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Variation in the Structure and Bacteriophage-Inactivating Capacity of *Salmonella anatum* Lipopolysaccharide as a Function of Growth Temperature

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Growth temperature affects both the structure and the phage-inactivating capacity of *Salmonella anatum* A1 lipopolysaccharide. Whereas *S. anatum* cells normally synthesize smooth lipopolysaccharide when grown at physiological temperature (37°C), a partial smooth-rough transition occurs when cells are grown at low temperature (20 to 25°C). The synthesis at low growth temperature of lipopolysaccharide molecules lacking O-antigen was detected both by increased sensitivity of cells to the rough-specific bacteriophage Felix O-1 and by fractionation of oligosaccharides derived from lipopolysaccharide by mild acid hydrolysis. Growth temperature-induced changes in the structure of *S. anatum* A1 lipopolysaccharide also affected its ability to inactivate ϵ^{15}, a bacteriophage that binds initially to the O-antigen portion of the molecule. Purified lipopolysaccharide prepared from cells grown at low growth temperature exhibited a higher in vitro phage-inactivating capacity than did lipopolysaccharide prepared from cells grown at physiological temperature (37°C).

The lipopolysaccharide of smooth gram-negative bacterial strains is an amphipathic molecule that contains three structurally discernible regions: the O-antigen, an electrically neutral, linear chain polysaccharide; the R-core, an electrically charged oligosaccharide containing phosphate and ethanolamine; and lipid A, a diglucosamine disaccharide substituted with fatty acids. Lipid A anchors the lipopolysaccharide molecule within the outer membrane. The hydrophilic R-core and O-antigen portions of the molecule extend outward from the cell surface into the aqueous environment (11, 16).

Extensive studies have been performed on the mechanism of lipopolysaccharide biosynthesis in salmonella (13, 24). Synthesis of O-antigen is known to occur independently of the lipid A-R-core moiety; oligosaccharide repeating units are formed on C_{55} polyisoprenoid lipid carriers located in the cytoplasmic membrane and subsequently polymerized into long O-antigen chains (19, 23). The final step in lipopolysacharide biosynthesis involves the joining of polymerized O-antigen to the R-core-lipid A component, followed by translocation of the completed molecule to the outer membrane. The mechanism of translocation of lipopolysaccharide molecules from the cytoplasmic membrane to the outer membrane is irreversible (14). It is also somewhat nonspecific; mutants that synthesize incomplete lipopolysaccharide molecules lacking O-antigen still translocate normal amounts of these products to the outer membrane (17).

There have been several reports of structural heterogeneity in the lipopolysaccharide of smooth gram-negative organisms which suggest that incomplete or otherwise abnormal lipopolysaccharide molecules are occasionally synthesized even in wild-type organisms (9; C. Walker, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K119, p. 166). Nikaido (12) showed that the degree of structural microheterogeneity in *Salmonella typhimurium* lipopolysaccharide is influenced by the carbon source. In this paper, we present data showing that the structure and composition of *Salmonella anatum* lipopolysaccharide vary as a function of growth temperature. In addition, our studies show that these changes have significant ramifications for the cell in terms of its sensitivity to various bacteriophages.

MATERIALS AND METHODS

Bacteria and bacteriophages. The wild-type strain *S. anatum* A1 and its rough mutant derivative R2 have been described previously (21). Mutant R2 lacks O-antigen and is sensitive to phage Felix O-1. Mutant sus11 was derived from A1 by nitrosoguanidine mutagenesis (8). It was detected by screening

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clones for sensitivity to bacteriophage ts11 (see below) at nonpermissive temperature by using a nibbled colony technique (22). *S. typhimurium* D21 is a derivative of strain LT2, obtained by curing for a defective PB1 prophage (25).

Bacteriophages ϵ^{15}vir and Felix O-1 have been described previously (21). The isolation of ts11, a mutant of ϵ^{15}vir, is described elsewhere (M. McConnell, A. Reznick, and A. Wright, Virology, in press). Phage ts11 is temperature sensitive in its ability to form plaques on *S. anatum* A1 cells, due to a block in DNA ejection.

Chemicals and enzymes. [^{3}H]glucose (4.5 Ci/mmol) was obtained from New England Nuclear Corp. ^{14}C-labeled glucose-fructose mixture (200 mCi/mmol) was prepared as previously described (15). Sephadex G50 was obtained from Pharmacia Fine Chemicals, Inc., and DEAE-cellulose was from Schleicher & Schuell Co. Fluorodinitrobenzene was from Eastman Kodak Co., and 2-thiobarbituric acid was supplied by Mallinckrodt. All other chemicals were standard products of reagent grade. DNase I was from Worthington Biochemicals Corp., and RNase A was from Sigma Chemical Co.

Microbiological and biochemical methods. Growth of cells in LB broth, labeling of cells with radiaoctive glucose, assay of bacteriophages on LB agar plates, and preparation of phage stocks by the confluent plate method have been described previously (22; McConnell et al., in press). Phage were purified from cell lysates by centrifugation in cesium chloride block gradients and sucrose gradients.

The procedure used for measuring in vitro inactivation of phage plaque-forming units by purified lipopolysaccharide involved incubation of phage-lipopolysaccharide suspensions in 5 mM Tris-chloride buffer (pH 7.8) containing 1 mM $MgSO_4$. At appropriate time intervals, samples were diluted 100-fold into ice-cold 5 mM Tris-chloride (pH 7.8) containing 1 mM EDTA and 1 μg of Triton X-100 per ml and then plated with *S. anatum* A1 cells on LB plates.

Lipopolysaccharide was obtained from nucleic acid-free cell wall preparations by using the phenol-water extraction method of Westphal et al. (20). It was purified by repeated sedimentation in a preparative ultracentrifuge.

Analysis of acetic acid-hydrolyzed lipopolysaccharide by column chromatography. Sephadex chromatography of water-soluble oligosaccharides obtained from lipopolysaccharide by acetic acid hydrolysis was performed by the method of Osborn et al. (14). Purified radioactive lipopolysaccharide preparations were mixed with 1 mg of nonlabeled carrier lipopolysaccharide, acetic acid was added to a final concentration of 0.125 N, and the samples were heated at 100°C for 30 min. Samples were neutralized with pyridine and centrifuged at high speed (81,000 × *g*) for 2 h, thus pelleting the lipid A portion of the lipopolysaccharide molecules and leaving the solubilized oligosaccharides in the supernatant. The lipid A pellet was resuspended in 0.125 N acetic acid, and the hydrolysis procedure was repeated through the high-speed-centrifugation step. The two supernatants were combined and concentrated to dryness with a rotoevaporator. The oligosaccharide material was suspended in 1 ml of water, and the pH was adjusted to 7.5 with NH_4OH. Neutral oligosaccharide fragments were removed by fractionation on a column (0.9 by 5.0 cm) of DEAE-cellulose at 4°C. After application of the sample to the column, the radioactive material was eluted in stepwise fashion by successively washing first with water and then with 10-ml volumes of 0.05, and 0.25 M pyridinium acetate solutions all buffered at pH 5.3. Most of the radiaoctivity (60 to 70%) eluted in the 0.05 and 0.25 M pyridinium acetate washes. These washes were pooled, concentrated to dryness, and suspended in 10% glycerol containing blue dextran marker dye. The sample was chromatographed at a rate of 0.2 ml/min on a column (0.9 by 50 cm) of Sephadex G50 (fine) that had been pre-equilibrated with 50 mM NH_4HCO_3 at 4°C. Fractions (0.5 ml) were collected, and 0.2 ml of each fraction was assayed for ^{3}H and ^{14}C radioactivity by using Bray scintillation fluid and a Beckman LS-235 scintillation counter.

Chemical assays. Rhamnose was measured by the method of Dische (4), 2-keto-3-deoxyoctonate was measured by the method of Aminoff (2), and primary amine was measured by the method of Ghuysen and Strominger (6). Standards used in these assays were L-rhamnose (Calbiochem), 2-keto-3-deoxyoctonate (Sigma), and ethanolamine (Sigma), respectively. Phosphate was determined by the method of Ames (1).

Lipopolysaccharide concentration was determined by measuring 2-keto-3-deoxyoctonate. A value of three 2-keto-3-deoxyoctonate residues per lipopolysaccharide molecule was used. 2-Keto-3-deoxyoctonate concentrations, determined experimentally, were multiplied by a factor of 1.5 since the thiobarbituric acid assay detects only two of the three 2-keto-3-deoxyoctonate residues present in lipopolysaccharide (5).

RESULTS

Variation of lipopolysaccharide structure with growth temperature of cells. Bacteriophage Felix O-1 is a useful probe for detecting changes in lipopolysaccharide structure. Its primary receptor site is the terminal *N*-acetylglucosamine unit of the lipopolysaccharide R-core. Thus, it can efficiently infect rough salmonella strains which have a complete R-core but lack O-antigen. Smooth strains in which all or most of the R-core units carry long O-antigen side chains adsorb Felix O-1 very poorly or not at all (10).

S. anatum is a smooth strain to which Felix O-1 adsorbs poorly and thus infects with very low efficiency. However, Table 1 shows that the plating efficiency of Felix O-1 on *S. anatum* was influenced by temperature. At 30°C or lower the phage formed plaques more efficiently than at higher temperatures. The phage itself is not temperature sensitive because it forms plaques on a rough mutant of *S. anatum* (strain R2) with equal efficiency at all temperatures used in the above experiment (unpublished data).

The above temperature effect suggested to us

TABLE 1. *Plaque-forming efficiency of Felix O-1 phage as a function of incubation temperature*[a]

Cell type	Plaque-forming efficiency at:				
	23°C	30°C	34°C	38°C	42°C
Control (*S. anatum* R2)	1.0	1.0	1.0	1.0	1.0
S. anatum A1	10^{-3}	2×10^{-3}	10^{-6}	10^{-6}	10^{-6}
S. anatum sus11	0.22	0.11	0.02	2×10^{-4}	2×10^{-5}

[a] Phage were mixed with cells, plated on LB plates, and then incubated at the indicated temperatures. The Felix O-1-sensitive strain *S. anatum* R2 gave equal numbers of Felix O-1 plaques at all the temperatures tested.

that growth temperature might influence the structure of the *S. anatum* lipopolysaccharide and, in particular, the O-antigen component. We therefore examined the nature of lipopolysaccharides isolated from *S. anatum* cells grown at different temperatures by fractionating acetic acid-hydrolyzed lipopolysaccharide on Sephadex columns. Mild acetic acid hydrolysis of lipopolysaccharide selectively cleaves ketodeoxyoctonate glycosidic linkages, releasing the oligosaccharide portion (R-core plus O-antigen) of the molecule from lipid A; the oligosaccharide products can be fractionated according to size on Sephadex columns. Figure 1 shows the results of a double-labeling experiment in which two lipopolysaccharide preparations extracted from *S. anatum* cells grown at either 21 or 33°C were mixed together; they were then hydrolyzed with acetic acid, and the oligosaccharide products were fractionated on Sephadex G50. The 21°C material exhibited a peak which comigrated with known R-core oligosaccharide prepared in the same manner from a mutant lacking O-antigen. There was much less, if any, R-core-sized material in the sample derived from the 33°C lipopolysaccharide. Figure 1 also indicates that the lipopolysaccharide of cells grown at 21°C contained a relatively larger proportion of R-core–O-antigen chains of sufficient length to be excluded in the void volume. Identical patterns of radioactivity were obtained upon reversing the isotopes used for labeling cells growing at 21 and 33°C (data not shown).

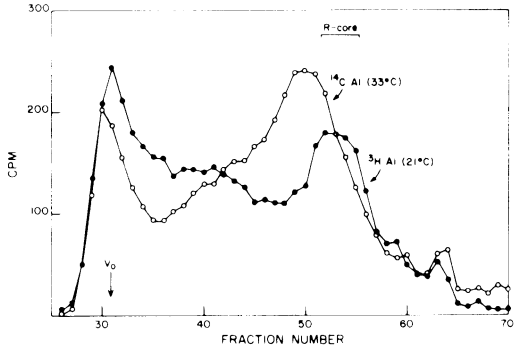

FIG. 1. *Sephadex chromatography of acetic acid-hydrolyzed lipopolysaccharide prepared from S. anatum A1 cells grown at 21 or 33°C. Lipopolysaccharide was extracted from cells grown in LB broth plus 1 μCi of [^{3}H]glucose or [^{14}C]glucose per ml at 21 and 33°C, respectively. The purified lipopolysaccharide preparations were combined and treated with acetic acid. The water-soluble oligosaccharides were chromatographed on DEAE-cellulose, and the material eluting in 0.05 and 0.25 M pyridinium acetate washes was combined and fractionated on Sephadex G50 as described in the text.*

Variation in phage-inactivating capacity of lipopolysaccharide as a function of growth temperature. Phage ϵ^{15} is inactivated by *S. anatum* lipopolysaccharide in the presence of a divalent cation (18). During inactivation of phage under these conditions, most of the phage DNA becomes susceptible to degradation by DNase. Our studies indicate that the capacity of purified *S. anatum* lipopolysaccharide to inactivate bacteriophage ϵ^{15} varies as a function of the temperature at which the cells were grown before extraction and purification of the lipopolysaccharide. The data presented in Fig. 2 show that lipopolysaccharide prepared from cells grown at low growth temperature was more effective in inactivating phage.

The mechanism of phage inactivation by lipopolysaccharide is not understood. The divalent cation requirement suggests that charged groups may be involved. We have found in preliminary experiments that lipopolysaccharide phosphate and primary amine content vary with the growth temperature of cells. At growth temperatures of 30 to 35°C, lipopolysaccharide molecules contain an average of about four phosphates and two primary amines, whereas at a growth temperature of 20°C, the average numbers are three and one, respectively. These values contrast with five phosphate groups and two ethanolamine residues for the average lipopolysaccharide molecule of *S. typhimurium*, as recently reviewed by Nikaido (13). A possible inverse relationship between phosphate content and ϵ^{15} phage-inactivating capacity of *S. anatum* lipopolysaccharide is suggested by additional data shown in Fig. 3. Figure 3A shows both lipopolysaccharide phosphate content and

phage-inactivating capacity plotted as a function of the temperature at which cells were grown before lipopolysaccharide extraction. A plot of the relative rates of lipopolysaccharide-induced phage inactivation as a function of lipopolysaccharide phosphate content gives the result shown in Fig. 3B.

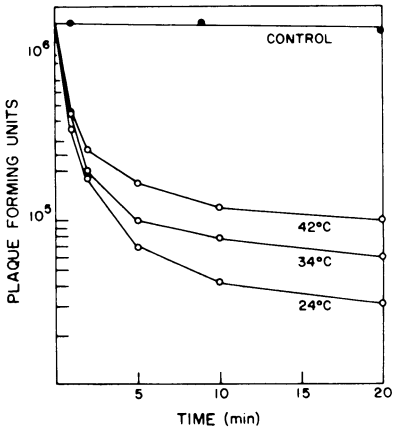

FIG. 2. *Phage inactivation by lipopolysaccharide prepared from S. anatum A1 cells grown at different temperatures. All reactions were carried out in Tris-chloride buffer (pH 7.8) plus 1 mM $MgSO_4$ and contained lipopolysaccharide at 2 μg of rhamnose equivalents per ml and 10^6 ϵ^{15}vir plaque-forming units per ml. Reactions were terminated by diluting samples 100-fold into cold 5 mM Tris-chloride buffer (pH 7.8) containing 1 mM EDTA. Free plaque-forming units were determined by plating on LB plates with S. anatum A1 cells. In the control, phage were incubated with lipopolysaccharide prepared from S. typhimurium strain D21.*

Studies on *S. anatum* sus11, a mutant with altered lipopolysaccharide structure and increased phage-inactivating capacity. The results in the preceding sections show that growth temperature-induced variation in the structure of *S. anatum* lipopolysaccharide influences its capacity to inactivate phages Felix O-1 and ϵ^{15}. The nature of lipopolysaccharide-induced inactivation of phage ϵ^{15} was further studied by using bacteriophage ts11, a mutant of ϵ^{15}vir which is temperature sensitive for DNA ejection (McConnell et al., in press), to screen for bacterial mutants altered in the site(s) required for triggering DNA ejection. *S. anatum* mutants that are sensitive to ts11 at 37°C were isolated from a mutagenized cell population by using a nibbled colony screening method, and one such mutant, sus11, was used for further study.

At 37°C, phage ts11 does not form plaques on *S. anatum* A1 cells, but forms plaques on sus11 cells with high efficiency (plating efficiencies, 3 × 10^{-5} and 0.41, respectively, relative to the ts11 plating efficiency observed at 30°C). In contrast, the parental phage, ϵ^{15}vir, forms plaques with equal efficiency at both 30 and 37°C. Biochemical assays indicate that suppression of the ts11 mutant phenotype by sus11 cells at 37°C is at

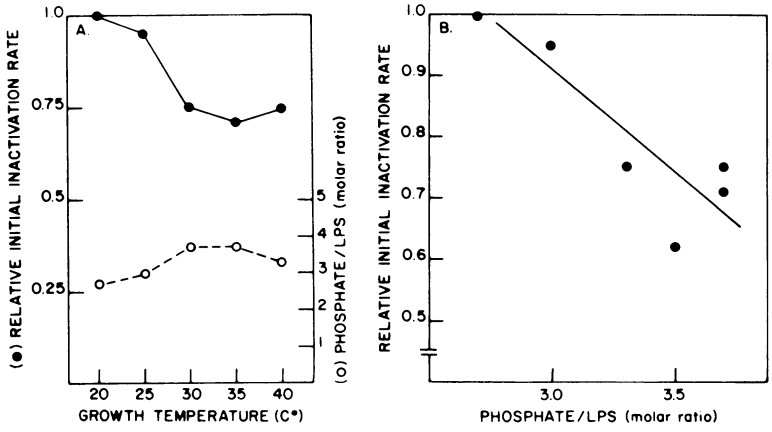

FIG. 3. *Phosphate content and phage-inactivating capacity of lipopolysaccharide (LPS) as a function of cell growth temperature. (A) Lipopolysaccharide phosphate contents and relative phage inactivation rates as a function of the growth temperature of cells before lipopolysaccharide extraction. Inactivation of phage by lipopolysaccharide was measured at 37°C in 5 mM Tris-chloride buffer (pH 7.8) containing 1 mM $MgSO_4$. The reactions contained phage at 10^6 plaque-forming units per ml and lipopolysaccharide at 0.5 μg of rhamnose equivalents per ml and were terminated by 100-fold dilution into cold 5 mM Tris-chloride buffer (pH 7.8) containing 1 mM EDTA and 1 μg of Triton X-100 per ml; they were then titrated by plating with S. anatum A1 cells at 30°C on LB plates. (B) Same data further analyzed by plotting phage-inactivating capacities of lipopolysaccharide preparations as a function of their phosphate contents. The graph also includes data obtained from experiments involving lipopolysaccharide prepared from cells grown at 15°C.*

the level of phage DNA ejection (unpublished data). In vitro studies involving purified lipopolysaccharide extracted from sus11 and *S. anatum* A1 cells show that sus11 lipopolysaccharide is more efficient than A1 lipopolysaccharide in triggering inactivation of both ts11 and ϵ^{15}vir phage (data not shown).

Felix O-1 formed plaques more efficiently on sus11 relative to parental *S. anatum* A1 at all temperatures tested (Table 1), indicating that sus11 cells contain a higher proportion of R-cores in which the terminal *N*-acetylglucosamine residue is accessible to Felix O-1 phage. This was confirmed by Sephadex fractionation of water-soluble oligosaccharides derived from sus11 lipopolysaccharide (isolated from sus11 cells grown at 33°C). The sus11 oligosaccharide profile was identical to the 21°C *S. anatum* profile shown in Fig. 1.

Preliminary data (not shown) indicate that lipopolysaccharide phosphate content is abnormally low in sus11 cells grown at 37°C, the temperature at which the temperature-sensitive defect in the plaque-forming ability of phage ts11 is suppressed. The changes in lipopolysaccharide structure resulting from the sus11 mutation therefore qualitatively resemble those changes that occur in the lipopolysaccharide of normal cells that are grown at low temperature.

DISCUSSION

Lipopolysaccharide isolated from *S. anatum* A1 cells grown at 20°C contains a greater proportion of R-core units unsubstituted by O-antigen than does lipopolysaccharide isolated from *S. anatum* A1 cells grown at 30 to 35°C. This change has been detected both by increased sensitivity of cells to the rough-specific bacteriophage Felix O-1 and by Sephadex chromatography of water-soluble oligosaccharide products formed by acetic acid hydrolysis of purified lipopolysaccharide. Chemical analyses indicate that the average lipopolysaccharide molecule from low-growth-temperature cells is deficient in phosphate and primary amine, two components normally found in the R-core regions of lipopolysaccharide molecules. A mutant of *S. anatum*, sus11, has a lipopolysaccharide which appears to be similar in structure to that of wild-type cells grown at low temperature (20°C).

sus11 lipopolysaccharide and lipopolysaccharide from *S. anatum* A1 cells grown at 20°C are both more effective in causing in vitro inactivation of phage ϵ^{15} than is lipopolysaccharide prepared from *S. anatum* A1 cells grown at 37°C. The increased phage-inactivating capacities of sus11 and low-growth-temperature wild-type lipopolysaccharides may be due to altered charge densities on these molecules resulting from the changes in their phosphate and primary amine contents. Interestingly, the DNA ejection mechanism of bacteriophage ϵ^{15} is highly sensitive to both divalent cation concentration and pH (McConnell et al., in press), thus making it likely that ionic interactions are involved.

It is possible that growth temperature-dependent alterations in the structure of *S. anatum* A1 lipopolysaccharide result from a decrease in the activity of R-core–O-antigen ligase relative to other enzymes involved in lipopolysaccharide biosynthesis. Such a situation would allow some lipopolysaccharide molecules to be translocated to the surface of the cell before receiving O-antigen. The variable O-antigen chain lengths observed in the lipopolysaccharide of *S. anatum* A1 cells grown at physiological temperature (37°C) suggest the absence of a tight coordinate regulation of the enzymes (or structures) responsible for O-antigen polymerization, ligation of R-core and O-antigen, and lipopolysaccharide translocation to the outer surface of the cell. Studies on mutant strains of *S. typhimurium* lend credence to this idea; *rfa* mutant strains defective in R-core synthesis still synthesize polymeric O-antigen linked to polyisoprenoid carrier lipid even though there is no R-core to which it can be joined (3, 7). Studies on leaky *rfa* mutants of *S. typhimurium* indicate that O-antigen polymers grow unusually large when ligation of R-core and O-antigen is occurring at less than the normal rate due to reduced amounts of normal R-core substrate (H. Nikaido, personal communication). Furthermore, strains which fail to synthesize and/or polymerize O-antigen because of mutations in *rfb* genes still transport normal amounts of rough lipopolysaccharide to the cell surface (17).

In summary, our major conclusion from this work is that lipopolysaccharide structure varies with temperature. There is a smooth-rough transition that is temperature dependent. One of the cellular properties affected by the structural variation of the surface is sensitivity to bacteriophages. Cells growing at low temperature are sensitive to bacteriophages that use both O-antigen (ϵ^{15}) and R-core (Felix O-1) as receptor, whereas cells growing at high temperature are sensitive only to those that have an O-antigen receptor. Although we have not determined the effect of temperature on lipopolysaccharide in other organisms, it seems likely that the effect is a general one. Studies on lipopolysaccharide structure and function should be conducted with this possibility in mind.

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