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Involvement of the Galactosyl-1-Phosphate Transferase Encoded by the *Salmonella enterica rfbP* Gene in O-Antigen Subunit Processing

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***rfbT* of *Salmonella enterica* LT2 was previously thought, together with *rfaL*, to be involved in the ligation of polymerized O antigen to core-lipid A, and three mutants were known. We report the mapping of the mutations to *rfbP*, the galactosyl-1-phosphate transferase gene, which is now shown to encode a bifunctional protein. The mutations which have the former *rfbT* phenotype are referred to as *rfbP(T)*. We also show that *rfbP(T)* mutants are not blocked in the ligation step as previously believed but in an earlier step, possibly in flipping the O-antigen subunit on undecaprenyl pyrophosphate from the cytoplasmic to periplasmic face of the cytoplasmic membrane.**

Lipopolysaccharides (LPS) are integral components of the outer membrane of gram-negative bacteria. They consist of three structural regions: the lipid A moiety, oligopolysaccharide core, and O antigen, each with its separate biosynthetic pathway. The genes for lipid A synthesis are mainly located at two loci at 2 and 4 min on the *Escherichia coli* chromosome (36), and presumably at corresponding positions on the *Salmonella enterica* chromosome. Core is synthesized on lipid A by products of *rfa* genes (24). O antigen is synthesized quite separately on a lipid carrier, undecaprenyl pyrophosphate (UndPP). The mature O antigen is then transferred to core-lipid A to form LPS before being translocated to the outer membrane. The biosynthesis of LPS has been reviewed recently (39).

O antigen is a polysaccharide with a repeat unit (O unit), which in *S. enterica* comprises three to six sugar residues. In group B, the O unit is a tetramer, Abe-Man-Rha-Gal (13). As a polysaccharide, O antigen is not a primary gene product but rather an assemblage of sugar moieties, each the product of a specific biosynthetic pathway. The genes encoding the enzymes of those pathways, or parts of pathways, specific to O-antigen synthesis, plus the genes for the sugar transferases which assemble the single O unit, are located in the *rfb* gene cluster, which maps close to *his* at 42 min on the *S. enterica* chromosome (14, 22, 44). The O polymerase which welds the single O units into polysaccharide O antigen is encoded by *rfc*, which in *S. enterica* LT2 maps at 32 min (24) and has been sequenced (3).

Biosynthesis of O antigen has been studied in *S. enterica* group B strain LT2. Synthesis of O antigen starts with transfer of galactosyl-1-phosphate from GDP-galactose to undecaprenyl phosphate by galactosyl-1-phosphate transferase (galactosyl-P-P-undecaprenol synthetase). Other sugar residues are then transferred sequentially to form the complete O unit. Single O units are polymerized into O polysaccharide, which is then transferred from UndPP to core-lipid A (Fig. 1). The O units must be synthesized on the cytoplasmic face of the inner membrane because all of the nucleotide sugars are synthesized by cytosolic enzymes; polymerization of the repeat O units as well as the transfer of O antigen to core-lipid A occurs at the periplasmic face of the inner membrane (25, 28). The mechanism by which the UndPP-linked single O unit is translocated to the periplasmic face of inner membrane is unknown.

In *rfa* mutants which do not have complete core, O antigen is synthesized and accumulated as UndPP-linked O antigen in the cytoplasmic membrane (1). UndPP-linked O antigen has been referred to as O hapten, and we will use this term to include all antigenic UndPP-linked O-antigen intermediates. O hapten differs from LPS in solubility and other properties (24). However, mutants in one of the genes in the *rfa* gene cluster, *rfaL*, accumulate O hapten but synthesize core which appears to be complete on the basis of chemical composition, serological properties, and ability to act as a receptor for bacteriophage Felix O (FO) (bacteriophage FO is a *Salmonella*-specific phage that requires core terminating in *N*-acetylglucosamine [the complete core] as the adsorption site [24]). *rfaL* mutants apparently are unable to transfer O antigen to the core, and this was verified by in vitro tests (24). It was concluded that *rfaL* is involved in O-antigen ligation, the transfer of the O antigen from UndPP to core-lipid A (24). The *rfaL* genes of *S. enterica* LT2 and *E. coli* K-12 have recently been sequenced (18, 23).

In the study reported herein, we found that M-antigen and O-antigen intermediates could be confused and that a brief description of M antigen is needed. *E. coli* K-12 and *S. enterica* LT2, like many enterobacteriaceae, produce M antigen or colanic acid, a capsular repeat-unit polysaccharide (8, 9, 12). The synthesis of M antigen is encoded by the *cps* gene cluster in *E. coli* and *S. enterica* (46, 48). LPS core, O antigen, and M antigen are the only biosynthetic products of *E. coli* K-12 and *S. enterica* LT2 known to contain galactose, and only the two repeat units (of O antigen and M antigen) are synthesized on UndPP.

Bacteriophage P22 uses polymerized group B O antigen as its receptor and has been used to isolate *rfb* and *rfa* mutants. Three P22-resistant mutants of strain LT2 isolated by Wilkinson et al. (51) were characterized as phage FO sensitive, phage P22 resistant, and producing O hapten (51). These mutants, like *rfaL* mutants, accumulate O hapten and make complete core-lipid A which accepts O antigen in vitro, but mapped near *his* at a locus named *rfbT* (51); however, it was not shown if *rfbT* was in the major *rfb* locus. Both *rfaL* and *rfbT* genes were believed being involved in O-antigen ligation (24), with *rfbT* considered to be for O-unit recognition and *rfaL* providing specificity for core recognition (47).

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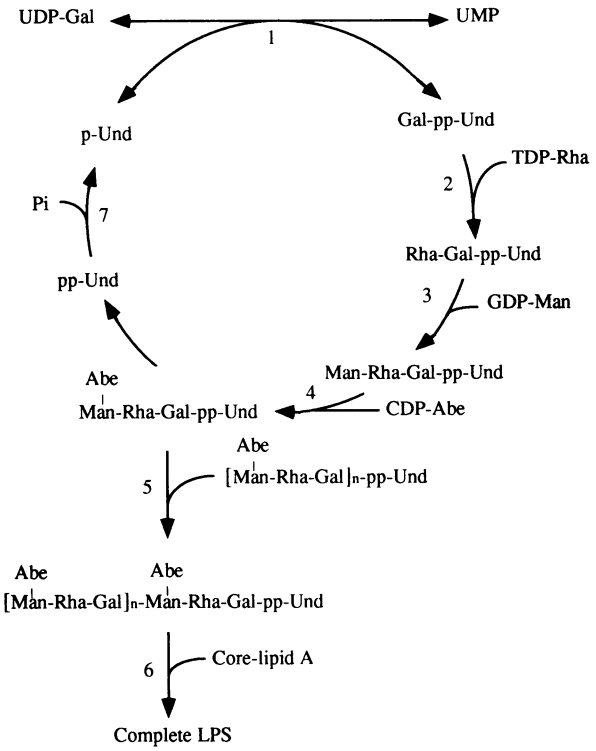

FIG. 1. Pathway of O-antigen biosynthesis of a group B *S. enterica* strain (28, 49). Steps 1 through 4 are catalyzed by galactosyl-1-phosphate, rhamnose, mannose, and abequose transferases, respectively. Step 5 is catalyzed by O-antigen polymerase encoded by *rfc*. Step 6 is the transfer step which was thought catalyzed by the proteins encoded by *rfaL* and *rfbT*. Abbreviations: Abe, abequose; Gal, galactose; Man, mannose; Rha, rhamnose; Und, UndPP; pp, pyrophosphoryl; LPS, lipopolysaccharide.

We report that the *rfbT* mutation maps within *rfbP*, the galactosyl-1-phosphate transferase gene, and that the mutants do not accumulate UndPP-linked long-chain O antigen as found in *rfaL* strains. This later result suggests that *rfbT* is not concerned with transfer of the O antigen from UndPP to core-lipid A as previously believed. We also show that these mutants accumulate material which may be UndPP-linked O unit and suggest that the function affected could be the flipping of UndPP-linked O unit from the cytoplasmic face to the periplasmic face of the cytoplasmic membrane.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. *galE* and *pmi* derivatives of SL1196, SL1197, and selected control strains were constructed as follows. Chromosomal DNA of P9138 or P9306, which has a transposon closely linked to *galE* or *pmi*, respectively, was transformed into these strains by electroporation. Transformants were isolated by selection on MacConkey-galactose or MacConkey-mannose plates supplemented with the appropriate antibiotic.

pPR1381 was made by cloning the *Hin*dIII fragment from positions 19.76 to 21.76 of the LT2 *rfb* cluster (14) into the *Hin*dIII site of pGEM-7z(+) (Promega). pPR1459 was made by replacing with a Cm^r gene a 2.0-kb *Pst*I fragment located in the middle of the 15.7-kb *Eco*RI insert of pPR648 (46), which covers the entire *cps* region of *S. enterica* LT2. P9340, a *cps*-deficient derivative of P9318, was made by transforming the insert of pPR1459 into P9318 and selecting for the Cm^r transformants which arise by incorporation of the insertion mutation into the chromosome through homologous recombination.

Chemicals, enzymes, and antisera. Restriction enzymes and T4 DNA ligase were from Boehringer Mannheim and Pharmacia. UDP-galactose, GDP-mannose, decaprenol phosphate, and D-[1-^{14}C]mannose were purchased from Sigma. D-[6-^{3}H] galactose was from Amersham. dTDP-rhamnose and dTDP-L-[U-^{14}C]rhamnose were kindly provided by L. Lindqvist (21, 22). *S. enterica* O4 and O5 antisera were from Wellcome Diagnostics.

DNA methods and computer analysis. DNA isolation, restriction digestion, and ligation were as described by Sambrook et al. (42). Electroporation was performed with a Pulser Controller (Bio-Rad) as instructed by Bio-Rad. Sequence analysis was carried out by using the ANGIS system at the University of Sydney, which incorporates several sets of programs. PEPPLOT and PLOTSTRUCTURE in the Genetics Computer Group package (6) were used to identify transmembrane segments and predict protein secondary structure. The method of Eisenberg et al. (7) was used to calculate hydrophobic moments. The programs BLAST, MATCH, and STATSEARCH, based on the method of Wilbur and Lipman (50), were used to search data banks. BESTFIT, also from the Genetics Computer Group package and based on the local homology algorithm of Smith and Waterman (45), was used to do the comparison between proteins. The parameters for running BESTFIT were a gap weight of 3.0 and a gap length of 1.0. Program CLUSTAL (10) was used to generate multiple protein sequence alignment.

[^{3}H]galactose labelling of O antigen and intermediates. One-milliliter overnight cultures were inoculated into 10 ml of L broth and grown for 3 h at 37°C with shaking before addition of 10^{-3} μmol (40 μCi) of [^{3}H]galactose and unlabelled galactose to bring the level to 5×10^{-3} μmol. Incubation was continued for 1 min, and cells were collected by centrifugation after cooling of the culture on ice for 10 min.

For pulse-chase experiments, cells were labelled for 1 min as described above before 10 μmol of unlabelled galactose was added to the culture. Incubation was continued for another 10 min, and cells were collected as before.

LPS, LPS core, and O hapten were extracted by the method of McGrath and Osborn (28). Labelled cells were first washed twice with 2-ml volumes of cold 50% acetone. The acetone-washed cells were resuspended in 0.5 ml of water and extracted twice with 45% phenol at 70°C (twice for 15 min each time). LPS and LPS core were precipitated from the aqueous phase with cold 65% ethanol–10 mM sodium acetate. Free O antigen, O units, and O-antigen intermediates which had been cleaved from UndPP by hot phenol remained in the ethanol supernatant. This supernatant was evaporated by freeze-drying and resuspended in 400 μl of 10% CsCl, 100 μl of which was subjected to gel filtration on a Sephadex G-15 column (1.5 by 40 cm), using 50 mM ammonium acetate (pH 7.0) as the packing and elution buffer. The elution rate was 85 μl/min. Fractions of 0.5 ml were collected, and radioactivity was counted in 5 ml of ASC II (Amersham) in a MINAXI liquid scintillation counter.

In vitro synthesis of O-unit intermediates. [^{14}C-Rha]-Gal-decaprenol pyrophosphate (DPP), [^{14}C-Man]-Rha-Gal-DPP, and [^{14}C-Rha]-Man-Man-Gal-DPP intermediates were synthesized in vitro, using the cell envelope fractions of P9298, P9299, and P4706, respectively, as sources of the enzymes (22). P9298 and P9299 contain plasmids carrying strain LT2 *rfb* genes for

TABLE 1. Bacterial strains

Strain	Laboratory stock no.	Characteristics	Reference
S. enterica LT2 (serovar typhimurium) strains			
SL1654	P9003	*hsdL trpB2 nml flaA66 rpsL xylT404, ilv-452 metE554 metA22 hsdA*	33
his-388	P9029	Δ(*hisD-metG*)	32
his-642	P9073	Δ(*hisI-rfbK*)	32
SGSC647	P9138	*zbi-812*::Tn*10 galE*	43
SL1196	P9285	*hisD27 trpA8 tolC-30 H1-a, H2-e,n,x, gal-437 xyl-412 rfbT*	51
SL1197	P9286	*hisD27 trpA8, tolC-30 H1-a H2-e,n,x gal-437 xyl-412 rfbT*	51
	P9298	P90316 carrying plasmids pPR686 and pPR1208	22
	P9299	P9036 carrying plasmids pPR686 and pPR1303	22
SL3848	P9300	*rfaL447 gal-851*	41
TT10251	P9306	*pmi*::Mud1-8	11
	P9317	As P9286, *pmi*	This study
	P9318	As P9073, *zbi-812*::Tn*10 galE*	This study
	P9320	As P9285, *zbi-812*::Tn*10 galE*	This study
	P9321	As P9286, *zbi-812*::Tn*10 galE*	This study
	P9322	As P9300 *zbi-812*::Tn*10 galE*	This study
	P9324	P9285 carrying plasmid pPR1381	This study
	P9325	P9286 carrying plasmid pPR1381	This study
	P9340	As P9318, *cps*::Cm^r	This study
E. coli K-12	P4706	P3127 carrying plasmid pPR1329	22

galactose and rhamnose transferases and for galactose, rhamnose, and mannose transferases, respectively; P4706 contains a plasmid carrying the transferase genes of strain M67 for synthesis of the group C2 O-unit intermediate (Rha-Man-Man-Gal) (22). The preparation of cell envelope fractions and the synthesis of these intermediates were as described by Osborn et al. (35) and Liu et al. (22). The oligosaccharides were cleaved from DPP by hydrolysis in 50 mM trifluoroacetic acid (100 °C for 30 min), and the trifluoroacetic acid was then dried by evaporation under a stream of N_2. The oligosaccharides which had not been cleaved were removed by repeated washing with *n*-butanol.

Other methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining of LPS for visualizing LPS was carried out as described by Brown et al. (2). Galactose transferase activity was assayed as described by Liu et al. (22). O4 and O5 O-antigen epitopes were detected by slide agglutination of bacteria as described by Leinonen (20).

RESULTS

The *rfbT* mutation is in the *rfbP* gene. Strains SL1196, SL1197, and SL1198 were isolated and characterized as *rfbT* mutants by Wilkinson et al. in 1972 (51), and no other such mutants have been reported since then. We carried out bacteriophage sensitivity and slide agglutination tests. Strain SL1198 showed resistance to both FO and P22 phages; thus, we suspect a secondary mutation in the *rfa* region in this strain. Strains SL1196 and SL1197 showed the expected pattern (P22 resistant and FO sensitive). We show below that the mutations map to *rfbP* and were therefore named *rfbP4451(T)* and *rfbP4452(T)*, respectively. Strain SL1196 did not agglutinate with O4 and O5 antisera, whereas strain SL1197 gave weak slide agglutination, suggesting that *rfbP4452(T)* may be a leaky mutation.

The whole *rfb* gene cluster of *S. enterica* LT2 has been cloned and sequenced, and most of the genes have been identified in this laboratory (14, 22, 38). To locate the *rfbT* mutations, a series of clones was transformed into SL1196 and SL1197. Several of these strains gave a phage sensitivity pattern identical to that of wild-type strain P9003 (P22 sensitive and FO sensitive) and strong positive slide agglutination with O4 and O5 antisera. pPR1381 is the smallest clone which complements the mutations, and strains P9324 and P9325, which are SL1196 and SL1197, respectively, carrying pPR1381, were studied further.

Whole cell membrane of SL1196, SL1197, P9324, and P9325 was prepared and analyzed by silver staining after SDS-PAGE. Strain SL1196 produces no detectable O antigen containing LPS, but strain SL1197 gives a weak band which runs at the position expected for LPS with a single O unit. It appears that *rfbP4452(T)* is leaky and produces a small amount of O antigen. Strains P9324 and P9325 give a ladder pattern of O-antigen distribution similar to that of wild-type strain P9003, although the amount of high-molecular-weight LPS is less than that in strain P9003 (wild-type) preparations (Fig. 2).

pPR1381 carries the right-hand end of the strain LT2 *rfb* gene cluster from positions 19.7 to 21.7, and the only complete gene located in this region is *rfbP*, which encodes the galactosyl-1-phosphate transferase (14). The *rfbT* mutations thus appeared to be complemented by *rfbP*; we therefore conclude that there is no separate *rfbT* gene, but that the *T* mutations are in *rfbP*, and refer to the mutations as *rfbP(T)*. We considered the possibility that we had previously made a sequencing error (14) and that there are two genes in the *rfbP* area. We therefore resequenced this area and confirmed the sequence as published. We also used a clone of *rfbP* made from PCR-amplified DNA (kindly provided by Dan Liu), as the clones discussed above carry incomplete open reading frames adjacent to *rfbP*. This clone also complemented the T$^-$ phenotype, showing that only *rfbP* is required for the T function.

***rfbP(T)* mutants have galactosyl-1-phosphate transferase activity.** The generally accepted function of RfbP is to transfer galactosyl-1-phosphate to UndP (galactosyl-1-phosphate transferase function) (14). Galactosyl-1-phosphate transferase activities of strains SL1196 and SL1197 were assayed by using the whole membrane fraction of overnight cultures. SL1196 and SL1197 have about 5 to 7% of the activity of P9003 (Table 2). Thus, RfbP of these two strains retains galactose phosphate transferase activity, as expected since it is needed to synthesize

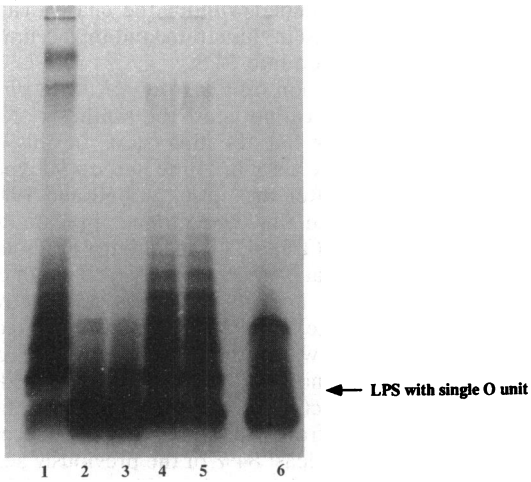

FIG. 2. LPS profiles of various strains in silver-stained SDS-PAGE. Whole membrane fractions were boiled for 5 min, then treated with proteinase K, and analyzed by SDS-PAGE with LPS-specific silver staining. Lanes: 1, P9003; 2, SL1196; 3, SL1197; 4, P9324 (SL1196 carrying pPR1381); 5, P9325 (SL1197 carrying pPR1381); 6, P9073.

O hapten, but there is a reduction in activity or amount of enzyme.

It is possible that the T block, which leads to the accumulation of O hapten, has a feedback effect on *rfbP* transcription and that the reduced galactosyl-1-phosphate transferase activity is due to a reduced level of enzyme rather than reduced enzyme kinetics. We therefore introduced a *galE* mutation which would prevent accumulation of any O-unit intermediate in these mutants in the absence of exogenous galactose (see below). It was found that in the wild-type strain, the *galE* mutation has little effect on the level of galactosyl-1-phosphate transferase activity, but that in the *rfbP(T)* mutants, the level of galactosyl-1-phosphate transferase activity increased about twofold (Table 2). It appears that there is some feedback effect due to accumulation of O hapten but that this is not the major cause of the T mutant effect on galactosyl-1-phosphate transferase.

***rfbP(T)* strains do not accumulate UndPP-linked long-chain O antigen.** To further characterise the T function of this protein, the quantity and properties of the O hapten accumulated in *rfbP(T)* strains were determined by using *galE* derivatives of *rfbP(T)* strains to label the O hapten, which was then isolated and analyzed by chromatography.

The use of *galE* mutants to specifically label O antigen was pioneered by Kent and Osborn (16, 17) and has been extensively used since then (15, 19, 25, 27, 29, 34, 35). The *galE* gene encodes galactose epimerase, which catalyzes the reversible interconversion of UDP-glucose and UDP-galactose. Galactose is a constituent of O antigen, LPS core, and the M-antigen capsule (24, 26) and in each case is transferred from UDP-galactose. The M-antigen capsule is made only under certain physiological conditions such as low temperature, high concentration of salts, and excess of fermentable sugars (26). Thus, under the usual conditions of growth, bacteria do not produce the M-antigen capsule, and the O antigen and core of *galE* strains can be specifically labelled by adding radioactive galactose to the medium (17). *galE* mutants of strains SL1196, SL1197, P9073, and P9300 were isolated (see Materials and Methods) and named P9320, P9321, P9318, and P9322, respectively. They all showed sensitivity to galactose, confirming that they are *galE* mutants. All four strains show the same phage pattern as that of their parent strains when grown on galactose-glucose media.

TABLE 2. Galactose transferase activity of *rfbP(T)* strains

Strain	Relevant genotype	In vitro transferase activity (cpm of [^{14}C]UDP-galactose incorporated into UndPP/30 min/200 ng of protein)
P9029	Δ(*hisD-rfb*)	18
SL1196	*rfbP4451(T)*	209
SL1197	rfbP4452(*T*)	140
P9320	*rfbP4451(T) galE*	548
P9321	*rfbP4452 (T) galE*	577
P9003	Wild type	3,378
P9138	*galE*	3,165

Pulse and pulse-chase experiments were carried out with D-[6-^{3}H]galactose to label O antigen, and after extraction, free O antigen and intermediates which had been cleaved from UndPP were separated on a Sephadex G-15 column which had been calibrated by using synthetic disaccharide, trisaccharide, and tetrasaccharide. Significant peaks, not all present in preparations from all strains, were seen at fractions 58, 61, 65, 76, 95, and labelled 1 to 5 in Fig. 3. Peak 1 runs at the same position as blue dextran and is thought to be long-chain O antigen; peak 5 runs at the same position as galactose and presumably is galactose. Other peaks were identified, as discussed below, as polymerized M antigen (peak 2), single O unit and M unit (peak 4), and presumably a short chain O antigen (peak 3).

In the wild-type strain, P9138, 5,120,000 cpm (about 2.2×10^{-4} μmol of [^{3}H]galactose) was incorporated into the cells in a 10-ml culture after a 1-min pulse. After phenol and water extraction of acetone-washed cells, 3,600,000 and 167,000 cpm (70 and 3%, respectively, of the incorporated radioactivity) were found in the 65% ethanol precipitate (represents complete LPS and LPS core) and supernatant (represents material originally linked to UndPP), respectively (Table 3). The material in the supernatant was then applied to the G-15 column (Fig. 3); most of the radioactive material runs at the same position as blue dextran (peak 1) and must be long-chain O antigen as expected. A chase-pulse experiment was then carried out. Counts recovered as UndPP-linked material decreased from 167,000 to 92,000 (a reduction of 75,000 cpm) (Table 3), while that in LPS increased from 3,600,000 to 3,710,000 (an accretion of 110,000, 35,000 cpm more than was lost from hapten). Thus, at least 68% of the radioactivity incorporated into LPS during the chase was derived from previously accumulated O hapten, and the remainder was from free [^{3}H]galactose which had entered the pool. This finding demonstrates an efficient chase of the previously accumulated hapten into LPS and a rapid flow of the free galactose through hapten into LPS.

In the *rfaL* strain, P9322, 1,150,000 cpm (22% of the amount found in the wild type) was found incorporated into the cells after the pulse, and 21 and 62% of that were recovered from the 65% ethanol precipitate and supernatant, respectively (Table 3). The ethanol-soluble material was excluded from the G-15 column as peak 1 (Fig. 3) and is thought to be the long-chain O antigen expected to accumulate. Note that this strain with a block in ligase activity has accumulated 10-fold more UndPP-associated long-chain O antigen than is found in the wild type (Fig. 3). After chasing, the amounts of radioactivity recovered from the pellet and the ethanol supernatant

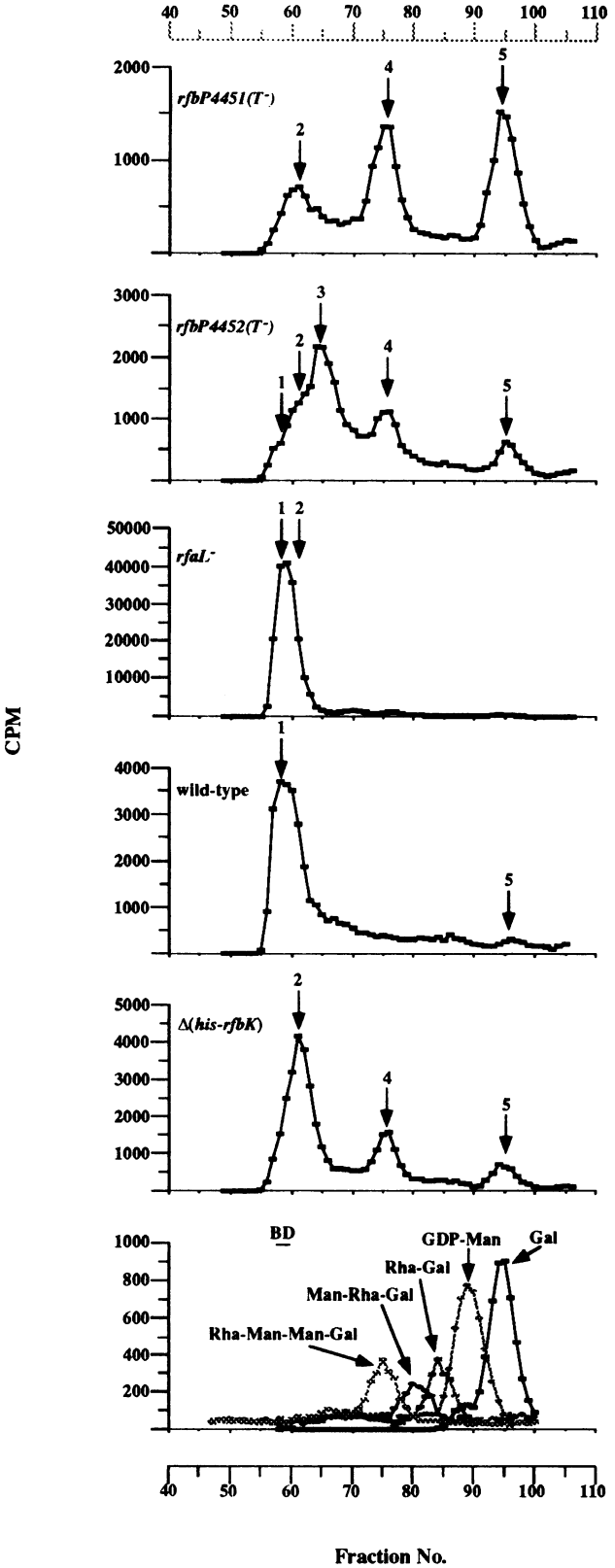

FIG. 3. Gel filtration of free O-antigen (M-antigen) intermediates accumulated by strains P9138 (wild type), P9320 (*rfbP4451(T)*], P9321 [*rfbP4452(T)*], P9322 (*rfaL*), and P9318 [Δ(*his-rfbK*)] in the pulse-label experiment carried out as described in Materials and Methods. In vitro-synthesized oligosaccharides were used as standards. UndPP-linked O antigens were extracted with 45% hot phenol, and the ethanol supernatants were taken to dryness on a rotary evaporator and dissolved in 400 μl of 10% CsCl. Samples of 100 μl were applied to a Sephadex G-15 column. The elution positions of galactose and GDP-mannose were determined by using [^{14}H]galactose and GDP-[^{14}C] mannose. The positions of disaccharide, trisaccharide, and tetrasaccharide were determined by using ^{14}C-labelled in vitro-synthesized oligosaccharides. The excluded volume, determined with blue dextran (BD), is indicated. Abbreviations are as in Fig. 1.

were similar to those obtained without the chase (Table 3). The ligation step is blocked in this strain, and the accumulated O hapten cannot be chased into LPS.

Strain P9318 has a deletion from *hisI* to *rfbK*, with *rfb* genes *rfbP* and *rfbK* deleted; it cannot start the synthesis of the O antigen, as RfbP carries out the first step. In this strain, 1,370,000 cpm (27% of the amount found in the wild type) was incorporated into cells after the pulse, and 50 and 19.0% of that were recovered from the 65% ethanol precipitate and supernatant, respectively (Table 3). The finding of radioactivity in ethanol-soluble material is unexpected, as this strain cannot synthesize O antigen or any intermediates; radioactivity is expected in the precipitate, as LPS core contains galactose. A pulse-chase experiment was then carried out, and counts recovered from the ethanol-soluble material decreased from 260,000 to 42,000 (a reduction of 218,000 cpm), while those in the precipitate increased from 686,000 to 893,000 (an accretion of 207,000 cpm). Thus, at least 84% of the previously accumulated ethanol-soluble material was chased into material which is insoluble in 65% ethanol. The ethanol-soluble materials from both the pulse and pulse-chase experiments were applied to the G-15 column, and it was found that the previously accumulated material (peaks 2, 4, and 5; Fig. 3) was fully chased through into ethanol-insoluble material (Fig. 4). The ethanol-soluble material from this strain must be something other than O hapten, as this *rfbP* strain cannot start synthesis of O antigen. Peak 5 presumably is galactose.

As galactose is also a constituent of the M-antigen capsule which is synthesized by polymerization of oligosaccharide repeating units linked to UndPP, and as the UndPP-linked intermediates of the M-antigen capsule will be extracted in the same way as UndPP-linked O antigen, the radioactivity seen here could represent them (although the M antigen is not synthesized under normal growth conditions). In an attempt to block synthesis of M antigen and thereby simplify interpretation of the data, we made a *cps* partial deletion derivative of strain P9318 by replacing a 2-kb chromosomal segment located in the *cps* gene cluster with a Cm^r gene. A pulse-chase experiment was carried out with this strain, P9340, and the ethanol-soluble material was then applied to the same G-15 column. A large amount of material ran as peak 4, with none at peak 2 (Fig. 4), indicating that the replacement of a *cps* gene(s) with the Cm^r gene causes the material in peak 4 to no longer be converted to peak 2 material. We conclude that the ethanol-soluble materials (peaks 2 and 4) found in strain P9318 do represent M-antigen capsule intermediates, as they are affected by a *cps* mutation, with peak 2 being polymerized M antigen and peak 4 being a single unit (M unit). Strain P9318 must be at least partly derepressed for capsule production, although it is not mucoid. As the *cps*::*cml* mutation exacerbated the problem of M-antigen intermediate accumulation, we did not transfer the mutation into the *rfbP(T)* and other strains. We then made several attempts to make a full deletion of the *cps* region in our strains, as the presence of peaks thought to be due to M-antigen intermediates complicated the

TABLE 3. Incorporation of [^{3}H]galactose by *galE* strains under pulse and pulse-chase conditions

Strain	Relevant genotype	Total counts incorporated	Pulse experiment				Pulse-chase experiment			
			65% ethanol supernatant		65% ethanol pellet		65% ethanol supernatant		65% ethanol pellet	
			[^{3}H]galactose (cpm)	% of total	[^{3}H]galactose (cpm)	% of total	[^{3}H]galactose (cpm)	% of total	[^{3}H]galactose (cpm)	% of total
P9320	*rfbP4451(T)*	1,390,000	113,000	8	982,000	71	124,000	9	961,000	69
P9321	*rfbP4452(T)*	1,770,000	148,000	8	1,490,000	84	44,000	2.5	1,610,000	91
P9138	Wild type	5,120,000	167,000	3	3,600,000	70	92,000	2	3,710,000	72
P9322	*rfaL*	1,150,000	715,000	62	241,000	21	707,000	62	232,000	20
P9318	*rfbP rfbK*	1,370,000	260,000	19	686,000	50	42,000	3	893,000	65

results with *rfbP(T)* mutants discussed below, but without success.

In the *rfbP4451(T)* strain, P9320, 1,390,000 cpm (27% of the amount of the wild type) was incorporated after the pulse, and 71 and 8% of that were found in the 65% ethanol precipitate and supernatant, respectively (Table 3). The radioactivity in the ethanol precipitate is much less than for the wild-type strain (Table 3), as expected given the absence of complete LPS molecules in this strain. As it was believed that *rfbT* was involved in the same ligation reaction as *rfaL*, we expected this strain to accumulate a similar amount of the ethanol-soluble material as did the *rfaL* strain. However, the ethanol supernatant contained only 15% of the radioactivity found in the *rfaL* strain. When applied to the G-15 column, this ethanol-soluble material gave the same three peaks (2, 4, and 5) as seen in the *rfbP rfbK* deletion strain P9318 (Fig. 3). The materials in peaks 2 and 4 may again represent M-antigen intermediates or could be O-antigen intermediates. A pulse-chase experiment was then carried out, and the amounts of radioactivity recovered from the ethanol precipitate and supernatant were similar to those obtained after the 1-min pulse (Table 3). The ethanol-soluble material of the pulse-chase experiment was also applied to the G-15 column, and all peaks present in the pulse experiment were found to be still present after the chase. The count in peak 2 decreases about twofold, but the count in peak 4 increased about 10%. We conclude that the materials in peak 2 of this strain represents the M-antigen intermediate, as it runs as an M-antigen intermediate and can be chased through. As peak 4 cannot be chased through, we conclude that the material in peak 4 of this strain must represent an intermediate of the O antigen, although it runs at the same position as the M unit. Peak 4 is at a position between the trisaccharide (Man-Rha-Gal, the precursor of the tetrasaccharide O unit of strain LT2) and tetrasaccharide (Rha-Man-Man-Gal) standards, and the O-antigen intermediate running at peak 4 in this strain could be the tetrasaccharide O unit (Abe-Man-Rha-Gal) of strain LT2. We conclude that the *rfbP4451(T)* strain cannot synthesize long-chain O antigen, and it must be blocked in a step prior to polymerization in the O-antigen synthetic pathway.

In the leaky *rfbP4452(T)* strain, P9321, 1,770,000 cpm (35% of the amount found in the wild type) was incorporated into the cells after the pulse, and 84 and 8% of that were recovered from the ethanol precipitate and supernatant, respectively (Table 3). More radioactivity was found in the precipitate of this strain than in that of the *rfbP4451(T)* strain, presumably because the *rfbP4452(T)* mutation in this strain is leaky and the strain synthesizes some complete LPS. The ethanol-soluble fraction had materials in peaks 1 through 5. Peaks 1 and 2 are present as distinct shoulders on peak 3, which in the *rfbP4451(T)* strain was detectable as a shoulder on peak 2 but is not otherwise seen. Peak 4, the presumptive O unit, is present but at a lower amount than in the *rfbP4451(T)* strain. These data can be interpreted on the assumption that the *rfbP4452(T)* mutation has the same block as the *rfbP4451(T)* mutation but is leaky, with less than maximal accumulation of UndPP-linked O unit and some full-length O antigen. The peak 3 material is perhaps short-chain-length O antigen. Peak 2 presumably comprises a capsule intermediate. After a pulse-chase, counts recovered from the ethanol-soluble material decreased from 148,000 to 44,000 (a reduction of 104,000 cpm), while those in LPS increased from 1,490,000 to 1,610,000 (an accretion of 120,000 cpm, 16,000 cpm more than was lost from the ethanol-soluble material). Thus, at least 87% of the radioactivity incorporated into LPS was derived from previously accumulated ethanol-soluble material. The effective chase of the previously accumulated hapten into LPS in this leaky *rfbP4452(T)* strain also indicates that the ligase function is normal in this strain.

Strains P9306 (*pmi*) and P9317, the *pmi* derivative of SL1197 [*rfbP4451(T)*], were labelled with D-[1-^{14}C]mannose and extracted with hot phenol. The ethanol-soluble materials from these strains were applied to the same G-15 column and gave results similar to those obtained with strains P9138 and P9321, respectively, using D-[6-^{3}H]galactose (data not shown), showing that all peaks contain mannose or fucose (GDP-mannose is a precursor of GDP-fucose). Mannose and fucose are the components of O antigen and M antigen, respectively. This results confirms that the UndPP-linked products discussed above must be O antigen or M antigen.

Location of the T-function domain in the *rfbP(T)*-encoded protein. The *rfbP* gene has been previously sequenced in this laboratory and is 1,428 bp in length (14). Analysis of the derived amino acid sequence reveals four transmembrane segments located at the N terminus and another located in the middle of the protein. The C-terminal half of this protein, including the last transmembrane segment, is homologous to the whole amino acid sequence of ExoY of *Rhizobium meliloti* (37), with 48.7% identity and 66.1% similarity (Fig. 5). Mutation of the *exoY* gene results in the lost of ability of *R. meliloti* to transfer galactose onto a lipid carrier, and ExoY is thought to be a galactosyl-1-phosphate transferase (40); thus, it is likely that the C-terminal half of RfbP (from about residue 252 to the C-terminal end) is the galactosyl-1-phosphate transferase domain.

DISCUSSION

We have shown that the *rfbT* gene, previously thought to encode a component of the ligase which links O antigen to core-lipid A, is neither a separate gene nor involved in ligation. The two *rfbT* mutations available were complemented by the

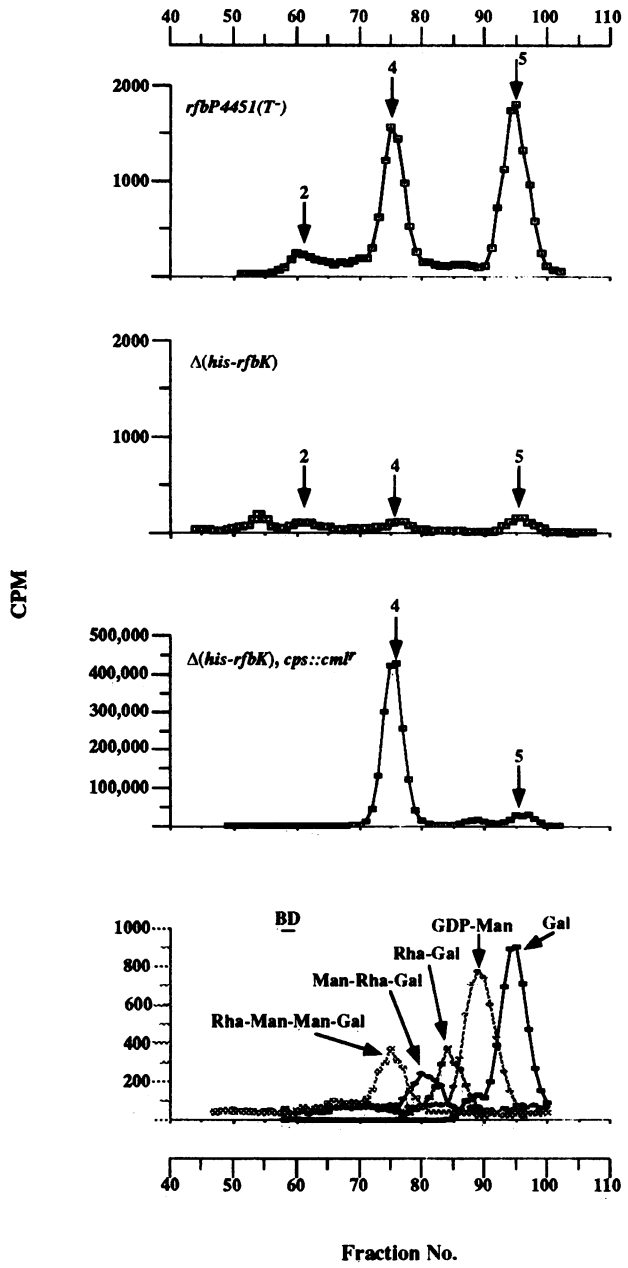

FIG. 4. Gel filtration of free O-antigen (M-antigen) intermediates accumulated by strains P9320 [*rfbP4451(T)*], P9318 [Δ(*his-rfbK*)], and P9340 [Δ(*his-rfbK*) *cps*::Cmlᴿ] in the pulse-chase experiment. In vitro-synthesized oligosaccharides were used as controls. The experiment was carried out as described in Materials and Methods. All other conditions are as for Fig. 3.

rfbP gene encoding the galactosyl-1-phosphate transferase. We conclude that the mutations must be in the *rfbP* gene and refer to them as *rfbP(T)* mutations.

In vivo studies using radioactive precursor showed that the *rfbP4451(T)* strain did not synthesize the UndPP-linked long-chain O antigen accumulated in *rfaL* mutants but instead accumulated a lower-molecular-weight intermediate. The *rfbP4452(T)* mutant also accumulated a very small amount of higher-molecular-weight material and is presumed to be leaky. The absence of long-chain O antigen does not fit the model of

```
RfbP  M---DNIDNKY---NPQLCKIFLAISDLIF--FNLALWFSLGCVYF--IFDQVQRFIPQD  50
GumD  MllaDlssatYttssPrLlskysAaaDLvlrvFdLtmvvasGliayrivFg---twvPaa  57

RfbP  QLDTRVITHFILSVVCVGWFWIRLRHYTIRKPFWYELKEIFRTIVI--------FAIFDL  102
GumD  pyrvaiaTtllySViCfalF----plYrswrg-----rgllselVvlggafggvFAlFav  108

RfbP  -ALIAFTKWQFSRYVWVFCWTFALILVPFF--RALTKHLLNKL---GIWKKKTIILGSGQ  156
GumD  hALIvqvgeQvSR-gWVglW-FvggLVslvaaRtLlrgfLNhLrtqGvdvqrvvvvGlrh  166

RfbP  NARGAYSALQSEEMMGFDVIAFFDTD---ASDAEINMLPVIKDTEIIWDLNRTGDVHYI-  212
GumD  pvmkishyLsrnpwvGmnmvgyFrTpydlAvaeqrqgLPclgDpdelieylknnqVeqvw  226

RfbP  LAYEYTELEKTHFWLRELSKHHCRSVTVVPSFRGLPLYNTDMSFIFSHEVMLLRIQNNLA  272
GumD  islplgErdhikqlLqrLdrypi-nVklVPdlfvfgLlNqsaeqIgSvpVinLR-Qggvd  284
ExoY                                    mksatrsattaffipqetgai  21

RfbP  KRSSRFLKRTF-DIVCSIMILIIASPLMIYLWYKVT-RDGGPAIYGHQRVGRHGKLFPCY  330
GumD  rdnyfvvakalqDkilaviaLmglwPLMlaiavgVkmsspGPvffrqrRhGlgGreFymf  344
ExoY  rpiggisKRsF-DvliaIlaLIalSPLfllvmglVkfsDGGsifYGHrRiGhnGqtFkCl  80

RfbP  KFRSMVMNSQEVLKELLANDPIARAEWEKDFKLKNDPRITAVGRFIRKTSLDELPQLFNV  390
GumD  KFRSMrvhddh-------gttIqqAt-------KNDtRITrfGsFlRrsSLDELPQiFNV  390
ExoY  KFRtMmeNgdrVLqEffksnPaAyeEWrttrKLqdDPRvTvVGsvlRKlSLDELPQLlNi  140

RfbP  LKGDMSLVGPRPIVSDELERYCDDVDYYLM---AKPGMTGLW-QVSG-RNDV----DYDT  441
GumD  LgGsMSiVGPRPhaaqhnthYeklinhYmqrhyvKPGiTG-WaQVnGfRgetpelrtmkk  449
ExoY  irGeMSiVGPRPvVeDELElYdsaaefYLr---srPGlTGLW-QiSG-RNDV----sYaT  191

RfbP  RVYFDSWYVKNWTLWNDIAILFKTAKVVLRRDGAY  476
GumD  RiqyDldYirrWsLWlDIrIivlTAveVLgqktAY  484
ExoY  RVaFDthYVqNWsLlaDlvIvFKTipaVclsrGsY  226
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FIG. 5. Alignment of deduced amino acid sequence of RfbP with those of GumD and ExoY, using the program CLUSTAL with a gap penalty of 10. Amino acids of GumD and ExoY identical to those of RfbP(T) are in uppercase.

rfbT and *rfbL*, both being involved in ligation of O antigen to core-lipid A (24, 47), since if that were the case, both *rfaL* and *rfbP(T)* strains would accumulate UndPP-linked long-chain O antigen. *rfbP(T)* mutants must then be blocked in a step in the O-antigen biosynthetic pathway prior to ligation.

The O-antigenic material which accumulated in the *galE rfbP4451(T)* strain, P9320 (Fig. 3, peak 4), ran at a position on the column different from that of a trisaccharide (Man-Rha-Gal, the precursor of the tetrasaccharide O unit of strain LT2), but close to that of the O unit of group C2 strains (Rha-Man-Man-Gal) (Fig. 3) and cannot be chased through (Fig. 4). The O unit of *S. enterica* group B strains is also a tetrasaccharide (Abe-Man-Rha-Gal) but differs in sugar composition and linkages between sugars, and the molecular shape may be different from that of the group C2 O unit. It would not be surprising if the mobilities of these two tetrasaccharides were slightly different, and we conclude that the low-molecular-weight material accumulated by mutant *rfbP4451(T)* is most likely the O unit of group B. *rfc* is the only gene required for polymerization of O units (3, 24, 31), and as the ladder pattern of LPS produced by strain P9324 (strain SL1196 containing pPR1381) shows that the *rfc* gene is normal in this strain, we conclude that the UndPP-linked single O units cannot access the polymerization and ligation sites. The synthesis of O units on UndPP occurs at the cytoplasmic face of inner membrane, but the polymerization of O units and ligation both occur at the periplasmic face of the inner membrane. One possible explanation is that in this strain, the flipping of UndPP-linked O unit from the cytoplasmic face to the periplasmic face of the inner membrane is blocked, as that would prevent polymerization of single O unit or ligation (to the lipid A-core) since both occur on the periplasmic face.

The *rfbP4452(T)* strain, SL1197, showed weak agglutination with O4 and O5 antisera and produced a small amount of LPS; we concluded that the mutation in this strain is leaky. After a 1-min label, it accumulated more low-molecular-weight UndPP-linked materials than the wild-type strain but also accumulated materials (peak 3) which are presumed to be O polysaccharide with fewer O units than the full-length O

antigen, suggesting that the block is not absolute. The presence of peak 3 in this strain supports the suggestion that the limiting step is flipping rather than the polymerization or ligation (to core-lipid A) step, because accumulation of O antigen with full-chain-length or single O unit is expected if the limiting step was ligation or polymerization, respectively.

The data were complicated by the presence of what appears to be M unit and M antigen in some strains running at positions identical or close to those of O unit and O antigen, respectively. However, we feel confident that the material identified as O unit (peak 4) in the *rfbP4451(T)* strain is indeed O unit, as it is not chased through, whereas in the same strain, the M antigen (peak 4) can be chased through. There is no reason to expect a mutation in *rfb* to block processing of M-antigen intermediates.

We conclude that *rfbP* encodes a bifunctional protein and that the T function may be concerned with the flipping of the UndPP-linked single O unit from the cytoplasmic face to the periplasmic face of the inner membrane, but this conclusion must be regarded as tentative pending more detailed analysis.

The discovery of a second function for *rfbP* raised the question as to whether there was an error in our original *rfbP* sequencing data. We have resequenced the *rfbP* gene and found no error. The deduced amino acid sequence of *rfbP(T)* shows good homology with those of *gumD* and *exoY* (Fig. 5). *gumD* encodes a sugar transferase involved in the synthesis of extracellular polysaccharide of *Xanthomonas campestris* (4, 30), and it can be aligned with entire *rfbP(T)* with 28.4% identity and 50.9% similarity at the amino acid level; the C-terminal part of RfbP(T) shows good homology with ExoY, which is a galactosyl-1-phosphate transferase in *R. meliloti*. The alignment with GumD supports our belief that there is only one gene in the *rfbP* region and that RfbP is a bifunctional protein. The alignment with ExoY indicates that the C-terminal end is the galactosyl-1-phosphate transferase domain. We suggest that GumD may also be bifunctional, with its N-terminal part involved in translocation of a lipid-linked oligosaccharide intermediate in *X. campestris*.

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