Accumulation of a Lipid-Linked Intermediate Involved in Enterobacterial Common Antigen Synthesis in *Salmonella typhimurium* Mutants Lacking dTDP-Glucose Pyrophosphorylase

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The heteropolysaccharide chains of enterobacterial common antigen (ECA) are composed of linear trisaccharide repeat units having the structure $\rightarrow 3)-\alpha\text{-Fuc4NAc-(1}\rightarrow 4)-\beta\text{-D-ManNAcA-(1}\rightarrow 4)-\alpha\text{-D-GlcNAc-(1}\rightarrow$. Mutants of *Salmonella typhimurium* lacking the structural gene for dTDP-glucose pyrophosphorylase (*rfbA*) are severely impaired in their ability to synthesize dTDP-glucose, which is a precursor of dTDP-4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc), the donor of Fuc4NAc residues for ECA synthesis. These mutants synthesize only trace amounts of ECA, and they are hypersensitive to sodium dodecyl sulfate (SDS). Incubation of $\Delta rfbA$ mutants with [^3H]N-acetylglucosamine ([^3H]GlcNAc) resulted in the accumulation of radioactivity in N-acetyl-D-mannosaminuronic acid (ManNAcA)-GlcNAc-pyrophosphorylundecaprenol (lipid II), the putative acceptor of Fuc4NAc residues in ECA synthesis. Lipid II did not accumulate in either wild-type cells or in *rff* mutants unable to synthesize ManNAcA. Both the accumulation of lipid II and the synthesis of trace amounts of ECA were abolished when $\Delta rfbA$ mutants were grown in the presence of the antibiotic tunicamycin. Tunicamycin also prevented the SDS-mediated lysis of the mutants. SDS-resistant derivatives of $\Delta rfbA$ mutants were isolated that were no longer able to synthesize trace amounts of ECA. Characterization of these derivatives revealed that they were defective in various steps of ECA synthesis leading to the synthesis of lipid II. The data support the conclusion that accumulation of lipid II is responsible in some way for the hypersensitivity of $\Delta rfbA$ mutants to SDS.

Enterobacterial common antigen (ECA) is a cell-surface glycolipid produced by all members of the family *Enterobacteriaceae* (12, 14, 20). The carbohydrate portion consists of a linear heteropolysaccharide composed of N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-mannosaminuronic acid (ManNAcA), and 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc) (9). These sugars are linked to form trisaccharide repeat units having the structure $\rightarrow 3)-\alpha\text{-Fuc4NAc-(1}\rightarrow 4)-\beta\text{-D-ManNAcA-(1}\rightarrow 4)-\alpha\text{-D-GlcNAc-(1}\rightarrow$ (9). All bacteria belonging to the *Enterobacteriaceae* synthesize the haptenic form of ECA, which consists of individual heteropolysaccharide chains linked to a phospholipid aglycone (3, 5, 14). In addition, certain rough mutants also possess ECA heteropolysaccharide chains that are covalently linked to the core region of lipopolysaccharide (14, 20).

Previous studies have established that GlcNAc-pyrophosphorylundecaprenol (lipid I) and ManNAcA-GlcNAc-pyrophosphorylundecaprenol (lipid II) are intermediates in ECA synthesis (1, 21). In addition, more recent experiments (unpublished data) have provided evidence that lipid II serves as the acceptor of Fuc4NAc residues from the donor dTDP-Fuc4NAc. However, nothing is yet known concerning the mechanisms involved in polysaccharide chain elongation or the transfer of ECA chains to phospholipid or lipopolysaccharide.

The genetic determinants of ECA synthesis include genes in the *rfe* and *rff* gene clusters located at min 83 and 85 of the *Salmonella* and *Escherichia coli* chromosomes, respectively (10, 11, 15, 27). In addition to these loci, synthesis of normal amounts of ECA in *Salmonella typhimurium* (group B) depends on a determinant located in the *his*-linked *rfb* region (11, 13). Thus, deletions in the structural gene for dTDP-

glucose pyrophosphorylase (*rfbA*) in *S. typhimurium* preclude synthesis of wild-type levels of dTDP-glucose, which is a precursor of dTDP-Fuc4NAc. Accordingly, $\Delta rfbA$ mutants synthesize trace amounts of ECA, and it has been suggested that the UDP-glucose pyrophosphorylase of these mutants is capable of catalyzing the synthesis of sufficient amounts of dTDP-glucose to account for the ECA^{trace} phenotype (6). In contrast to group B organisms, the structural gene for dTDP-glucose pyrophosphorylase appears to be located in the *rfe* gene cluster of *Salmonella montevideo* (group C₁) (6).

Mutants possessing the ECA^{trace} phenotype are hypersensitive to sodium dodecyl sulfate (SDS), and they readily acquire secondary suppressor mutations in the *rff* gene cluster (13). We present evidence in this study that the synthesis of trace amounts of ECA in $\Delta rfbA$ mutants of *S. typhimurium* is accompanied by the accumulation of lipid II. The data also indicate that the accumulation of lipid II is responsible for the hypersensitivity of these mutants to SDS.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. typhimurium* strains relevant to the present study are listed in Table 1. Cultures were grown with vigorous aeration in Proteose Peptone (Difco Laboratories, Detroit, Mich.)-beef extract medium (24) containing 0.2% glucose (medium A). Strain SH5150-2 *ilv*⁺ *rff*⁺ *his*-809 was constructed by introduction of the *rff*⁺ allele from the HfrK1-2 donor SA464 *serA*13 *rfa*-3058 (26) into the *S. typhimurium* recipient SH5150 *ilv*-1190 *rff*-4270 *his*-809 (13) by conjugation and selection for the closely linked *ilv*⁺ allele. Transconjugants were then screened for their sensitivity to SDS. The presence of the *rff*⁺ allele in the *ilv*⁺ SDS-sensitive transconjugant SH5150-2 was confirmed by demonstrating the ability of the strain to

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TABLE 1. Strains of *S. typhimurium* used

Strain	Relevant characteristics	Source or reference
PR122	<i>hisF1009 trpB2 metA22 xyl-1 rpsL galE nagB F⁻</i>	23
SH4892	<i>rfb-4020 his-5046 Str^r</i>	H. Nikaido
SH5150	<i>rff-4270 ilv-1190 his-809</i> (extended deletion in the <i>his-rfb</i> region)	P. H. Mäkelä
SH5150-2	<i>ilv⁺ rff⁺</i> recombinant from cross HfrK1-2 × SH5150	This study
SH5187	<i>his-399</i> (extended deletion in the <i>his-rfb</i> region)	P. H. Mäkelä
SA464 (HfrK1-2)	<i>serA13 rfa-3058</i> (CW, 0-117)	26

synthesize trace amounts of ECA, as determined by Western blot (immunoblot) analysis, and the occurrence in cell extracts of wild-type levels of the enzyme activities involved in the conversion of UDP-GlcNAc to UDP-ManNAcA. SDS-resistant derivatives of SH5150-2 were isolated by plating out cells from cultures growing in medium A onto Proteose Peptone-beef extract agar plates containing 0.1% SDS followed by incubation of the plates at 37°C. SDS-resistant derivatives occurred at a frequency of 1.9×10^{-5} .

Radiochemicals, chemicals, and reagents. UDP-*N*-acetyl-D-[6-³H(N)]glucosamine (18.5 Ci/mmol) was purchased from Dupont, NEN Research Products (Boston, Mass.). *N*-Acetyl-D-[1-³H]glucosamine (2.84 and 3.4 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill.). Tunicamycin (mixture of isomers A, B, C, and D) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Mouse anti-ECA monoclonal antibody MAB898 (immunoglobulin G2a) was kindly provided by D. Bitter-Suermann, Johannes-Gutenberg University, Mainz, Federal Republic of Germany (19). Authentic β -D-ManNAcA-(1 $\rightarrow$ 4)-D-GlcNAc was the generous gift of H. Paulsen, University of Hamburg, Hamburg, Federal Republic of Germany.

Incorporation of [³H]GlcNAc into lipid I and lipid II in vivo. Cultures (20 ml) were grown in medium A overnight at 37°C. Cells from the overnight cultures were used to inoculate fresh medium A (60 ml), and growth was allowed to continue until the culture reached an A_{600} of 0.4. The cells were harvested by centrifugation (25,000 $\times g$, 10 min, 25°C), resuspended in 6 ml of fresh medium A containing [³H]GlcNAc (18 μ Ci, 2.84 Ci/mmol), and incubated at 37°C with shaking for an additional 30 min. The labeled cells were subsequently poured over 2.5 g of crushed ice, harvested by centrifugation (25,000 $\times g$, 15 min, 4°C), and washed with 6 ml of cold 0.9% saline. In experiments in which the effects of tunicamycin were evaluated, the drug was added to cultures to give a final concentration of 10 μ g/ml unless indicated otherwise. The washed pellets were extracted successively with 6 ml of 95% ethanol and 6 ml of acetone and subsequently dried in vacuo. The extracted cells were suspended in 1.25 ml of chloroform-methanol (3:2, vol/vol), and the suspension was stirred vigorously for 15 min at room temperature. Particulate matter was removed by centrifugation (25,000 $\times g$, 15 min, 25°C), and the supernatant solution was mixed thoroughly following the addition of 0.25 ml of 4 mM MgCl₂. The lower organic phase was reextracted with 0.25 ml of the upper phase resulting from a mixture of four parts of chloroform-methanol (3:2, vol/vol) and one part of 4 mM MgCl₂. Following a brief centrifugation, the lower chloroform-rich phase was removed and evaporated to dryness

under a stream of nitrogen. The residue was suspended in chloroform-methanol-water (10:10:3, by volume) and analyzed by ascending chromatography on EDTA-treated SG-81 paper employing solvent A (see below for a definition of solvents used). Radioactive regions of the chromatogram were cut into 1-cm sections, and the sections were soaked in 0.5 ml of 1.25% SDS for 6 to 8 h at 42°C and analyzed by liquid scintillation counting with toluene-Liquifluor-Bio-Solv (22).

Analysis of water-soluble component released from lipid II by treatment with mild acid. Strain SH5187 was grown in medium A (200 ml) to an A_{600} of 0.4. The cells were harvested by centrifugation, resuspended in 25 ml of fresh medium A containing [³H]GlcNAc (75 μ Ci, 2.84 Ci/mmol), and incubated with shaking at 37°C for 30 min. Subsequent processing of the cells for extraction of radioactive lipid II was done as described above except that the volume of all reagents was increased fourfold. The extracted lipid was resuspended in methanol-toluene (1:1, by volume) and partially purified by DEAE-cellulose chromatography as outlined below. The fractions composing the major peak of radioactive material were pooled and evaporated to dryness. The residue was dissolved in 1.0 ml of chloroform-methanol (2:1, vol/vol) and extracted with 0.2 ml of water. The resulting lower organic phase was reduced to dryness, and the radioactive lipid was incubated with 0.3 ml of 0.1 N HCl at 100°C for 15 min. The hydrolysate was then taken to dryness in vacuo over NaOH pellets and P₂O₅. The residue was dissolved in 0.5 ml of water, and portions were analyzed by paper chromatography and high-voltage paper electrophoresis. The remainder was applied to a small column (1 ml) of Dowex 50 X 4(H⁺), and the column was washed with 1.5 ml of water. The eluant was lyophilized, and the residue containing the decationized oligosaccharide was dissolved in 1 ml of water to which was added 10 ml of 5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, pH 4.75. The mixture was incubated at room temperature for 3 h with stirring, and the pH was maintained at 4.75 by the addition of 0.01 N HCl. Aqueous NaBH₄ (2 M, 500 μ l) was added to the mixture over 30 min, and the pH was maintained at 7.0 by the addition of HCl. The solution was subsequently incubated overnight at 4°C with stirring. Sodium ions were removed by passage of the solution over a small column (2 ml) of Dowex 50 X 4(H⁺). The column was washed with water, and the eluant was evaporated to dryness. Borate was removed as volatile methyl borate by repeated evaporation to dryness in the presence of acidic methanol, and the sample was dissolved in 1 ml of water. The radioactive oligosaccharide was subjected to a second reduction by the addition of solid NaBH₄ to the solution to give a final concentration of 1 M, and the reaction mixture was incubated for 36 h at 4°C. Subsequent removal of sodium and borate ions was done as described above. The reduced oligosaccharide was purified by preparative paper electrophoresis and incubated with 4 N HCl at 100°C for 26 h. The hydrolysate was then reduced to dryness in vacuo over NaOH pellets and P₂O₅. The residue was dissolved in water and analyzed by descending paper chromatography employing solvent C.

Assay for in vitro synthesis of lipid I. The preparation of cell envelopes, as well as the composition of reaction mixtures and the conditions used for the in vitro synthesis of [³H]GlcNAc-labeled lipid I, have been described previously (1).

Assay for synthesis of UDP-ManNAcA from UDP-GlcNAc. Bacteria were grown until the late exponential phase in 200

ml of medium A at 37°C with shaking. The cells were harvested by centrifugation and washed with 0.9% cold saline. The pellet was suspended in 1.0 ml of 30 mM Tris hydrochloride (pH 7.6), and the cells were disrupted by sonication with a Sonifier cell disrupter equipped with a micro-tip probe (Heat Systems-Ultrasonics, Inc.). Particulate matter was removed by centrifugation ($175,000 \times g$, 1 h, 4°C), and the soluble fraction was used as the source of enzymes in reaction mixtures. Net synthesis of UDP-ManNAcA from UDP-GlcNAc was assayed by the method of Ichihara et al. (4) as modified by Lew et al. (6). Protein was determined by the method of Lowry et al. (8) with bovine serum albumin as the standard.

Western blot analyses. Western blot (immunoblot) analyses were conducted as previously described (2, 21) with mouse anti-ECA monoclonal antibody MAb898 (immunoglobulin G2a).

Chromatographic and electrophoretic procedures. Paper chromatography was done with Whatman no. 3 and SG-81 papers. SG-81 papers were pretreated by dipping them in EDTA (0.068 M, pH 7.7) and allowing them to dry at room temperature. The EDTA-treated papers were activated at 110°C for 10 min immediately prior to use. The following solvent systems were used (by volume): solvent A, chloroform-methanol-water-ammonium hydroxide (88:48:10:1); solvent B, isobutyric acid-1 N ammonium hydroxide (5:3); solvent C, butanol-pyridine-water (6:4:3).

Paper electrophoresis was done on Whatman no. 3 paper with pyridine-acetic acid-water (1:10:289, by volume), pH 3.5. Electrophoresis was conducted at 50 V/cm with cooling with a Savant flat plate electrophoresis apparatus.

Radioactive compounds were detected by sectioning papers and analyzing the sections by liquid scintillation counting as described above. Nonradioactive sugars were detected on papers with alkaline silver nitrate (28).

DEAE-cellulose (Whatman DE-52, acetate form) was prepared as described by Rouser et al. (25). The poured columns were washed thoroughly with chloroform-methanol-water (2:3:1, by volume) followed by methanol prior to sample application. After sample application, columns were developed with a linear gradient of ammonium acetate (0 to 0.15 M) in chloroform-methanol-water (2:3:1, by volume).

RESULTS

Synthesis of trace amounts of ECA in $\Delta rfbA$ mutants. The relative amounts of ECA in ECA-positive strains and in ECA^{trace} mutants lacking the *rfa* gene were determined by Western blot analysis (Fig. 1). ECA polymers were readily detected in SDS extracts obtained from intact cells of strain PR122 (*rfa*⁺). In contrast, only trace amounts of ECA were detected in extracts obtained from strains SH5187 ($\Delta rfbA$) and SH5150-2 ($\Delta rfbA$). In addition, the synthesis of ECA by ECA^{trace} strains was abolished by tunicamycin, as was also the case for the synthesis of ECA by ECA-positive strains (Fig. 1) (1, 21).

Accumulation of lipid II in $\Delta rfbA$ mutants. Previous experiments established that lipid II is an intermediate in ECA synthesis (1), and recent experiments indicate that lipid II serves as the acceptor of Fuc4NAc residues (P. D. Rick, unpublished experiments). Thus, experiments were conducted to examine the possibility that lipid II accumulates in mutants defective in the synthesis of dTDP-Fuc4NAc, the donor of Fuc4NAc residues in ECA synthesis. Incubation of strain SH5150-2 ($\Delta rfbA$) with [³H]GlcNAc resulted in the accumulation of isotope in a chloroform-methanol-soluble

FIG. 1. ECA levels in ECA⁺, ECA^{trace}, and ECA⁻ strains of *S. typhimurium* as determined by Western blot analysis. Cultures were grown overnight in medium A at 37°C in the presence and absence of tunicamycin as indicated below. Washed cells were extracted with 2% SDS (100°C), and the SDS-soluble fraction was examined by Western blot analysis with mouse anti-ECA monoclonal antibody. The arrow indicates the position of the dye front. Additional details are provided in Materials and Methods. Lanes: 1, strain PR122 (plus tunicamycin); 2, strain PR122 (minus tunicamycin); 3, strain SH5187 (plus tunicamycin); 4, strain SH5187 (minus tunicamycin); 5, strain SH5150-2 (plus tunicamycin); 6, strain SH5150-2 (minus tunicamycin); 7, strain SH5150 (minus tunicamycin). Molecular weights are shown on the left (K, 10³).

component (compound X) having the same chromatographic mobility as lipid II when analyzed by ascending chromatography on SG-81 paper (Fig. 2C). In addition, the synthesis of compound X was inhibited by tunicamycin. Compound X did not accumulate in strain SH4892 (wild type) (Fig. 2A) or in strain SH5150, which also lacks the *rfa* gene, but which is unable to synthesize lipid II owing to a lesion in the *rff* gene cluster (Fig. 2B). It is interesting to note that the inability of strain SH5150 to synthesize lipid II results in the accumulation of lipid I. However, in contrast to the tunicamycin-sensitive accumulation of lipid II in strain SH5150-2, the accumulation of lipid I in the parent strain SH5150 was not abolished by tunicamycin. Strain SH5150 synthesizes an Ra-chemotype lipopolysaccharide lacking only the O-antigen region. Accordingly, the inability of tunicamycin to abolish lipid I synthesis is in agreement with previous results which demonstrated that the drug is unable to inhibit ECA synthesis in strains possessing a complete lipopolysaccharide core region (21). As discussed later, it is possible that the accumulation of lipid II by strain SH5150-2 results in increased permeability of the outer membrane to tunicamycin, thereby facilitating access of the drug to its target.

Partial purification of compound X was accomplished by DEAE-cellulose chromatography. Accordingly, DEAE-cellulose chromatography of the chloroform-methanol-soluble fraction obtained from [³H]GlcNAc-labeled cells revealed a single peak of radioactivity that eluted from the column at the same concentration of ammonium acetate (0.09 M) as did authentic lipid II (data not shown). Mild acid hydrolysis of the isotopically labeled material resulted in the quantitative recovery of radioactivity in a single water-soluble compo-

FIG. 2. Accumulation of $[^3\text{H}]\text{GlcNAc}$ -containing intermediates in ΔrfbA mutants. Cells were grown to the mid-logarithmic phase in medium A at 37°C in the presence (○) or absence (●) of tunicamycin. $[^3\text{H}]\text{GlcNAc}$ was added to the cultures, and labeling was carried out for 30 min. Washed cells were extracted with chloroform-methanol (3:2, by volume), and chloroform-methanol-soluble radioactive material was analyzed by ascending chromatography on SG-81 paper with solvent A. Additional details are provided in Materials and Methods. (A) Strain SH4892; (B) strain SH5150; (C) strain SH5150-2.

ment that exhibited the same mobility as authentic $\beta\text{-D-MannNAcA-(1}\rightarrow\text{4)-D-GlcNAc}$ when analyzed by both descending paper chromatography (Fig. 3) and paper electrophoresis (Fig. 4). Further characterization of the radioactive compound released by mild acid hydrolysis was facilitated by reduction of the compound with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and NaBH_4 . The reduced compound was electrophoretically neutral (data not shown), and treatment of this compound with strong acid (4

FIG. 3. Paper chromatography of the oligosaccharide released from radioactive compound X by mild acid hydrolysis. Radioactive compound X was extracted from strain SH5187 following incubation of the cells with $[^3\text{H}]\text{GlcNAc}$, and the labeled compound was purified by DEAE-cellulose chromatography. The water-soluble radioactive component released from labeled compound X by treatment with mild acid (0.1 N HCl, 100°C , 15 min) was analyzed by descending paper chromatography with solvent B. The location of standard $\beta\text{-D-MannNAcA-(1}\rightarrow\text{4)-D-GlcNAc}$ is indicated. Additional details are provided in Materials and Methods.

N HCl, 100°C) released approximately equal amounts of radioactive glucosaminitol (1,800 dpm) and mannosamine (1,590 dpm). The only other radioactive component detected after strong acid hydrolysis of the fully reduced compound was unhydrolyzed material which accounted for approximately 18% of the total radioactivity. The data presented above strongly support the identification of compound X as lipid II.

Relationship between accumulation of lipid II and SDS sensitivity of $\text{ECA}^{\text{trace}}$ mutants. Earlier studies demonstrated that mutants of *S. typhimurium* possessing the $\text{ECA}^{\text{trace}}$ phenotype are hypersensitive to SDS (13). Thus, experiments were conducted to investigate the possibility that the accumulation of lipid II in these mutants is responsible for their increased sensitivity to this detergent. Addition of SDS (0.05% final concentration) to cultures of SH5150-2 during mid-logarithmic-phase growth resulted in cell death owing to lysis (Fig. 5). However, since tunicamycin inhibits the synthesis of lipid I (1) and thereby precludes the accumulation of lipid II (Fig. 2), the ability of tunicamycin to protect cells against the effects of SDS was examined. The SDS-mediated lysis of cells was prevented by growing cells in the presence of tunicamycin (Fig. 5), and the growth rate of tunicamycin-treated cells after the addition of SDS was only somewhat reduced in comparison with that of tunicamycin-treated cells to which no SDS was added. These data support the conclusion that the accumulation of lipid II contributes to the SDS hypersensitivity of mutants possessing the $\text{ECA}^{\text{trace}}$ phenotype.

Characterization of SDS-resistant derivatives of strain SH5150-2. Mutants of *S. typhimurium* possessing the $\text{ECA}^{\text{trace}}$ phenotype readily accumulate suppressor mutations that render them both resistant to SDS and $\text{ECA}^{\text{trace}}$ negative (13). Previous characterization of several strains possessing such suppressor mutations indicated that the mutations were all located in the *rff* gene cluster (13); this region includes the genes involved in UDP-ManNAcA synthesis (7) as well as the structural gene for the transferase

FIG. 4. High-voltage paper electrophoresis of the oligosaccharide released from radioactive compound X by mild acid hydrolysis. Tritium-labeled compound X oligosaccharide was obtained as described in the legend to Fig. 3. High-voltage paper electrophoresis was done as described in Materials and Methods. The location of standard β -D-ManNAcA-(1 $\rightarrow$ 4)-D-GlcNAc is indicated.

that catalyzes the transfer of ManNAcA from UDP-ManNAcA to lipid I (2). However, the specific biochemical defects in ECA synthesis owing to the suppressor mutations were not identified in these studies. Accordingly, biochemical characterization of SDS-resistant and ECA-negative derivatives of strain SH5150-2 was done to define more specifically the nature of the suppressor mutations (Table 2).

Characterization of three independent and randomly selected SDS-resistant and ECA-negative derivatives revealed that one of the derivatives, SDS-resistant derivative 2, was indeed defective in the synthesis of UDP-ManNAcA. The inability of this derivative to synthesize lipid II resulted in the accumulation of lipid I; the accumulation of lipid I was also observed in strain SH5150, which also possesses a lesion in the *rff* gene cluster (Table 2; Fig. 2). Similarly, the accumulation of lipid I in SDS-resistant derivative 2 was only partially inhibited by tunicamycin (data not shown).

Characterization of SDS-resistant derivative 3 revealed a new class of suppressor mutation that rendered the mutant ECA-negative owing to an apparent defect in the synthesis of lipid I. No incorporation of radioactivity into lipid I was observed when membrane preparations of this derivative were incubated with UDP-[3 H]GlcNAc under standard reaction conditions. Thus, the suppressor mutation in this mutant appears to result in a defect in the transferase that catalyzes the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to undecaprenyl phosphate. However, neither the chromosomal location of the mutation nor that of the structural gene for the transferase has yet been determined.

SDS-resistant derivative 1 was found to be defective in the synthesis of both UDP-ManNAcA and lipid I. Although it seems likely that the ECA-negative phenotype of this derivative is due to multiple mutations, the existence of a single mutation that affects synthesis of both UDP-ManNAcA and lipid I has not been excluded.

DISCUSSION

Earlier studies in this laboratory established that GlcNAc pyrophosphorylundecaprenol (lipid I) and ManNAcA-Glc-

FIG. 5. Effect of tunicamycin on the SDS sensitivity of strain SH5150-2. Cultures of strain SH5150-2 (ECA^{trace}) were grown overnight at 37°C in medium A containing tunicamycin (20 μ g/ml). The cells were then washed, diluted, and resuspended in fresh medium A either lacking or containing tunicamycin (15 μ g/ml) as indicated. Cultures were grown to an A_{600} of 0.4 to 0.5, and SDS was added as indicated to give a final concentration of 0.05% (wt/vol). The A_{600} and the number of viable cells in each culture were monitored throughout the experiment. Additional details are provided in Materials and Methods.

Nac-pyrophosphorylundecaprenol (lipid II) are intermediates in the synthesis of ECA (1, 21). This information, together with a knowledge of the structure of ECA (9), suggests that lipid II serves as the acceptor of Fuc4NAc residues in the assembly of ECA polysaccharide chains. The results of experiments presented in the present study support this conclusion; the data clearly demonstrate the accumulation of lipid II in deletion mutants of *S. typhimurium* defective in the synthesis of dTDP-Fuc4NAc, the donor of Fuc4NAc residues in ECA synthesis. These mutants possess deletions in the *his*-linked *rff* region that extend through the *rffA* locus, the structural gene for dTDP-glucose pyrophosphorylase (6, 13). Thus, the mutants are unable to synthesize wild-type levels of dTDP-glucose, which is a precursor of dTDP-Fuc4NAc (6). However, Δ *rffA* mutants that are not defective in the synthesis of lipid II are still capable of synthesizing trace amounts of ECA, and it has been suggested that this might be due to the ability of UDP-glucose pyrophosphorylase to catalyze the synthesis of sufficient amounts of dTDP-glucose to account for the ECA^{trace} phenotype (6). Lipid II accumulation was not observed in wild-type strains or in *rff* mutants defective in the synthesis of UDP-ManNAcA, the donor of ManNAcA residues.

Mutants that possess the ECA^{trace} phenotype are hypersensitive to SDS (13), and the data presented in this investigation suggest that their increased sensitivity to this detergent is specifically related to the accumulation of lipid II. Thus, lipid I was found to accumulate in Δ *rffA* *rff* mutants

TABLE 2. Biochemical characterization of SDS-resistant derivatives of strain SH5150-2 (ECA^{trace})^a

Strain	Relevant genotype (phenotype) ^b	Incorporation of [³ H]GlcNAc into lipid I and lipid II in vivo (dpm)		Incorporation of [³ H]GlcNAc into lipid I in vitro (dpm)	Enzyme sp act of UDP-GlcNAc→UDP-ManNAcA nmol/h per mg of protein
		Lipid I	Lipid II		
SH4892	Wild type (ECA ⁺)	1,000	900	1,851	13.85
SH5150	$\Delta rfbA$ <i>rff</i> (ECA ⁻)	13,000	900	1,100	1.35
SH5150-2	$\Delta rfbA$ (ECA ^{trace})	100	10,700	943	8.94
SDS-resistant derivatives:					
1	$\Delta rfbA$ <i>rff</i> ? (ECA ⁻)	1,700	1,200	104	0.92
2	$\Delta rfbA$ <i>rff</i> (ECA ⁻)	21,900	1,400	1,287	1.85
3	$\Delta rfbA$? (ECA ⁻)	600	750	83	8.35

^a SDS-resistant derivatives of strain SH5150-2 were selected by plating cells out on Proteose Peptone-beef extract agar containing 0.1% SDS as described in Materials and Methods. Experimental details concerning the in vivo incorporation of [³H]GlcNAc into lipid I and lipid II, the in vitro incorporation of [³H]GlcNAc into lipid I, and assays for the conversion of UDP-GlcNAc to UDP-ManNAcA are provided in Materials and Methods.

^b ECA was determined by Western blot analysis.

unable to synthesize lipid II owing to defects in the synthesis of UDP-ManNAcA (Fig. 2; Table 2). However, the accumulation of lipid I did not render these mutants hypersensitive to SDS. In contrast, the accumulation of lipid II by strain SH5150-2 $\Delta rfbA$ *rff*⁺ was accompanied by marked sensitivity of the mutant to SDS. Previous investigations established that tunicamycin inhibits the synthesis of lipid I and thereby precludes the synthesis of lipid II (1). Accordingly, tunicamycin abolished the accumulation of lipid II by strain SH5150-2, and the tunicamycin-treated cells were significantly more resistant to SDS. Indeed, tunicamycin prevented the SDS-mediated lysis of strain SH5150-2, and addition of SDS to cultures of the mutant growing in the presence of the drug only resulted in a decrease in growth rate. Although it seems reasonable to assume that lipid II accumulates in the cytoplasmic membrane of mutants possessing the ECA^{trace} phenotype, the site of lipid II accumulation in these mutants has not yet been determined.

It is now well established that the synthesis of lipopolysaccharide lacking a complete inner core region is accompanied by pronounced alterations in outer membrane protein and phospholipid composition and organization; such alterations are responsible for the increased sensitivity of these deep-rough mutants to hydrophobic antibiotics and other agents (16-18). For mutants that possess the ECA^{trace} phenotype, the available evidence suggests the possibility that accumulation of lipid II also effects alterations in the outer membrane that result in increased permeability to SDS and other anionic detergents (13). However, the nature of these alterations, as well as the basis for the pronounced difference between the effects of lipid I and lipid II accumulation on the sensitivity of the mutants to SDS, remain to be established. It seems likely that these alterations are distinct from those which occur in deep-rough mutants since deep-rough mutants are considerably more sensitive to gentian violet, erythromycin, and a variety of other antibiotics than are mutants that possess the ECA^{trace} phenotype (13).

The isolation of SDS-resistant derivatives of $\Delta rfbA$ mutants with apparent defects in the synthesis of UDP-ManNAcA and/or lipid I provided indirect support for the conclusion that the accumulation of lipid II is responsible for the hypersensitivity of the mutants to SDS. Alternatively, it is also possible that the increased sensitivity of these mutants to SDS is related to the synthesis of trace amounts of ECA rather than to the accumulation of lipid II. The latter model would predict that selection for SDS-resistant derivatives would also yield mutants defective in either the transfer of Fuc4NAc to lipid II or the polymerization of ECA

trisaccharide repeat units. In this regard, it is of interest that mutants of *S. typhimurium* defective in the synthesis of the mannosyl and abequosyl residues of O-antigen are genetically unstable, and it has been suggested that this is due to the deleterious accumulation of lipid-linked O-antigen intermediates (29). However, it is not known whether these mutants are hypersensitive to SDS or other agents.

Previous experiments demonstrated that tunicamycin is only effective in inhibiting the in vivo synthesis of ECA in mutants of *E. coli* and *S. typhimurium* that possess an incomplete lipopolysaccharide outer core region (19). Accordingly, the synthesis of ECA by mutants of *S. typhimurium* that possess an Ra-chemotype lipopolysaccharide is unaffected by tunicamycin, whereas the drug completely inhibits ECA synthesis in mutants that synthesize an Rc-chemotype lipopolysaccharide. Thus, it was somewhat surprising to find that tunicamycin was able to partially inhibit lipid I accumulation in strain SH5150 (Fig. 2) since this strain synthesizes an Ra-chemotype lipopolysaccharide. In addition, the accumulation of lipid I by SDS-resistant derivative 2 was also partially inhibited by tunicamycin (data not shown). These observations suggest the possibility that lipid I accumulation in some way renders the outer membrane of these mutants leaky with respect to tunicamycin permeability without significantly affecting their sensitivity to SDS. Alternatively, it is possible that the outer membranes of all strains possessing an Ra-chemotype lipopolysaccharide are slightly permeable to tunicamycin. Furthermore, since the intracellular concentration of the drug achieved in these strains is apparently insufficient to abolish ECA synthesis, the limited permeability of the outer membrane of these strains to the drug would not be as easily detected as is the case with ECA-negative mutants that accumulate lipid-linked intermediates.

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