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Localization of the Terminal Steps of O-Antigen Synthesis in *Salmonella typhimurium*

BARBARA C. McGRATH AND M. J. OSBORN*

Department of Microbiology, University of Connecticut Health Center, Farmington, Connecticut 06030

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Previous immunoelectron microscopic studies have shown that both the final intermediate in O-antigen synthesis, undecaprenol-linked O polymer, and newly synthesized O-antigenic lipopolysaccharide are localized to the periplasmic face of the inner membrane (C. A. Mulford and M. J. Osborn, Proc. Natl. Acad. Sci. USA 80:1159–1163, 1983). In vivo pulse-chase experiments now provide further evidence that attachment of O antigen to core lipopolysaccharide, as well as polymerization of O-specific polysaccharide chains, takes place at the periplasmic face of the membrane. Mutants doubly conditional in lipopolysaccharide synthesis [*kdsA*(Ts) *pmi*] were constructed in which synthesis of core lipopolysaccharide and O antigen are temperature sensitive and mannose dependent, respectively. Periplasmic orientation of O antigen:core lipopolysaccharide ligase was established by experiments showing rapid chase of undecaprenol-linked O polymer, previously accumulated at 42°C in the absence of core synthesis, into lipopolysaccharide following resumption of core formation at 30°C. In addition, chase of the monomeric O-specific tetrasaccharide unit into lipopolysaccharide was found in similar experiments in an O-polymerase-negative [*rfc kdsA*(Ts) *pmi*) mutant, suggesting that polymerization of O chains also occurs at the external face of the inner membrane.

Biosynthesis of the O antigen of *Salmonella typhimurium* is a complex process catalyzed by glycosyltransferases of the inner (cytoplasmic) membrane (13). The oligosaccharide repeating unit is formed by sequential transfer of galactose-1-phosphate (galactose-1-P), rhamnose, mannose, and abequose residues from the respective nucleotide sugars to the carrier lipid, undecaprenyl phosphate (UndP) (Fig. 1). Monomeric tetrasaccharide units are polymerized into O-specific polysaccharide chains, which are then transferred from UndP to the independently synthesized core lipopolysaccharide. Finally, the O-antigenic lipopolysaccharide product is translocated from the inner membrane to the external leaflet of the outer membrane.

Information on the topology of O-antigen assembly within the inner membrane is fragmentary, but available evidence suggests that the pathway is transmembrane (12). Nucleotide sugar precursor pools and enzymes for early steps in core lipopolysaccharide synthesis are believed to be cytosolic (1, 15). On the other hand, immunoelectron microscopy has established that newly synthesized O-positive lipopolysaccharide is transiently localized at the extracytoplasmic (periplasmic) surface of the inner membrane as an intermediate in translocation to the outer membrane (11, 12). These studies also showed that UndP-linked O-antigen polymer accumulates stably at the periplasmic face of inner membrane when transfer to lipopolysaccharide is blocked by a mutation in core biosynthesis. The latter finding strongly suggested that the final transfer of O antigen to lipopolysaccharide takes place at the periplasmic face of the inner membrane. However, the possibility could not be excluded that the observed accumulation of periplasmically oriented undecaprenol-linked O polymer in the mutant strain represents a dead-end side product of the interrupted pathway of lipopolysaccharide assembly.

In order to address this question more directly, we have now carried out pulse-chase experiments with doubly conditional mutants in which core lipopolysaccharide synthesis and O-antigen formation can be separately controlled. The results described here support the previous conclusion that attachment of O antigen to lipopolysaccharide takes place at the periplasmic face of the inner membrane and in addition provide evidence that O-antigen polymerase is also periplasmically oriented.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* BCM1 is a *kdsA*(Ts) derivative of SL3540 (*galE pmi trp met*), which was constructed by P1-mediated cotransduction of *kdsA*(Ts) with a closely linked transposon Tn*10* (16). BCM2 was similarly derived from a *pmi* derivative of the *rfc* strain G5710. G5710 was isolated as a spontaneous *rfc* mutant of G30 (*galE*) and identified by phage pattern, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of its lipopolysaccharide, and enzymatic assay of O polymerase. The *pmi* mutation was isolated after nitrosoguanidine mutagenesis by selection on MacConkey-mannose plates and enzyme assay. All *pmi* mutants were maintained in *galE* backgrounds in order to minimize secondary mutation to the O-antigen-negative (*rfb*) phenotype.

Growth conditions and medium. Cultures were grown at 30°C with vigorous aeration. Unless otherwise specified, a synthetic rich medium (MRSS medium) was used, containing M9 salts, 0.2% ribose, and a complete mixture of amino acids, purines, pyrimidines, and vitamins (10). This medium was used to avoid trace amounts of galactose present in peptone-based media.

Pulse-chase procedure. Cultures were grown at 30°C to a density of approximately 5×10^8/ml; the temperature was then quickly raised by addition of 1 volume of fresh 65°C medium, and incubation was continued at 42°C for 30 min. Synthesis of lipopolysaccharide ceases within 10 to 15 min at this temperature because of the thermosensitive *kdsA* mutation. Nonradioactive galactose (5 μM for BCM1, 20 μM for BCM2) and ^{3}H- or ^{14}C-labeled mannose (at the concentra-

* Corresponding author.

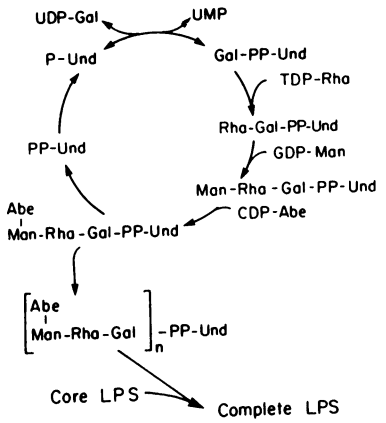

FIG. 1. Pathway of O antigen biosynthesis. Abbreviations: Gal, D-galactose; Rha, L-rhamnose; Man, D-mannose; Abe, abequose; Und, undecaprenol; PP, pyrophosphoryl; LPS, lipopolysaccharide.

tions indicated in Results) were then added, and the incubation was continued for 15 min. At the end of the pulse period, the culture was split into two portions, one of which was shifted to 30°C for chase while the other remained at 42°C. At zero time of chase and at the indicated times thereafter, 10-ml samples were removed into centrifuge tubes containing 0.5 ml of 50 mM DNP (2,4-dinitrophenol) and 2 g of ice. All samples were kept on ice thereafter. Cells were recovered by centrifugation in a Sorvall RC2B centrifuge (10,000 rpm, 5 min, 4°C) for analysis as described below.

For determination of mannose incorporation into polymeric intermediates and lipopolysaccharide in BCM1, labeled cells were first washed twice with 2 ml of cold 50% acetone to remove low-molecular-weight materials. Acetone-washed cells were resuspended in 1 ml of 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5) containing 50 to 100 mg of carrier lipopolysaccharide and were extracted with 45% phenol at 70°C as previously described (5, 12). Lipopolysaccharide was precipitated from the aqueous phase with cold 65% ethanol–10 mM sodium acetate (5), and radioactivity was counted. Nonlipopolysaccharide O antigen remained in the ethanol supernatant, and radioactivity in the supernatant was counted without further fractionation.

Formation of undecaprenol-linked tetrasaccharide in BCM2 was routinely measured by extraction into *n*-butanol. Cells were washed by centrifugation with 2 ml of saline containing 2.5 mM DNP and extracted with 1.5 ml of *n*-butanol saturated with 50 mM KCl by vigorous agitation on a Vortex mixer for 30 s (5). The butanol phase was washed twice with 1.0 ml of 50 mM KCl, and radioactivity was counted. Lipopolysaccharide present in the aqueous phase and interface material of the initial butanol extraction was recovered by precipitation with 2 volumes of ethanol, and radioactivity was counted. In experiments in which tetrasaccharide-lipid was to be recovered for further analysis, $CHCl_3$-methanol (2:1) was used for extraction instead of butanol.

Analytical procedures. Gel filtration was done with a column of Sephadex G50 medium (1 by 45 cm) with 50 mM ammonium acetate, pH 7.0. DEAE chromatography was carried out with 2-ml columns of DE52 in 5-ml syringes. The columns were washed with 10 ml of 500 mM pyridinium acetate, pH 4.5, and H_2O before use. Samples were applied in 2 ml of H_2O, and the columns were washed with 9 ml of H_2O. Elution was carried out sequentially with 10 ml each of 50, 100, 200, and 500 mM pyridinium acetate, pH 4.5.

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (8) with 12% acrylamide. Aqueous phases from phenol or butanol extractions were concentrated by lyophilization. Residual phenol was removed before lyophilization by extraction with diethyl ether. Gels were treated with En^{3}Hance (New England Nuclear [NEN]), dried, and autoradiographed with Kodak X-Omat film at −70°C for 3 to 5 days.

Radioactive samples were counted in a Beckman LS2800 liquid scintillation counter. Aqueous samples brought to 0.5 ml with H_2O and counted in 5 ml of either Liquiscint (NEN) or Polyfluor (Packard). Samples in butanol were counted in 10 volumes of the same scintillant. Samples in $CHCl_3$-methanol were taken to dryness in glass vials and counted in 0.5 ml of H_2O as above.

Materials. Radioactive sugars were purchased from NEN.

RESULTS

Transfer of previously accumulated undecaprenol-linked O antigen to newly synthesized core lipopolysaccharide. When attachment of O-antigen chains to lipopolysaccharide is blocked by mutation in synthesis of the acceptor lipopolysaccharide core, polymeric undecaprenol-linked O-antigen intermediates accumulate rapidly at the periplasmic face of the inner membrane (11). To test directly whether this material is a true intermediate in the assembly pathway, we carried out pulse-chase experiments in a mutant, BCM1, which is conditionally defective in synthesis of both core lipopolysaccharide and O antigen. Core biosynthesis is rendered temperature sensitive by a *kdsA*(Ts) mutation (16), while formation of O antigen is dependent on the presence of exogenous mannose because of a mutation in the gene for phosphomannose isomerase (*pmi*). The strain also contains a *galE* mutation, and exogenous galactose is therefore required for synthesis of both O antigen and the complete core lipopolysaccharide which acts as the acceptor of O-antigen chains. Accumulation of labeled undecaprenol-linked O antigen at the periplasmic face of the inner membrane can be initiated by addition of radioactive mannose (plus nonradioactive galactose) to a culture growing at the nonpermissive temperature (42°C). If the accumulated material is, as postulated, the direct precursor of lipopolysaccharide O antigen, the radioactivity should be efficiently chased into lipopolysaccharide following removal of mannose and shift to the temperature permissive for formation of core lipopolysaccharide (30°C).

Experimental conditions for pulse and chase in BCM. In order to obtain satisfactory chase kinetics, it was necessary to establish conditions for efficient shutdown of O-antigen formation and prompt resumption of core lipopolysaccharide biosynthesis at the time of chase. Maximal accumulation of undecaprenol-linked O antigen (approximately 800 pmol of mannose or 25 to 30 pmol of polysaccharide per 10^{10} cells) is limited by the available pool of UndP in the inner membrane and is small compared with the potential size of intracellular mannose-phosphate and GDP-mannose pools. For this reason, chase by addition of excess nonradioactive mannose proved unsatisfactory. Acceptable chase kinetics were obtained when the concentration of labeled mannose added for the pulse was reduced to 100 nM in order to shrink the size of intracellular pools. Under these conditions, incorporation of [^{3}H]mannose reached a plateau within 9 min at 42°C (Fig. 2); at plateau, 80 to 85% of the total added radioactivity was

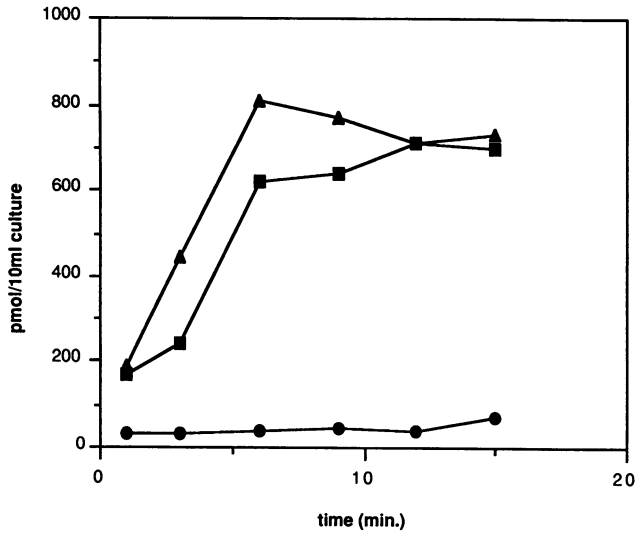

FIG. 2. Accumulation of undecaprenol-linked O polymer in BCM1 at 42°C. BCM1 was grown at 30°C and shifted to 42°C for 30 min as described in Materials and Methods. At this time, nonradioactive galactose (5 μM) and [^{3}H]mannose (100 nM, 100 μCi/μmol) were added, and 10-ml samples were removed at the indicated times for analysis as described in Materials and Methods. Symbols: ▲, acetone-washed cells; ■, undecaprenol-linked O antigen; ●, lipopolysaccharide.

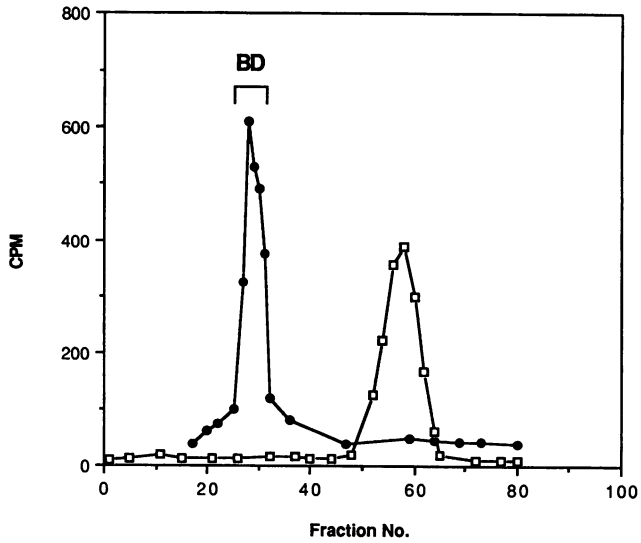

FIG. 3. Gel filtration of product accumulated by BCM1 at 42°C. BCM1 was labeled with [^{14}C]mannose (100 mCi/μmol) at 42°C as described in the legend to Fig. 1 and Materials and Methods. Undecaprenol-linked intermediates were extracted with 45% phenol at 70°C (see Materials and Methods), which also cleaves the saccharide chain from undecaprenol. The ethanol-soluble fraction was taken to dryness on a rotary evaporator at 20°C and dissolved in 5 mM HEPES buffer, pH 7.5. Approximately 10,000 cpm was applied to a Sephadex G50 column as described in Materials and Methods. Fractions were 0.5 ml. The excluded volume, determined with blue dextran (BD), is indicated. The elution position of monomer oligosaccharide was determined with enzymatically synthesized ^{3}H-trisaccharide (Man-Rha-Gal) (□). Radioactivity from [^{14}C]mannose is indicated by closed circles.

reproducibly incorporated into membrane-associated product. The product was isolated by phenol extraction and identified (6) as undecaprenol-linked O polymer by solubility in 65% ethanol, exclusion from Sephadex G50 (Fig. 3), and elution from DEAE-cellulose (data not shown).

Restoration of lipopolysaccharide core biosynthesis was fast and efficient after shift of the culture from 42 to 30°C under chase conditions. Synthesis of core was monitored by incorporation of [^{14}C]galactose into lipopolysaccharide in the absence of added mannose (Fig. 4). Incorporation into a phenol-extractable, ethanol-precipitable lipopolysaccharide product was barely detectable at 42°C. Significant incorporation commenced within 1 to 1.5 min after the shift to 30°C, and by 3 min it corresponded to synthesis of over 3 nmol of potential acceptor lipopolysaccharide.

Chase of previously accumulated undecaprenol-linked O antigen into lipopolysaccharide. A culture of BCM1 was labeled with [^{3}H]mannose for 15 min at 42°C, and the temperature was then shifted to 30°C. After a delay of approximately 1 min, corresponding to the lag in reinitiation of lipopolysaccharide core synthesis, radioactivity was rapidly and efficiently chased out of undecaprenol-linked intermediates and into lipopolysaccharide (Fig. 5A). No change was seen in the control culture maintained at 42°C over the period of chase (Fig. 5B). The half-time of chase at 30°C was approximately 1.5 min, and over 85% of the radioactivity incorporated during the pulse was recovered in the lipopolysaccharide product. Identification of the chase product was confirmed by SDS-polyacrylamide gel electrophoresis and autoradioography of the phenol-extracted products (Fig. 6). The nonlipopolysaccharide O antigen present at time zero of chase did not enter the gel; emergence of the ladder of bands characteristic of lipopolysaccharide O antigen (3, 12) paralleled the time course of chase, as shown in Fig. 5.

Transfer of previously accumulated undecaprenol-linked tetrasaccharide to newly synthesized core lipopolysaccharide.

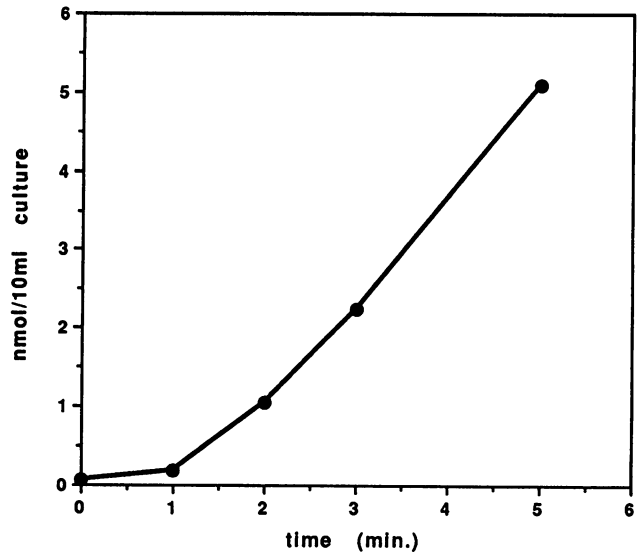

FIG. 4. Incorporation of [^{3}H]galactose into core lipopolysaccharide under pulse-chase conditions. BCM1 was incubated with [^{3}H]galactose (5 μM, 10 μCi/μmol) in the absence of mannose under standard conditions of pulse (42°C) and chase (30°C). Samples were removed at the indicated times after the shift to 30°C, and galactose incorporation into lipopolysaccharide was determined by phenol extraction and ethanol precipitation (see Materials and Methods).

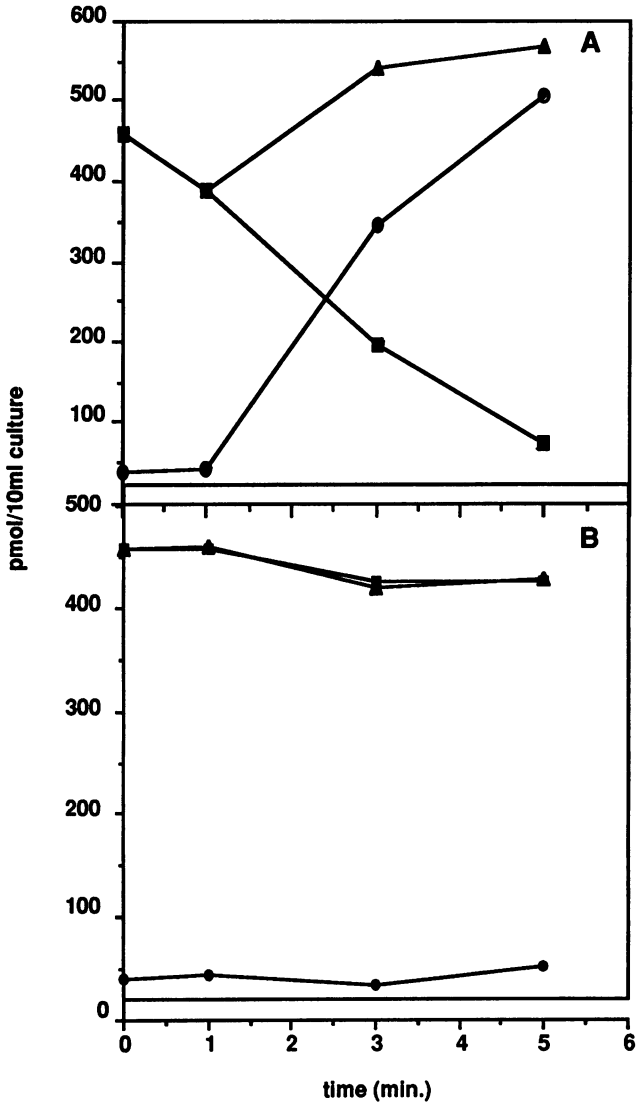

FIG. 5. Chase of previously accumulated undecaprenol-linked O polymer into lipopolysaccharide. Conditions for pulse-labeling with [^{3}H]mannose were as described in the legend to Fig. 1. Chase was carried out at 30°C (A) or 42°C (B). Symbols: ▲, phenol extract; ■, undecaprenol-linked O antigen; ●, lipopolysaccharide.

Mutants defective in O-antigen polymerase (*rfc* mutants) produce lipopolysaccharide that carries a single O-antigen repeat unit (4, 14, 17) and this species is also a prominent component of the O-antigen ladder seen in SDS gel electrophoresis of wild-type lipopolysaccharide. It was therefore of interest to compare chase of the tetrasaccharide monomer into lipopolysaccharide with that described above for polymer.

Strain BCM2, which is *pmi kdsA*(Ts) *galE rfc* (O-polymerase negative), was used for this purpose. Incorporation of radioactive mannose into butanol-soluble tetrasaccharide-lipid at 42°C (Fig. 7) followed a time course similar to that for polymer-lipid. Identification of the product as the monomeric tetrasaccharide was confirmed by mild acid hydrolysis and gel filtration (data not shown). It should be noted that the total amount of mannose incorporated into O-specific product was about 20-fold lower than in the *rfc*$^+$ derivative,

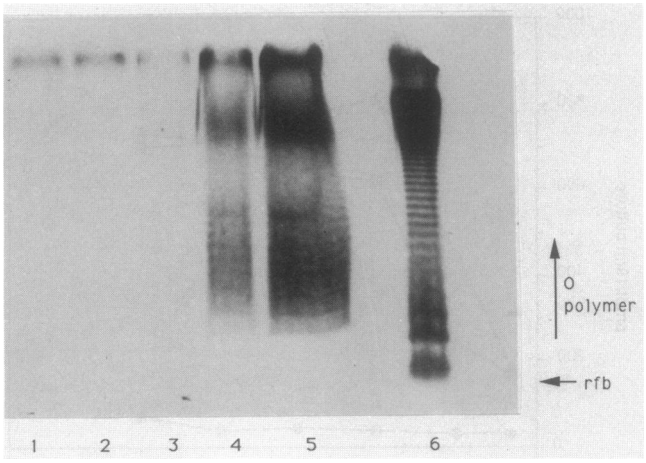

FIG. 6. SDS-polyacrylamide gel electrophoresis of the product of chase in BCM1. Pulse and chase were carried out under standard conditions except that [^{14}C]mannose (100 μCi/μmol) was used in place of [^{3}H]mannose. Samples were extracted with phenol as described in Materials and Methods, and the resulting aqueous phases were extracted with ether to remove residual phenol. Samples were lyophilized and dissolved in a minimal volume of 5 mM HEPES buffer, pH 7.5. An equal number of counts (approximately 10,000 cpm) were taken from each sample for electrophoresis. Lanes 1 to 5, 0, 0.5, 1, 3, and 5 min of chase at 30°C, respectively. Lane 6, Standard wild-type lipopolysaccharide labeled with [^{14}C]galactose. The arrow marked rfb indicates the position of the complete core lipopolysaccharide lacking O antigen.

consistent with formation of a monomeric rather than polymeric product. As a result, however, satisfactory chase was obtained only when the internal pool of labeled precursors remaining at the end of the pulse was minimized by reducing the external concentration of [^{3}H]mannose to 8 nM. Under these conditions, tetrasaccharide-lipid accounted for 60 to 70% of the total cell-associated radioactivity. A high concentration of glucose was also added at the time of shift to

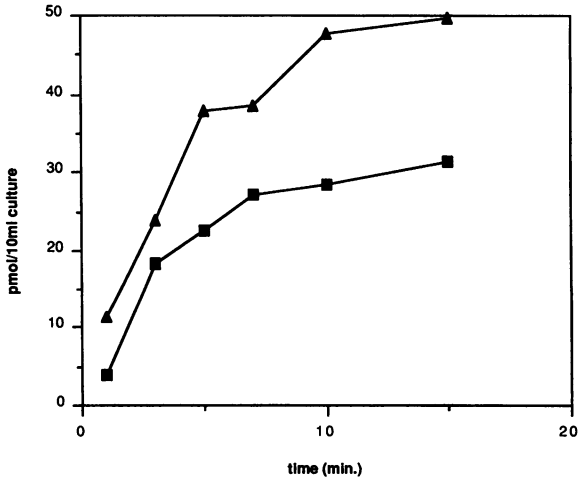

FIG. 7. Accumulation of undecaprenol-linked tetrasaccharide monomer unit at 42°C in BCM2. Conditions for pulse and for measurement of undecaprenol-linked oligosaccharide were as described in Materials and Methods; concentrations of [^{14}C]mannose (100 μCi/μmol) and nonradioactive galactose were 8.3 nM and 5 μM, respectively. Symbols: ▲, total cell-associated radioactivity; ■, lipid-linked oligosaccharide.

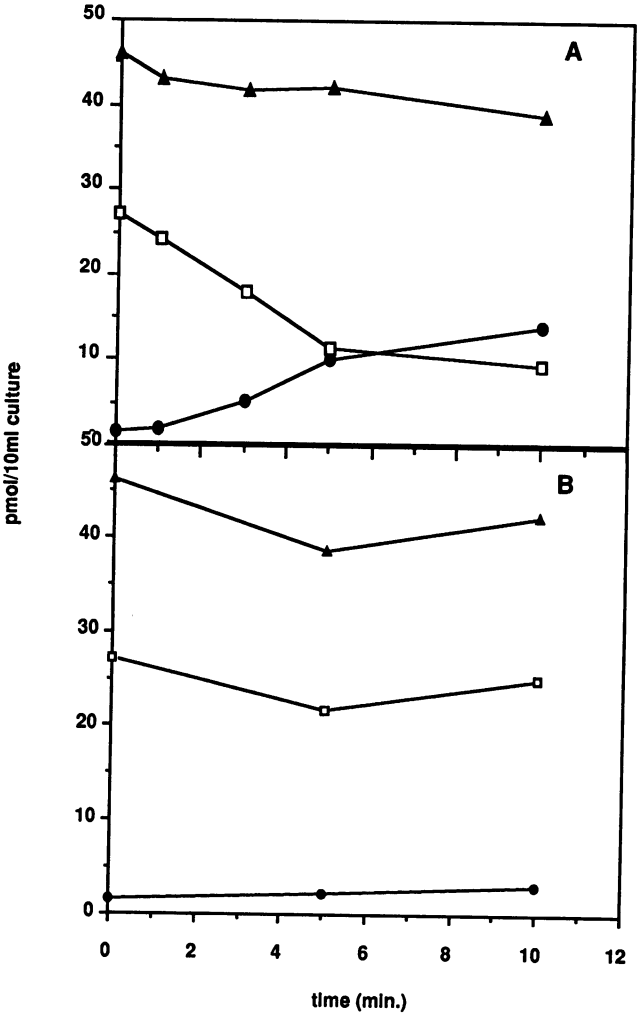

FIG. 8. Chase of previously accumulated undecaprenol-linked tetrasaccharide into lipopolysaccharide in BCM2. Standard pulse-chase conditions were used, with 20 μM nonradioactive galactose and 8.3 nM [^{14}C]mannose (100 μCi/μmol). Glucose (5 mM) was added at time zero of chase to minimize continued uptake of mannose. Chase was performed at 30°C (A) or 42°C (B). Symbols: ▲, total cell-associated radioactivity; □, undecaprenol-linked oligosaccharide; ●, lipopolysaccharide.

30°C in order to minimize further mannose uptake during chase without expanding internal pools.

With the above modifications of conditions, chase of radioactive undecaprenol-linked monomer into lipopolysaccharide began promptly after the shift to 30°C and continued at a more or less linear rate for at least 10 min (Fig. 8). The end product of the chase was characterized by phenol extraction, ethanol precipitation, and SDS-polyacrylamide gel electrophoresis (Fig. 9). The labeled product migrated as a single band at the position corresponding to lipopolysaccharide carrying a single O repeat unit. The apparent rate of transfer of monomer to acceptor lipopolysaccharide was, however, considerably slower than that observed for polymer, and even after 10 min, transfer was only 50 to 60% complete. The difference in the observed rate presumably reflects, at least in part, a preference of the O-antigen ligase enzyme for polymeric substrates.

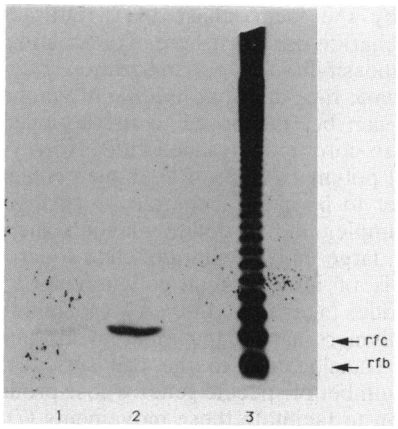

FIG. 9. SDS-polyacrylamide gel electrophoresis of the product of chase in BCM2. Pulse-chase conditions were as described in the legend to Fig. 7. Samples were taken at 0 and 15 min of chase. Phenol extraction and electrophoresis were carried out as described in the legend to Fig. 5. Lane 1, 0 min of chase; lane 2, 15 min of chase; lane 3, standard wild-type lipopolysaccharide. The arrow marked rfb indicates the position of the complete core lipopolysaccharide lacking O antigen. The arrow marked rfc indicates the position of core lipopolysaccharide carrying a single O-antigen tetrasaccharide unit.

DISCUSSION

The results presented here, in conjunction with previous immunoelectron microscopic evidence (11, 12) provide strong evidence that the final step in lipopolysaccharide assembly—transfer of O-antigen chains to core lipopolysaccharide by O-antigen ligase—takes place at the periplasmic face of the inner membrane. This conclusion is based on the observations that (i) undecaprenol-linked O polymer accumulates at the periplasmic face of the membrane under conditions in which it cannot be transferred to core lipopolysaccharide (11), (ii) transfer of previously accumulated polymer to newly synthesized acceptor core lipopolysaccharide is rapid and virtually complete (this work); and (iii) newly synthesized lipopolysaccharide O antigen transiently occupies the periplasmic face of the inner membrane as an intermediate in translocation to the outer membrane (11, 12). Although it has not been directly demonstrated that the lipid-linked O polymer is localized exclusively at the periplasmic side of the membrane, the finding that over 85% of the pulse-labeled intermediate was subsequently chased into lipopolysaccharide argues strongly that periplasmically oriented molecules are effective substrates for O ligase. The topology of the undecaprenol-mediated O-antigen pathway thus appears to resemble that of murein assembly, in which addition of new peptidoglycan units from undecaprenol-linked intermediates to the existing murein sacculus necessarily takes place at the periplasmic side of the membrane.

The available evidence suggests that polymerization of O-antigen chains also takes place at the periplasmic surface. The lipopolysaccharide of mutants lacking O-antigen polymerase contains a single O-specific oligosaccharide unit (17), and this species is also prominent in wild-type lipopolysaccharides (4, 14). Thus, the undecaprenol-linked monomer must be accessible to the active site of O-ligase. The conclusion that the monomeric intermediate is located, at least in part, at the periplasmic side of the membrane is

supported by the facile chase of O-tetrasaccharide into lipopolysaccharide observed here. The existing data do not exclude the possibility that polymerization actually occurs at the cytoplasmic face and that lipid-linked monomer as well as polymer can be transposed to the periplasmic side for attachment to core lipopolysaccharide. However, the alternative—that polymerization of O-antigen chains, as well as their transfer to lipopolysaccharide, is periplasmically oriented—is simpler and in addition avoids the necessity of transposing large polysaccharide chains across the lipid bilayer. It is of interest that polymerization of capsular polysaccharides takes place in the cytosol and that transport of these polymers across the inner membrane as well as subsequent translocation to the cell surface requires the action of a number of specific genes whose products presumably function to facilitate these movements (7). No genetic evidence has yet been adduced to suggest participation of analogous machinery in biogenesis of O antigen.

The topology of the early steps of O antigen synthesis leading to formation of undecaprenol-linked tetrasaccharide remains to be established. Indirect evidence (2) suggests that the oligosaccharide unit is initially assembled on the cytoplasmic side and subsequently transposed to the periplasmic face for polymerization. Studies are in progress to test this hypothesis and to determine more directly the orientation of the active site of the O-polymerase enzyme. The proposed pathway of lipopolysaccharide assembly also requires transmembrane transposition of the core lipopolysaccharide from its presumptive site of synthesis at the cytoplasmic face to the periplasmic face, where O chains are attached. That this transposition may be dependent on maintenance of membrane potential is suggested by recent observations that the uncoupler DNP inhibits transfer of O chains to the core by preventing access of newly synthesized core lipopolysaccharide to the ligase enzyme (9).

It is well established that the size distribution of O-antigen chains in lipopolysaccharide is nonuniform and apparently nonrandom (4, 14), but the biosynthetic mechanism responsible for the complex pattern of chain lengths observed in SDS-polyacrylamide gel electrophoresis is poorly understood. It is of interest that the chain length distribution observed here following chase of previously synthesized O chains into lipopolysaccharide paralleled the normal pattern rather closely. The results are not consistent with a model in which the distribution is determined primarily by chain length specificity of the O-ligase, since transfer of the accumulated intermediates to lipopolysaccharide was almost quantitative, or by simple competition between ligase and polymerase. The observations are in accord with the recent conclusion of Goldman and Hunt (3), based on mathematical modeling, that chain length specificity is determined by O polymerase; the mechanistic basis for the phenomenon remains unknown.

ACKNOWLEDGMENT

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