Evidence for Shorter Average *O*-Polysaccharide Chainlength in the Lipopolysaccharide of a Bacteriophage Felix 01-sensitive Variant of *Salmonella anatum* A1

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Prolonged culturing in the laboratory has resulted in the formation of a stable derivative of the smooth Group E bacterial strain, *Salmonella anatum* A1, that is sensitive to both the R-core-specific bacteriophage Felix 01 and *O*-polysaccharide-specific bacteriophage ϵ^{15} . The variant strain, designated *S. anatum* A1-1, exhibits a normal number of irreversible binding sites for ϵ^{15} but the relative quality and/or accessibility of those sites appears to be diminished. Infectious ϵ^{15} phage particles are released more rapidly from *S. anatum* A1-1 than from its parent under acidic pH conditions known to interfere with the phage DNA ejection step.

The purified lipopolysaccharide (LPS) of *S. anatum* A1-1 exhibits a reduced rhamnose/heptose ratio in chemical assays. Fractionation of this LPS on SDS-urea-polyacrylamide gels followed by silver staining reveals a narrower range of *O*-polysaccharide chain lengths relative to that of the parent (0 to 20 vs. 0 to 40 repeating units, respectively).

INTRODUCTION

Bacteriophage Felix 01 is a *Salmonella*-specific bacteriophage that requires lipopolysaccharide (LPS) R-core terminating in *N*-acetylglucosamine for optimal adsorption and infection (Felix & Callow, 1943; Lindberg & Hellerqvist, 1971). *Salmonella anatum* A1 is a Group E1 organism that is much less sensitive to bacteriophage Felix 01 than are most smooth *Salmonella* strains (Takacs & Nagy, 1973; Fey *et al.*, 1978; McConnell & Wright, 1979). Because mutant *rfb*- and *rfc*-like derivatives of *Salmonella anatum* A1 that synthesize *O*-polysaccharide-deficient LPSs are completely sensitive to bacteriophage Felix 01, one may infer that the *N*-acetylglucosamine receptor necessary for Felix 01 sensitivity occupies a more cryptic position in *Salmonella anatum* A1 LPS than it does in other smooth *Salmonella* species. The crypticity of Felix 01 receptor in *S. anatum* A1 LPS could be due to longer average *O*-polysaccharide chainlength, a higher percentage of R-cores substituted by *O*-polysaccharide side chains, or both.

In a recent investigation of the nature of the interaction between bacteriophage ϵ^{15} and its host cell, *Salmonella anatum* A1, at acidic pH (McConnell *et al.*, 1982), we serendipitously discovered a variant bacterial strain within our supposedly pure culture of *Salmonella anatum* A1. Although the variant strain (designated *S. anatum* A1-1) exhibited normal sensitivity to ϵ^{15} as determined by efficiency of plating, it was more resistant than its parent to ϵ^{15} infection at acidic pH and was also quite sensitive to bacteriophage Felix 01. In this paper we present a comparison of some of the properties of *S. anatum* A1 and *S. anatum* A1-1 cells and their purified LPSs. Our data show that the increased sensitivity to Felix 01 exhibited by *S. anatum* A1-1 cells

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is probably attributable, at least in part, to the synthesis by this strain of LPS molecules that contain relatively short *O*-polysaccharide side chains.

A preliminary report on these studies has appeared elsewhere (McConnell & Schoelz, 1981).

METHODS

Bacteria and phage. The parental *Salmonella anatum* A1 strain used in these studies was supplied to us as a lyophilized culture (dating from 1971) by Andrew Wright of Tufts University. It is referred to in the paper as *S. anatum* A1. *Salmonella anatum* A1-1 is a spontaneously-derived variant of *S. anatum* A1 that was first isolated in our laboratory (McConnell & Schoelz, 1981). It is fully sensitive to bacteriophage ϵ^{15} as measured by plating efficiency. *Salmonella anatum* R2, also derived from *S. anatum* A1, is auxotrophic for threonine and leucine, sensitive to bacteriophage Felix 01 and devoid of rhamnose (i.e. an Ra chemotype).

Bacteriophages ϵ^{15} vir and Felix 01 were provided to us by Andrew Wright and have been previously described (Felix & Callow, 1943; Lindberg & Holmes, 1969; Wright, 1971).

Microbiological techniques. Bacteria were routinely grown with aeration in trypticase soy broth at 37 °C. Phage stocks were prepared by the confluent lysis method on trypticase soy agar plates and stored at 4 °C in chloroform-saturated M9 buffer (Smith & Levine, 1964), following partial purification by differential centrifugation. The mid-exponential phase cells used in phage inactivation assays were routinely poisoned with azide (0.02%, w/v) 20 min before harvesting, then washed and resuspended in reaction buffer containing 0.02% azide. All other aspects of the phage inactivation assay were described by McConnell *et al.* (1982).

Maximum phage ϵ^{15} adsorption capacities of *S. anatum* strains were measured by mixing equal volumes of cells grown at 37 °C (2×10^8 c.f.u. ml⁻¹) and ϵ^{15} vir phage (2×10^{11} p.f.u. ml⁻¹) in azide-poisoned trypticase soy broth (final m.o.i. = 1000). After 30 min at 4 °C, bacteria (and adsorbed phage) were sedimented by centrifugation (9200 g) and the resulting supernatants were treated with chloroform and titrated for remaining free phage in order to calculate the number of phage adsorbed per cell.

Maximum phage inactivation capacities of *S. anatum* strains were measured by incubating phage and cells together for 2 h at 30 °C at phage/cell ratios ranging from 1 to 1000. The reaction buffer was 0.05 M-Tris (Calbiochem-Boehringer) adjusted to pH 7 with maleic acid (Baker) and supplemented with 0.001 M-MgSO₄ and 0.02% azide. Reactions were terminated by dilution into cold chloroform-saturated 0.05 M-Tris/maleate buffer (pH 5), then the mixtures were titrated to determine the concentration of free p.f.u. remaining and the total number of phage inactivated per cell.

Biochemical techniques. LPS preparations suitable for chemical analyses were obtained from cells that had been pretreated with lysozyme before phenol/water extraction (Westphal *et al.*, 1952; Johnson & Perry, 1976). Cells grown to mid-exponential phase in trypticase soy broth at 37 °C were harvested, then incubated for 24 h at 37 °C in a solution containing 0.05 M-Tris/HCl (pH 8), lysozyme (50 µg ml⁻¹), EDTA (0.001 M), and 0.02% azide before extraction with 45% (w/w) phenol at 72 °C. After a 30 min phenol extraction, the LPS-containing aqueous layer was dialysed exhaustively against 0.001 M-NaCl/0.02% azide at room temperature, then treated for 2 h at 37 °C with a 0.01 M-Tris (pH 8)/0.001 M-MgSO₄ buffer containing DNAase I and RNAase (both from Calbiochem) at final concentrations of 10 µg ml⁻¹ and 100 µg ml⁻¹, respectively. The nuclease-treated LPS suspensions were extracted once again with hot phenol and the LPS-containing aqueous layers dialysed as before against 0.001 M-NaCl/0.02% azide. SDS-polyacrylamide gel electrophoresis and silver staining of LPS preparations were performed according to published methods (Tsai & Frasch, 1982), except that the sample application buffer used was at a pH of 7.8 rather than 6.8.

Chemical analysis of rhamnose and heptose content in the various LPS preparations was done by the method of Dische (1955) using L-rhamnose (Calbiochem) and D-mannoheptulose (Sigma) as standards.

RESULTS AND DISCUSSION

A useful method for detecting 'partial roughness' (incomplete substitution of R-cores with *O*-polysaccharide) in so-called smooth strains of *Salmonella* is to measure their sensitivities to Felix 01, an R-core-specific bacteriophage. *Salmonella anatum* A1, unlike most species of smooth salmonellae, is quite resistant to Felix 01 (e.o.p. $\leq 10^{-6}$ at 37 °C). On the other hand, our newly-isolated ϵ^{15} -sensitive derivative of *S. anatum* A1, *S. anatum* A1-1, was found to plate bacteriophage Felix 01 quite efficiently, with e.o.p. values equalling those of *S. anatum* R2, an Ra chemotype derivative of *S. anatum* A1, at temperatures of 23 °C and 30 °C (Table 1). The parental *S. anatum* A1 strain also plated Felix 01 more efficiently at 23 °C, a phenomenon that has been previously documented (McConnell & Wright, 1979).

Table 1. Efficiency of plating of bacteriophage Felix 01 on *Salmonella anatum* strains A1 and A1-1 as a function of temperature

Bacteria were grown in trypticase soy broth at 37 °C, then plated with Felix 01 phage on trypticase soy agar plates at the temperatures indicated. Plating efficiency is relative to that on *S. anatum* R2, a rough derivative (Ra chemotype) of *S. anatum* A1. The results are means $\pm$ S.E.M., with the number of separate experiments shown in parentheses.

Host cell	Efficiency of plating of Felix 01:		
	at 23 °C	at 30 °C	at 37 °C
<i>S. anatum</i> A1-1	1.41* $\pm$ 0.29 (9)	0.98 $\pm$ 0.20 (7)	0.45 $\pm$ 0.08 (7)
<i>S. anatum</i> A1†	0.010 $\pm$ 0.007 (4)	4 $\pm$ 3 $\times 10^{-6}$ (3)	3 $\pm$ 2 $\times 10^{-6}$ (3)

* This value is higher than 1.0 because *S. anatum* R2 exhibits a decreased efficiency of plating of Felix 01 phage at 23 °C, relative to 30 °C and 37 °C (unpublished data).

† Revived from a lyophilized culture of *S. anatum* A1 (1971) provided to us by A. Wright (Tufts University).

The mutation that renders *S. anatum* A1-1 more sensitive to Felix 01 does not diminish the effectiveness of this strain as a host cell for ϵ^{15} , as evidenced by the following data: (1) identical e.o.p. values for ϵ^{15} on *S. anatum* A1-1 and its *S. anatum* A1 parent; (2) similar phage adsorption capacities (419 ± 14 phage per cell for A1-1 and 394 ± 42 for A1; $n = 3$, S.E.M.); and (3) similar phage-inactivating capacities (275 ± 104 phage per cell for A1-1 and 276 ± 83 for A1; $n = 4$, S.E.M.). Our measurement of about 275 inactivated ϵ^{15} phage per cell for both A1-1 and its A1 parent under saturating phage/cell ratios agrees reasonably well with Bayer's reported values for the number of inactivated ϵ^{15} phage per *S. anatum* A1 cell and for the number of adhesion sites that connect the inner and outer membranes of these organisms; adhesion sites are thought to serve as irreversible binding sites for several phages, including ϵ^{15} (Bayer, 1974, 1979; Bayer *et al.*, 1980).

While the number of irreversible binding sites for bacteriophage ϵ^{15} appears to be normal for *S. anatum* A1-1 cells, there is reason to believe that the affinity of those binding sites may be somewhat diminished. For example, bacteriophage ϵ^{15} is known to experience a block in DNA ejection when incubated with its host cell *S. anatum* A1 at pH 5, and under suitable reaction conditions, will be eluted from the cell surface as the *O*-polysaccharide receptor is destroyed by the enzymically-active glycosidase (McConnell, 1976; McConnell *et al.*, 1979, 1982). When ϵ^{15} phage were incubated with *S. anatum* A1-1 cells at pH 5, the phage were more rapidly released and the total percentage of free phage recovered was higher than in reactions involving the parent *S. anatum* A1 strain (Fig. 1). Such results would be expected if phage particles were experiencing increased repulsion at the secondary (irreversible) binding site because of alterations in its structure. Research from several laboratories suggests that the as yet undefined secondary binding site is located within the R-core/lipid A region of the *S. anatum* A1 LPS molecule (Takeda & Uetake, 1973; Kanegasaki & Tomita, 1976; McConnell *et al.*, 1979).

Studies on the composition and structure of purified LPS suggest an explanation for the increased sensitivity of *S. anatum* A1-1 cells to bacteriophage Felix 01. When LPSs purified from A1-1 and A1 cells were tested for their contents of rhamnose and heptose, two sugars found in the *O*-polysaccharide and R-core regions of the molecule, respectively, the rhamnose/heptose molar ratio of the *S. anatum* A1-1 LPS was found to be lower than that measured in the LPS of the original A1 parent strain (Table 2). Both mutant and parental LPSs appear *O*-polysaccharide-deficient when compared to LPS extracted from acetone-dried *S. anatum* A1 cells previously grown by Robbins and Uchida in 1961 for their studies on cell surface conversion by bacteriophage ϵ^{15} (Robbins & Uchida, 1962). The *S. anatum* A1 strain that they studied is the distant ancestor of our own *S. anatum* A1 strain and its A1-1 derivative.

Several laboratories have recently demonstrated the structural heterogeneity of smooth *E. coli* and *Salmonella typhimurium* LPS preparations by fractionating them on SDS-polyacrylamide gels (Goldman & Leive, 1980; Munford *et al.*, 1980; Palva & Mäkelä, 1980; Tsai & Frasch, 1982). Forty or more molecular species are routinely observed in such LPS preparations, each

Fig. 1. Kinetics of ϵ^{15} inactivation by cells of *S. anatum* A1 and *S. anatum* A1-1 at pH 5. Phage ϵ^{15} vir (10^{10} p.f.u. ml $^{-1}$) and cells of *S. anatum* A1 or A1-1 (10^8 c.f.u. ml $^{-1}$) were incubated together at 37 °C in 0.05 M-Tris buffer adjusted to either pH 5 or pH 7 with maleic acid and supplemented with 10^{-4} M-MgSO $_4$ and 0.02% NaN $_3$. Phage inactivation was determined by titrating free phage after dilution of samples into chloroform-saturated 0.05 M-Tris/maleate buffer (pH 5) supplemented with 0.001 M-EDTA. The titres of free phage were normalized with respect to control reactions in which *S. anatum* R2 cells (Ra chemotype) were present. Symbols: *S. anatum* A1-1 cells at pH 5 (○), and at pH 7 (□); *S. anatum* A1 cells at pH 5 (●), and at pH 7 (■).

Table 2. Rhamnose/heptose ratios in the purified LPS of *Salmonella anatum* strains A1 and A1-1

LPS was prepared from exponential-phase cells grown at 37 °C and assayed for rhamnose and heptose content as indicated in Methods. The values shown are the average rhamnose/heptose molar ratios from five separate assays, $\pm$ S.E.M.

<i>S. anatum</i> R2	<i>S. anatum</i> A1-1	<i>S. anatum</i> A1*	<i>S. anatum</i> A1†
0.13 $\pm$ 0.08	4.05 $\pm$ 0.63	5.76 $\pm$ 1.08	12.52 $\pm$ 2.21

* Source of cell inoculum used in the preparation of LPS was the lyophilized culture of *S. anatum* A1 dating from 1971.

† LPS extracted from acetone-dried *S. anatum* A1 cells dating from 1961.

one probably unique with respect to the length of its *O*-polysaccharide sidechain. When the LPS of *S. anatum* A1-1 was fractionated by SDS-polyacrylamide gel electrophoresis along with those of its A1 parent and its distant ancestor (A1 cells that were acetone-dried by Robbins in 1961) and the separated bands were made visible by silver staining, the fractionation patterns observed for the parental A1 LPS and the LPS extracted from Robbins' *S. anatum* A1 cells were rather similar in appearance (Fig. 2). This was surprising in view of the substantially higher rhamnose/heptose ratio of the latter (Table 2). The two *S. anatum* A1 LPS fractionation patterns were also somewhat similar to those previously reported for *E. coli* and *S. typhimurium*. In the case of the A1-1 LPS, however, only smaller LPS molecules with *O*-polysaccharides ranging from 0 up to about 20 repeating units in length were plainly visible. This is most obvious in Fig. 2(b) (tracks 1 and 2), in which the LPS samples were electrophoresed for a longer period. The decreased range of *O*-polysaccharide chain lengths in the A1-1 LPS was observed repeatedly using several batches of *S. anatum* A1-1 LPS prepared over a 30 month period. We confirmed that the silver-stained bands on our gels represented LPS molecules by pre-treating A1 LPS with ϵ^{15} phage (which possess *O*-polysaccharide-degrading glycosidase activity) before electrophoresis. This resulted in the elimination of the more slowly migrating bands (Fig. 3). The

Fig. 2. SDS-polyacrylamide-urea gel fractionation of *S. anatum* LPS. LPS samples were fractionated on 20% (w/v) polyacrylamide gels containing 4 M-urea and 0.1% SDS according to the method of Tsai & Frasch (1982), except that the sample application buffer had a pH of 7.8, rather than 6.8. Gel (a) contained: lane 1, *S. anatum* R2 LPS (0.2 nmol heptose equiv.); lane 2, *S. anatum* A1-1 LPS (1.0 nmol heptose equiv.); lane 3, *S. anatum* A1 LPS (1.0 nmol heptose equiv.); and lane 4, LPS prepared from Robbins' acetone-dried *S. anatum* A1 cells grown in 1961 (Robbins & Uchida, 1962) (1.0 nmol heptose equiv.). Gel (b) was electrophoresed several hours longer than gel (a) and contained: lane 1, *S. anatum* A1 LPS (1.0 nmol heptose equiv.); and lane 2, *S. anatum* A1-1 LPS (1.0 nmol heptose equiv.). The arrow marks the position of R-core in gel (b). The numbers refer to the probable number of *O*-polysaccharide repeating units present in a given band on the gel. The values of 34 and 47 were obtained by plotting the distance migrated by a given band vs. the number of *O*-polysaccharide repeating units present within the band, then extrapolating to determine the range of *O*-polysaccharide chainlengths represented by the broad, diffusely-staining, slowly-migrating band that is present in lane 1.

residual bands with mobilities approaching that of the R-core presumably represent LPS molecules with incompletely digested *O*-polysaccharides. We have previously suggested that an ϵ^{15} phage may need to interact simultaneously with R-core and an R-core-proximal remnant of the *O*-polysaccharide in order to eject its DNA (Wright *et al.*, 1980).

In summary, the evidence for decreased *O*-polysaccharide content in *Salmonella anatum* A1-1 LPS includes: (1) the increased sensitivity of A1-1 cells to the R-core-specific phage Felix 01; (2)

Fig. 3. Effects of pretreatment with ϵ^{15} phage particles on the fractionation pattern of *S. anatum* A1 LPS. The conditions for electrophoresis and silver staining were as described in Fig. 2. The *S. anatum* A1 LPS at 120 nmol heptose equiv. ml^{-1} was pretreated for 24 h at 37 °C with ϵ^{15} phage (10^{11} p.f.u. ml^{-1}) in 0.05 M-Tris/HCl buffer (pH 7) supplemented with 0.02% NaN_3 , before electrophoresis. Lane 1, *S. anatum* R2 LPS (0.25 nmol heptose equiv.); lane 2, *S. anatum* A1 LPS pretreated with ϵ^{15} phage (1.0 nmol heptose equiv.); and lane 3, non-treated *S. anatum* A1 LPS (1.0 nmol heptose equiv.).

the lower rhamnose/heptose ratio in A1-1 LPS; and (3) the SDS-polyacrylamide gel electrophoresis pattern of A1-1 LPS, which reveals molecules containing *O*-polysaccharide chains ranging from 0 to 20 repeating units in length, rather than the range of 0 to 47 *O*-polysaccharide repeating units observed in LPS molecules of the parental *S. anatum* A1 strain and those of other strains including *S. typhimurium* and *E. coli* (Goldman & Leive, 1980; Munford *et al.*, 1980; Palva & Mäkelä, 1980). The gel data provide strong proof that the lower rhamnose/heptose ratio of A1-1 LPS is due to a decrease in the average length of *O*-polysaccharide side chains rather than to a decrease in the proportion of R-cores substituted by *O*-polysaccharide, such as one observes in the LPS of wild-type *S. anatum* A1 cells grown at low temperatures (McConnell & Wright, 1979).

The explanation for the diminished size distribution range of *S. anatum* A1-1 *O*-polysaccharide side chains is not yet known. It is probably not caused by decreased activity of α -polymerase, the enzyme that polymerizes trisaccharide repeating units, since such a condition should

increase the relative proportion of LPS molecules containing only one *O*-polysaccharide repeating unit and this has not been observed. It might be argued that the less mobile bands which are present in fractionated *S. anatum* A1 LPS preparations but absent from those of A1-1 are not LPS molecules at all, but some haptenic form of *O*-polysaccharide; however, we think this highly unlikely since all known haptenic forms of *O*-polysaccharide have very low charge/mass ratios and should not be able to migrate into 20% SDS-polyacrylamide gels at all (Anaker *et al.*, 1964; Kent & Osborn, 1968; Jann *et al.*, 1970; Goldman *et al.*, 1982). On the other hand, haptenic *O*-polysaccharides, if present in varying quantities from one bacterial strain to another, could explain the curious results that we obtained in our studies of the LPS obtained from Robbins' acetone-dried *S. anatum* A1 cells that were grown in 1961; namely, that an LPS preparation with apparently two times more *O*-polysaccharide (from lipopolysaccharide plus *O*-haptene?) than is found in the LPS of our laboratory strain of *S. anatum* A1 (Table 2) could still yield a similar fractionation pattern on SDS-polyacrylamide gels (Fig. 2). We are continuing to investigate this phenomenon.

Finally, it is possible that the available space for accommodation of nascent *O*-polysaccharide chains on the outer surface of the cytoplasmic membrane is somehow reduced in the case of A1-1 cells, thereby limiting the maximum size that they can attain. We know of no data that argue either for or against such an hypothesis.

An important conclusion that can be drawn from these studies is that the transition of a laboratory-grown, Gram-negative bacterial strain from one that produces smooth LPS to one that produces rough LPS may occur very gradually, giving rise to subtly-altered, 'semi-smooth' variant organisms such as A1-1 along the way. Bacteriologists involved in long-term LPS studies of any kind should perhaps subject their smooth bacterial strains to multiple phage sensitivity tests when re-cloning them, taking care to include in their screening procedure a test for sensitivity to R-core-specific phages with long tail fibres whenever such are available.

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