediately after the shift, 1 ml of the culture maintained at 28°C and 1 ml of the culture maintained at 42°C were pulse-labeled for 1 min with carrier-free [2-^{3}H]glycerol (10 μCi, 10 Ci/mmol) at 28°C and 42°C, respectively. The incorporation of isotope was terminated by the addi-

tion of 1.0 ml of cold 10% trichloroacetic acid to each sample, and the precipitates were collected on membrane filters (Millipore Corp., type HA, 0.45 μm) by suction filtration. The precipitates were washed with cold 5% trichloroacetic acid, and the filters were then dried and counted as described in the accompanying paper (15). Pulse-labeling of each culture was carried out in the same manner at 30-min intervals over a period of 3 h.

Paper chromatography. Ascending chromatography was carried out on Whatman no. 1 paper employing 1-butanol–isobutyric acid–1 N NH_4OH (10:28:15, vol/vol/vol) as the solvent system. Isotopically labeled compounds were located on chromatograms by sectioning strips of the chromatograms into 1-cm segments and determining the amount of radioactivity in the segments as described below.

Counting procedures. Counting procedures were as described in the accompanying paper (15).

RESULTS

Characterization of temperature-resistant transductants. The inability to synthesize a complete lipid A at elevated temperatures by the temperature-sensitive KDO-8-P synthetase mutant, *S. typhimurium* PR32, is accompanied by cell death (15). Evidence that the temperature-sensitive mutation in KDO-8-P synthesis is intimately associated with the temperature-sensitive lethal phenotype was obtained by analysis of transductants of strain PR32 temperature-resistant in growth. Transductions were carried out with phage P22HT4 as described above, and temperature-resistant transductants were obtained at a frequency of approximately 10^{-5}. Examination of the KDO-8-P synthetase activity in crude extracts of two of the transductants revealed that enzyme activity was restored to wild-type levels when assayed at 37°C (Table 2). In addition, the transductants showed wild-type growth rates at 42°C (data not shown), and no accumulation of lipid A precursor was detected in either of the transductants when mid-log-phase cultures growing at 30°C were shifted to 42°C for 2 h. (Fig. 1).

Effect of exogenous D-arabinose-5-phosphate on cell viability and lipopolysaccharide synthesis. Previous studies have shown that the activity of the temperature-sensitive KDO-8-P synthetase of strain PR32 is significantly stabilized in cell-free extracts at elevated temperatures by relatively high concentrations of D-arabinose-5-phosphate (15). In addition, D-arabinose-5-phosphate is a substrate, but not an inducer, of the hexose-phosphate transport system (5), and prior induction of this system allows the transport of exogenous D-arabinose-5-phosphate (0.2 mM) to an intracellular concentration of about 11.5 mM (13). These observations suggested the possibility that exogenously supplied D-arabinose-5-phosphate might also effect stabilization of enzyme activity at nonpermissive temperatures in vivo and result in the rescue of both cell viability and lipopolysaccharide synthesis. The effect of exogenously supplied D-arabinose-5-phosphate

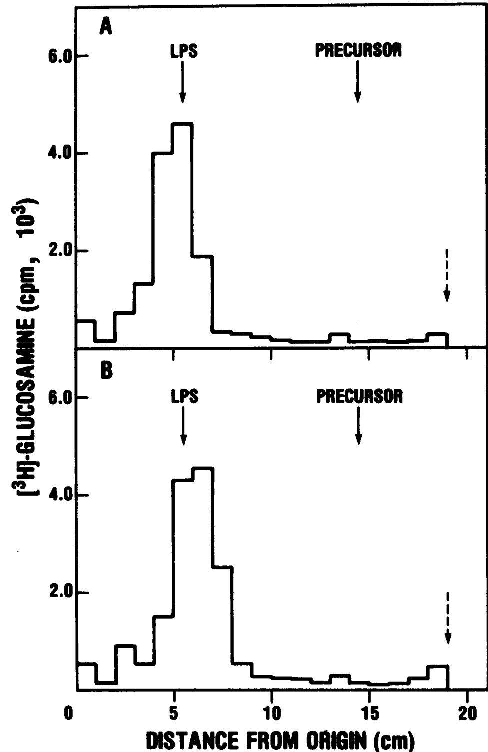

FIG. 1. Paper chromatography of radioactive material present in butanol-pyridinium acetate extracts of temperature-resistant transductants of strain PR32 incubated at 42°C in the presence of [^{3}H]glucosamine. Cultures were grown in PPBE broth to an absorbancy at 600 nm of 0.5 and then shifted to 42°C in the presence of D-[6-^{3}H]glucosamine (10 μCi/mmol). After 5 h at 42°C, 10 ml of each culture was extracted with butanol-pyridinium acetate as previously described (15), and the extracts were analyzed by ascending paper chromatography as described in the text. A, transductant 1; B, transductant 2. The mobilities of R_c-lipopolysaccharide and lipid A precursor are indicated by the solid arrows. Solvent fronts are indicated by the broken arrows.

TABLE 2. KDO-8-P synthetase activity of temperature-resistant transductants[a]

Strain	Sp act at 37°C (nmol/min per mg)
Mutant (PR32)	3.8
Donor (G30)	27.8
Parent (PR122)	33.3
Transductant 1	33.4
Transductant 2	35.1

[a] Enzyme activities were determined as previously described (13).

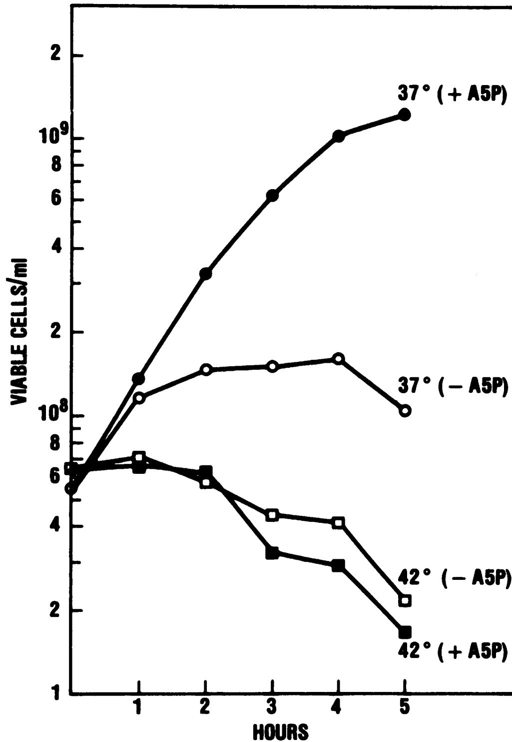

FIG. 2. Effect of exogenous D-arabinose-5-phosphate on the viability of strain PR32 at nonpermissive temperatures. Cultures were grown at 30°C in PPBE broth containing fructose-6-phosphate (0.1 mM) to a cell density of about 6×10^7 cells per ml. The cultures were then shifted to either 37 or 42°C in the presence or absence of D-arabinose-5-phosphate (0.2 mM) as indicated. Colony-forming units were determined at the indicated times by plating dilutions of the cultures onto PPBE agar plates and incubating the plates at 30°C for 36 h.

on the viability of strain PR32 at 37 and 42°C is shown in Fig. 2. The number of colony-forming units continued to increase normally when cells growing at 30°C were shifted to 37°C in media supplemented with D-arabinose-5-phosphate (0.2 mM). However, a shift of cultures to 37°C in the absence of exogenously supplied D-arabinose-5-phosphate resulted in a marked reduction in the rate of viable cell increase, and after 4 h viability began to decrease. In contrast, cell viability was not rescued by exogenously supplied D-arabinose-5-phosphate when cultures were maintained at 42°C.

The effect of exogenously supplied D-arabinose-5-phosphate on the relative rates of lipopolysaccharide and lipid A precursor synthesis at temperatures permissive (30°C) and nonpermissive (37°C, 42°C) for growth and viability is shown in Fig. 3. In the absence of D-arabinose-5-phosphate supplementation, the ratio of lipid A precursor to lipopolysaccharide increased from 2.7 at 30°C to 8.1 and 32.3 at 37 and 42°C, respectively. The addition of D-arabinose-5-phosphate (0.2 mM) to cultures maintained at either 30 or 37°C resulted in almost complete rescue of lipopolysaccharide synthesis. However, D-arabinose-5-phosphate supplementation of cultures maintained at 42°C resulted in only partial rescue of lipopolysaccharide synthesis, and the ratio of precursor to lipopolysaccharide was 0.92.

Relationship of the *kdsA* defect to expression of the temperature-sensitive lethal phenotype. To determine whether the temperature-sensitive lethal phenotype is due solely to expression of the

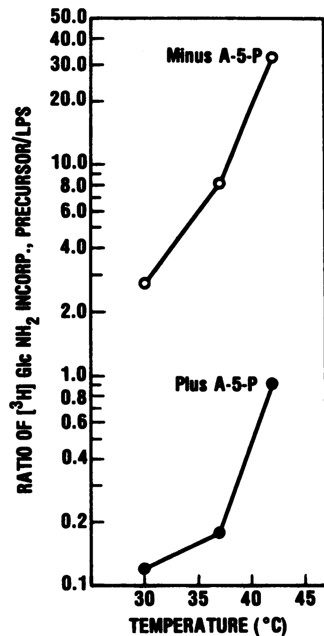

FIG. 3. Effect of temperature and exogenously supplied D-arabinose-5-phosphate on the synthesis of lipopolysaccharide and lipid A precursor in strain PR32. Cultures were grown in PPBE broth containing fructose-6-phosphate (0.1 mM) at 30°C to an absorbancy at 600 nm of 0.5. For each experiment at a given temperature, the cultures were then divided into two equal portions; one portion was supplemented with D-arabinose-5-phosphate (A-5-P) to a final concentration of 0.2 mM (●), and the other portion was not supplemented (O). Both cultures were then incubated at the indicated temperatures in the presence of D-[6-^{3}H]glucosamine (50 μM, 100 μCi/μmol) for 60 min. Samples (10 ml) of each culture were then extracted with butanol-pyridinium acetate as previously described (15), and the relative amount of radioactivity in lipopolysaccharide and lipid A precursor was determined after ascending paper-chromatography as described in the text.

TABLE 3. Effect of temperature on cell viability[a]

Strain	Relevant genotype	Mean % survival[b]
PR32	$kdsA_{PR32}$	7.7 ± 4.2
PRX20	$kdsA_{PRX20}$	72.0 ± 7
PR122	$kdsA^+$	85.0 ± 12.1
LT2 transductants	$kdsA_{PR32}$	83.4 ± 13.1

[a] Dilutions of log-phase cultures growing in PPBE broth at 30°C were plated out on PPBE agar plates and incubated at 42°C for 6 h. The plates were then incubated at 30°C, and the number of colonies observed after 36-48 h was compared with the number of colony-forming units observed when corresponding dilutions were plated out directly at 30°C.

[b] Values are based on 10 determinations. In the case of the LT2 transductants, the value represents the mean percent survival of 100 independent transductants.

$kdsA_{PR32}$ defect, the $kdsA_{PR32}$ allele was transduced into a wild-type genetic background, and the effect of expression of this allele on the viability of the resulting transductants was determined. Selection of $kdsA_{PR32}$ transductants was facilitated by insertion of the transposable element Tn*10*, which carries tetracycline resistance, into the chromosome of strain PR32 at a locus 90% cotransducible with the $kdsA_{PR32}$ allele (see above). It was then possible to move the $kdsA_{PR32}$ allele from strain PR32 into wild-type genetic backgrounds by P22 transduction and selection for Tet^r transductants.

When the above approach was employed to transduce the $kdsA_{PR32}$ allele into wild-type *S. typhimurium* LT2, Tet^r transductants were obtained at a frequency of 5×10^{-4}, and Tet^r was 88% cotransducible with temperature-sensitive growth at 42°C. The effect of nonpermissive temperature on the viability of 100 independent temperature-sensitive transductants is shown in Table 3. Temperature-sensitive $kdsA_{PR32}$ transductants of LT2 did not suffer a decrease in viability beyond that observed for either the parental strain (PR122) or the previously characterized *kdsA* mutant, strain PRX20 (14) after incubation at 42°C for 6 h. In contrast, strain PR32 suffered an approximate 90% loss of viability at 42°C, and the number of viable cells after 6 h was approximately 10-fold lower than observed for either the parental strain or the $kdsA_{PR32}$ transductants of LT2.

Thus, the defect in KDO-8-P synthesis alone does not appear to be sufficient for the observed temperature-sensitive lethal phenotype of strain PR32. Rather these results suggest that strain PR32 possesses a second unlinked defect or multiple unlinked defects whose lethal expression is dependent on the inability of the mutant to synthesize KDO at elevated temperatures.

Support for this conclusion was provided by introducing an independent temperature-sensitive lesion in KDO-8-P synthesis into the genetic background of strain PR32 and observing the effect of its expression on cell viability. Accordingly, the *kdsA* lesion of *S. typhimurium* HD50 was transduced into temperature-resistant $kdsA^+$ transductants of strain PR32 by employing the same strategy as described above for transduction of the *kdsA*PR32 allele into wild-type genetic backgrounds (see above). The expression of the $kdsA_{HD50}$ allele at 42°C in transductants possessing the genetic background of strain PR32 resulted in almost total loss of cell viability (Table 4). In contrast, expression of the same allele at 42°C in the wild-type genetic background of *S. typhimurium* LT2 did not result in greater than normal cell death.

The accompanying paper demonstrated that growth and viability of the parental strain PR122 are unaltered at 42°C (15). However, introduction of either the $kdsA_{HD50}$ or $kdsA_{PR32}$ alleles into the chromosome of strain PR122 establishes the temperature-sensitive lethal phenotype characteristic of the mutant strain PR32, and the respective *kdsA* derivatives of strain PR122 suffer a pronounced loss of viability at 42°C (Fig. 4). These observations suggest that the second unknown defect(s) is present in the parental strain and was not induced by the mutagenesis employed for the isolation of strain PR32. In addition, these data provide additional support for the conclusion that the lethal expression of the second defect(s) is dependent on the expression of the *kdsA* lesion at elevated temperatures.

Additional phenotypic characterization. The chromosomal location of the unlinked defect(s) has not been established, and attempts to define the biochemical basis for the temperature-sensitive lethal phenotype of strain PR32 have thus far proved unrevealing. The mutant strain does not leak RNase at 30°C as determined by plate assays (18), but significant leakage of RNase was observed when cells grown under permissive conditions were subsequently incubated at

TABLE 4. Effect of temperature on the cell viability of strains possessing the $kdsA_{HD50}$ allele[a]

Strain	Genetic background	Mean % survival[b]
Transductant A	LT2	76.2 ± 11.2
Transductant B	LT2	95.9 ± 14.7
Transductant C	PR32	1.5 ± 0.7
Transductant D	PR32	1.9 ± 1.7

[a] Cell viabilities at 42°C were determined as described in footnotes of Table 3. The $kdsA_{HD50}$ allele was transduced into *S. typhimurium* LT2 and $kdsA^+$ transductants of strain PR32 as described in the text.

[b] Values are based on eight determinations.

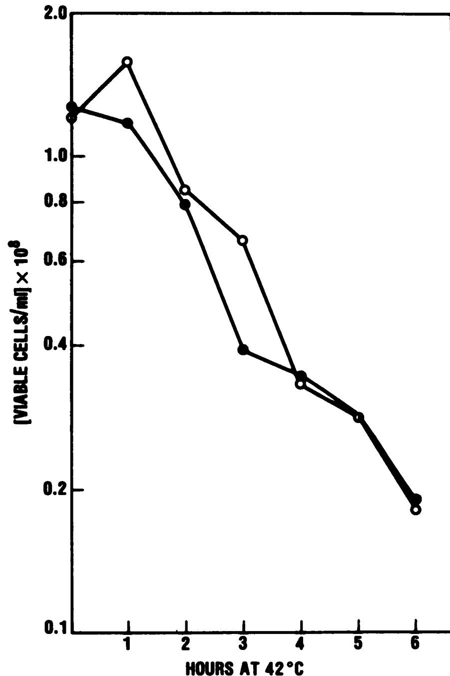

FIG. 4. Effect of temperature on the viability of $kdsA_{HD50}$ and $kdsA_{PR32}$ transductants of strain PR122. Cultures were grown with shaking in PPBE broth at 30°C to an absorbancy at 600 nm of 0.5 and then shifted to 42°C. Dilutions of each culture were plated out on PPBE agar at the indicated times, and the plates were maintained at 30°C. Colony-forming units were determined after incubation of the plates at 30°C for 36 h; $kdsA_{HD50}$ transductant (○), $kdsA_{PR32}$ transductant (●). Each point represents the average of three determinations.

42°C for 8 h (data not shown). However, similar results were observed with strain PRX20 (*kdsA*) and transductants of LT-2 possessing either the $kdsA_{HD50}$ or the $kdsA_{PR32}$ allele. In addition, the antibiotic sensitivities of strain PR32 were compared with those of $kdsA_{PR32}$ transductants of LT2 and temperature-resistant $kdsA^+$ transductants of PR32 by disk diffusion assay at 30°C. These experiments revealed that strain PR32 does not exhibit significantly increased sensitivities to the following antibiotics (concentrations per disk are given within parentheses): polymyxin B (300 U), gentamycin (10 μg), tobramycin (10 μg), kanamycin (30 μg), neomycin (30 μg), carbenicillin (100 μg), ampicillin (10 μg), methicillin (5 μg), cloxacillin (1 μg), cephalothin (30 μg), novobiocin (5 μg), bacitracin (10 U), rifampin (5 μg), and chloromycetin (30 μg) (data not shown).

The phospholipid compositions of strains PR32, PR122, LT2, and transductants of LT2 possessing either the *kdsA*HD50 or the *kdsA*PR32 allele were determined during exponential growth at 28°C and after a 3-h shift of cultures to 42°C. Strain PR32 contained somewhat less phosphatidylglycerol than the parental strain (PR122) during exponential growth at 28°C (Table 5). However, after 3 h at 42°C, the phosphatidylglycerol content of strain PR32 was strikingly reduced, and phosphatidylglycerol comprised only 7% of the total phospholipid. In contrast, there was comparatively little change in the phosphatidylglycerol content of strain PR122. The decrease in phosphatidylglycerol at 42°C was accompanied by a marked increase in cardiolipin which accounted for approximately 20% of the total phospholipid.

To determine whether the alterations in the phosphatidylglycerol and cardiolipin content of strain PR32 at 42°C were related to expression of the temperature-sensitive lethal phenotype, the phospholipid compositions of a $kdsA_{HD50}$ transductant of LT2 and a $kdsA_{HD50}$ transductant of a temperature-resistant $kdsA^+$ derivative of PR32 were determined at 28 and 42°C. After 3 h at

TABLE 5. Effect of temperature on the phospholipid compositions of $kdsA^-$ mutants and wild-type strains

Strain	Relevant genotype	Temp (°C)	Phospholipid composition[a] (% of total $CHCl_3$-soluble ^{32}P)		
			Phosphatidyl-ethanolamine	Phosphatidyl-glycerol	Cardiolipin
PR32	$kdsA_{PR32}$	28	82.4	12.8	4.8
		42	72.4	7.1	20.5
PR122	Wild type	28	81.4	17.6	1.0
		42	79.2	15.9	4.9
LT2 transductant[b]	$kdsA_{HD50}$	28	78.3	19.4	2.3
		42	77.6	8.8	13.6
PR32 transductant[b]	$kdsA_{HD50}$	28	82.3	15.1	2.6
		42	79.6	5.6	14.8
LT2	Wild type	28	85.2	13.3	1.5
		42	76.3	18.9	4.8

[a] Phospholipid compositions were determined as described in the text.
[b] The $kdsA_{HD50}$ allele was transduced into *S. typhimurium* LT2 and a $kdsA^+$ transductant of strain PR32 as described in the text.

42°C, the phosphatidylglycerol content of both transductants was reduced to approximately the same level as observed for strain PR32 (Table 5), and in each case a decrease in phosphatidylglycerol content was accompanied by marked increase in cardiolipin. However, expression of the *kdsA*$_{HD50}$ allele in LT2 does not result in a significant loss of cell viability (Table 4). Thus, although expression of the *kdsA* allele appears to be responsible for the observed alterations in the content of phosphatidylglycerol and cardiolipin in cells incubated under nonpermissive conditions, these alterations do not appear to be related to the expression of the temperature-sensitive lethal phenotype.

No significant alteration in the rate of total phospholipid synthesis was observed in cultures of PR32 after a shift of exponentially growing cells to nonpermissive conditions. Rates of total phospholipid synthesis were determined for cultures maintained under permissive and nonpermissive conditions for various periods of time by measuring the amount of isotope incorporated into cold trichloroacetic acid-insoluble material after pulse-labeling 1-ml samples with [2-^{3}H]glycerol for 1 min as described above. Cultures pulse-labeled after 3 h at 42°C incorporated 2.1 × 10^5 cpm per optical density unit, whereas cultures growing exponentially at 28°C incorporated 2.5 × 10^5 cpm per optical density unit.

DISCUSSION

Mutants of *S. typhimurium* conditionally defective in the synthesis of a fully acylated and KDO-substituted lipid A region of lipopolysaccharide do not grow under nonpermissive conditions, but they remain viable and resume growth after a shift back to permissive conditions (7, 14, 15). The accompanying communication describes the isolation and characterization of a novel mutant of *S. typhimurium* temperature sensitive in the synthesis of KDO-8-P (15). Unlike all other mutants conditionally defective in the synthesis of KDO-8-P, the inability of the new mutant to synthesize KDO at elevated temperatures is accompanied by cell death.

The results presented here clearly indicate that expression of the *kdsA* lesion at elevated temperatures is required for expression of the temperature-sensitive lethal phenotype. The following lines of evidence support this conclusion. (i) Temperature-resistant transductants of strain PR32 possess wild-type KDO-8-P synthetase activity, and they synthesize a fully acylated and KDO-substituted lipid A region at elevated temperatures without detectable accumulation of lipid A precursor. (ii) The mutant suffers a loss of viability concomitant with a complete inhibition of lipopolysaccharide synthesis when cultures are maintained at 37°C. However, D-arabinose-5-phosphate stabilization of the mutant KDO-8-P synthetase in vitro at 37°C (15) and viability of the mutant, as well as lipopolysaccharide synthesis, are rescued in vivo at 37°C by exogenously supplied D-arabinose-5-phosphate. (iii) Transduction of the *kdsA*HD50 allele into temperature-resistant *kdsA*$^{+}$ derivatives of strain PR32 reestablishes the original temperature-sensitive lethal phenotype. This observation also suggests that the lethal phenotype is not uniquely associated with expression of the *kdsA*PR32 allele.

Although the defect in KDO synthesis is a prerequisite for the temperature-sensitive loss in cell viability, the inability to synthesize a complete lipid A is not in itself sufficient cause for lethality. The data presented here suggest that strain PR32 possesses an additional unknown mutation, or possibly multiple lesions, whose lethal expression is dependent on the inability of the mutant to synthesize a complete lipid A at elevated temperatures. Thus, transduction of the *kdsA*$_{PR32}$ allele into *S. typhimurium* LT2 renders the transductants temperature sensitive in growth, but the transductants undergo growth stasis at elevated temperatures. Similarly, expression of the *kdsA*$_{HD50}$ allele in the genetic background of *S. typhimurium* LT2 also results in growth stasis. However, expression of the *kdsA*$_{HD50}$ allele at 42°C in the genetic background of strain PR32 results in a complete loss of viability. These observations further suggest that the second lesion in strain PR32 is unlinked to the *kdsA* locus which maps at 39 min on the *S. typhimurium* chromosome (7). In addition, it appears that the second lesion was not induced by the chemical mutagenesis employed for the isolation of strain PR32 since the introduction of either the *kdsA*$_{HD50}$ or *kdsA*$_{PR32}$ alleles into the chromosome of the parental strain PR122 establishes the temperature-sensitive lethal phenotype characteristic of strain PR32. The point at which the second defect was introduced into the parental strain or its progenitors is currently under investigation.

The effect of exogenously supplied D-arabinose-5-phosphate on the relative rates of synthesis of lipopolysaccharide and lipid A precursor by strain PR32 at 30, 37, and 42°C is almost identical to the results obtained with a previously described temperature-sensitive *kdsA* mutant, *S. typhimurium* PRX22H9 (14). Examination of strain PRX22H9 revealed a progressive increase in precursor/lipopolysaccharide ratios which closely paralleled an increase in generation times as the growth temperature was increased. At growth temperatures of 37°C and below, both growth and synthesis of lipopolysaccharide in strain PRX22H9 was rescued by exogenously supplied D-arabinose-5-phosphate. At 42°C

where the KDO-8-P synthetase of this mutant was essentially inactive, D-arabinose-5-phosphate was unable to rescue either growth or the defect in lipid A synthesis. These observations suggest that the growth stasis observed in strain PRX22H9 and related mutants is primarily related to the disruption of essential cellular functions due either to the accumulation of lipid A precursor in the cytoplasmic membrane or outer membrane (11) or to the absence of a fully acylated and KDO-substituted lipid A region or to both. Although the mechanism of growth stasis is entirely unknown, it seems likely that the role of temperature in establishing growth stasis in temperature-sensitive *kdsA* mutants is restricted to its immediate effect on the activity of the KDO-8-P synthetase of these mutants. In contrast, the role of elevated temperature on expression of the lethal phenotype of strain PR32 appears to be more complex. The ratio of lipid A precursor synthesis to lipopolysaccharide synthesis by strain PR32 is approximately threefold greater at 30°C in the absence of exogenously supplied D-arabinose-5-phosphate than at 42°C with supplementation. However, growth and viability are unaffected at 30°C, whereas the partial rescue of lipopolysaccharide synthesis at 42°C by exogenously supplied D-arabinose-5-phosphate is not accompanied by a rescue of viability. These results suggest the possibility that the second lesion in strain PR32 may also be temperature sensitive, but that its expression, as related to its effect on cell viability, is silent without concomitant expression of the *kdsA* defect. Thus, expression of the temperature-sensitive lethal phenotype may require accumulation of lipid A precursor at temperatures nonpermissive for the second defect. Alternatively, nonlethal expression of the second defect may occur independent of temperature, but the coupled interaction of the second lesion with the *kdsA* defect as a prerequisite for lethality may require elevated temperatures. In this event cell death would not be observed at lower temperatures where the synthesis of a fully acylated and KDO-substituted lipid A region is nevertheless substantially impaired.

The chromosomal location of the unlinked mutation has not been mapped, and preliminary attempts to characterize the mutant phenotype have not yet provided insights into the biochemical basis for the observed temperature-sensitive loss in viability. Synthesis of a fully acylated and KDO-substituted lipid A is substantially impaired during growth of strain PR32 at 30°C. However, the accumulation of lipid A precursor under these conditions is not accompanied by leakage of RNase. In addition, the sensitivity of the mutant to antibiotics is not increased beyond that observed for transductants of LT-2 possessing the $kdsA_{PR32}$ allele or for temperature-resistant $kdsA^{+}$ transductants of PR32. Thus, the functional integrity of the outer membrane does not appear to be markedly altered under conditions which are only semipermissive for synthesis of lipopolysaccharide.

It is attractive to speculate that at elevated temperatures the combined expression of the *kdsA* lesion and the unlinked mutation(s) leads to an alteration of some essential physiological function of the outer membrane or to a defect in outer membrane assembly. However, we have no experimental support for this conclusion. Cells of the mutant elongate into filaments after a shift to nonpermissive temperatures, but electron microscopy of cells held at nonpermissive conditions has not revealed any obvious abnormalities in the ultrastructure of the cell envelope. In addition, expression of either the $kdsA_{PR32}$ allele or the $kdsA_{HD50}$ allele in strain PR32 results in a marked decrease in the level of phosphatidylglycerol and a pronounced increase in cardiolipin under conditions which result in cell death. However, the alterations in phospholipid composition appear to be unrelated to the temperature-sensitive lethal phenotype since expression of these alleles at elevated temperatures in other genetic backgrounds results in similar alterations in phospholipid composition without the occurrence of cell death.

The relationship between the observed alteration in phospholipid composition and expression of the *kdsA* lesion at elevated temperatures is entirely unknown. In this regard, it is interesting to note that Nishijima et al. (9, 10) have isolated temperature-sensitive lethal mutants of *Escherichia coli* which form filaments and are defective in phosphatidylglycerol synthesis at 42°C. These mutants are altered at two genetic loci designated *pgsA* and *pgsB*. The *pgsA* locus defines the structural gene for phosphatidylglycerophosphate synthetase whereas the biochemical role of the *pgsB* locus is not known. However, lesions in both loci are required for significant depletion of phosphatidylglycerol and expression of the temperature-sensitive lethal phenotype. Moreover, the observation that depletion of phosphatidylglycerol at 42°C is accompanied by altered lipid A synthesis in these mutants is extremely intriguing, and these investigators have postulated that phospholipid metabolism and lipopolysaccharide synthesis are somehow coupled. However, the nature of this metabolic relationship remains to be established.

Mutants conditionally defective in KDO-8-P synthesis translocate lipid A precursor poorly from the cytoplasmic membrane to the outer membrane and significant accumulation of precursor occurs in the cytoplasmic membrane (11).

These observations raise the possibility that translocation of precursor in strain PR32 may be decreased to a greater extent than observed in previously described mutants. In this event, the temperature-sensitive lethal phenotype of strain PR32 might be due to the disruption of essential cytoplasmic function(s) as a result of increased accumulation of precursor in the cytoplasmic membrane.

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LITERATURE CITED

1. **Ames, G. F.** 1968. Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. J. Bacteriol. **95**:833–843.
2. **Bligh, E. G., and J. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–918.
3. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, p. 13–26. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **Dulbecco, R., and M. Vogt.** 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. **99**:167–182.
5. **Eidels, L., P. D. Rick, N. P. Stimler, and M. J. Osborn.** 1974. Transport of D-arabinose-5-phosphate and D-sedoheptulose-7-phosphate by the hexose phosphate transport system of *Salmonella typhimurium*. J. Bacteriol. **119**:138–143.
6. **Lehmann, V.** 1977. Isolation, purification and properties of an intermediate in 3-deoxy-D-manno-octulosonic acid-lipid A biosynthesis. Eur. J. Biochem. **75**:257–266.
7. **Lehmann, V., E. Rupprecht, and M. J. Osborn.** 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*. Eur. J. Biochem. **76**:41–49.
8. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Nishijima, M., and C. R. H. Raetz.** 1979. Membrane lipid biogenesis in *Escherichia coli*: identification of genetic loci for phosphatidylglycerophosphate synthetase and construction of mutants lacking phosphatidylglycerol. J. Biol. Chem. **254**:7837–7844.
10. **Nishijima, M., and C. R. H. Raetz.** 1981. Characterization of two membrane-associated glycolipids from an *Escherichia coli* mutant deficient in phosphatidylglycerol. J. Biol. Chem. **256**:10690–10696.
11. **Osborn, M. J., P. D. Rick, and N. S. Rasmussen.** 1980. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Translocation and integration of an incomplete mutant lipid A into the outer membrane. J. Biol. Chem. **255**:4246–4251.
12. **Rick, P. D., L. W.-M. Fung, C. Ho, and M. J. Osborn.** 1977. Lipid A mutants of *Salmonella typhimurium*. Purification and characterization of a lipid A precursor produced by a mutant in 3-deoxy-D-manno-octulosonate-8-phosphate synthetase. J. Biol. Chem. **252**:4904–4912.
13. **Rick, P. D., and M. J. Osborn.** 1972. Isolation of a mutant of *Salmonella typhimurium* dependent on D-arabinose-5-phosphate for growth and synthesis of 3-deoxy-D-manno-octulosonate (ketodeoxyoctonate). Proc. Natl. Acad. Sci. U.S.A. **69**:3756–3760.
14. **Rick, P. D., and M. J. Osborn.** 1977. Lipid A mutants of *Salmonella typhimurium*. Characterization of a conditional lethal mutant in 3-deoxy-D-manno-octulosonate-8-phosphate synthetase. J. Biol. Chem. **252**:4895–4903.
15. **Rick, P. D., and D. A. Young.** 1982. Isolation and characterization of a temperature-sensitive lethal mutant of *Salmonella typhimurium* that is conditionally defective in 3-deoxy-D-manno-octulosonate-8-phosphate synthesis. J. Bacteriol. **150**:447–455.
16. **Rothfield, L., M. J. Osborn, and B. L. Horecker.** 1964. Biosynthesis of bacterial lipopolysaccharide. II. Incorporation of glucose and galactose catalyzed by particulate and soluble enzymes in *Salmonella*. J. Biol. Chem. **239**:2788–2795.
17. **Volk, W. A.** 1966. D-Arabinose-5-phosphate. Methods Enzymol. **9**:38–39.
18. **Yem, D. W., and H. C. Wu.** 1978. Physiological characterization of an *Escherichia coli* mutant altered in the structure of murein lipoprotein. J. Bacteriol. **133**:1419–1426.