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# Structure of a capsular polysaccharide isolated from *Salmonella enteritidis*

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**Abstract**—*Salmonella enteritidis* is a food-borne enteric human pathogen that can form a complex protective extracellular matrix. We describe here a component of this matrix which is distinct from other known salmonella extracellular polysaccharides such as cellulose and colanic acid. We have used glycosyl composition and linkage analysis, as well as 1D and 2D NMR spectroscopy to determine the structure of this polysaccharide. We propose that the primary saccharide in the *S. enteritidis* capsule has a branched tetrasaccharide repeating unit having the following structure: →3)-α-D-Gal*p*-(1→2)-[α-Tyv*p*-(1→3)]-α-D-Man*p*-(1→4)-α-L-Rha*p*-(1→. This structure is partially substituted on both tyvelose and galactose with a glucose-containing side chain. It further bears considerable similarity to the O antigen from this organism, a feature found in a number of other capsules from Gram-negative bacteria. In addition, we have detected fatty acids at levels that indicate the presence of a lipid anchor.


*Keywords:* Extracellular matrix; Capsule; *Salmonella*; O Antigen; Capsular polysaccharide; Glycolipid

## 1. Introduction

*Salmonella enteritidis*, a persistent food-borne enteric human pathogen, is capable of forming biofilms on abiotic surfaces.[1] This has major economic and health consequences.[2,3] Biofilm formation in *Salmonella* is associated with the multicellular and aggregative behavior variously described as rdar,[4] rugose,[5] or lacy.[6] This multicellular behavior is highly conserved amongst *Salmonellae*[7] and is characterized by the elaboration of thin, aggregative fimbriae (Tafi, curli;[8] cellulose;[9] and other uncharacterized exopolysaccharides).[10] Together, these components form the extracellular matrix that confers resistance to acid and bleach and facilitates environmental persistence.[5,11–13]

Indeed, many bacteria are capable of shielding themselves from environmental stresses, host immune responses and phagocytosis by the secretion of an apparently amorphous matrix of secreted polysaccharides.[14] Such saccharides are often classified into three categories: extracellular or 'slime' polysaccharides, which are only loosely associated with the bacterium, capsular polysaccharides, which are more tightly bound to the surface of the bacterium, and lipopolysaccharides, which form the basis of the outer leaflet of the Gram-negative outer membrane and are always anchored to the cell surface by lipid A.[15–17]

Capsular polysaccharides are a diverse group of molecules. *Escherichia coli* are known to produce over 80 distinct capsular polysaccharides, and these are often divided into two subgroups. Group 1 (as well as the

*Abbreviations*: SCPS, salmonella capsular polysaccharide; LPS, lipopolysaccharide; CPS, capsular polysaccharide; TX 100, Triton X100; GC/EIMS, gas chromatography electron ionization mass spectrometry; PMAA, partially methylated alditol acetate; gCOSY, gradient enhanced correlated spectroscopy; TOCSY, total correlation spectroscopy; gHSQC, gradient enhanced single quantum coherence; HMQC, heteronuclear multiple quantum coherence.

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chemically similar group 4) CPS molecules tend to be high-MW polysaccharides with low charge density and are sometimes linked to the cell surface via lipid A, while group 2 (as well as the chemically similar group 3) CPS molecules have lower MW, higher charge and can be linked to the outer membrane via phosphatidic acid.[16–19] Group 4 capsules used to be considered as a part of group 1,[15] but because they comprise structurally identical O-units they are now their own group and are known as the 'O-Ag capsules'.[17]

Note that covalent linkage to a lipid anchor does not seem to be an absolute requirement for CPS. For example, in group 2 capsular polysaccharides from *E. coli*, only 20–50% of the isolated saccharides are substituted with lipid,[20] while disruption of key LPS oligosaccharide core biosynthetic genes has no effect on the expression of K30 CPS in *E. coli*.[21] The repeating oligosaccharide subunits of some CPS molecules may actually resemble LPS as has been seen in several unrelated strains of bacteria, such as *E. coli* O111:B4 and *Vibrio ordalii*.[22–24] In *E. coli* O111 half the O-Ag is found as an LPS fraction, and the remainder is in an LPS-unlinked capsular form.[22]

While *E. coli* has a number of well-characterized capsular polysaccharides, cases in which salmonella capsular polysaccharides have been described are rare. Indeed, previous studies have failed to find CPS in *Salmonella minnesota* and *Salmonella typhimurium*.[23] *Salmonella typhi*, however, has a well-characterized capsular polysaccharide, the Vi antigen, which consists of a 2000 kDa homopolymer of (1→4)-2-acetamido-2-deoxy-α-D-galacturonic acid variably acetylated at C-3 and with no known lipid anchor.[25,26] *Salmonella enterica* serovar *typhimurium* has a recently described CPS, which contains glucose and mannose with lower levels of galactose, but the size, charge and lipid anchor (if any) of this CPS are as yet undefined.[27]

In our previous work, we have described a CPS from *S. enteritidis*, which is tightly associated with the bacterium.[28] This CPS is distinct from both Vi CPS and *S. enterica* CPS in that it consists of a repeating oligosaccharide containing both deoxyhexose and dideoxyhexose sugars. We also discovered a series of *yih* genes responsible for the assembly and transport of the molecule, which are distinct from the LPS biosynthetic operon and are conserved amongst *Salmonellae*. Mutation of these genes results in a phenotype that is sensitive to desiccation. We describe here the chemical structure of this CPS.

## 2. Results

### 2.1. Purification of salmonella capsular polysaccharide

Our efforts at purification of salmonella capsular polysaccharide (SCPS) have been directed towards two goals. First, we wished to purify the intact, lipidated SCPS so that we could examine its lipid anchor. Second, we desired to produce a pure, nonlipidated molecule, which is highly soluble, for glycosyl linkage and NMR studies. Both these goals were facilitated by the use of a bacterial strain that does not make cellulose, leaving only negatively charged components such as LPS as contaminants.

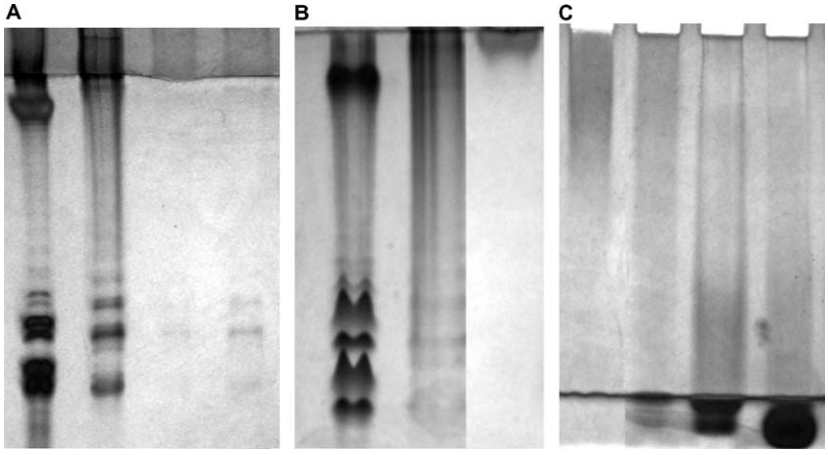


**Figure 1.** Analysis of salmonella capsular polysaccharide purifications by SDOC PAGE. Initial preparations of size exclusion purified salmonella capsular polysaccharide were repurified by anion-exchange chromatography and analyzed on 18% (gels A and B) or 4% polyacrylamide gels. Panel A represents a silver stain of *E. coli* O55:B5 LPS (lane 1), salmonella capsular polysaccharide starting material (lane 2), purified salmonella capsular polysaccharide after anion-exchange and polymyxin affinity chromatography (lane 3) and LPS from the anion-exchange 250–750 mM eluate (lane 4). Panel B shows analysis of salmonella capsular polysaccharide purification after hydrazinolysis. Lane 1 is *E. coli* O55:B5 standard LPS. Lane 2 is salmonella capsular polysaccharide starting material. Lane 3 is purified salmonella capsular polysaccharide. Panel C is a 4% acrylamide gel comparing FITC dextran standard of MW $2 \times 10^6$ Da (lane 1) with salmonella capsular polysaccharide purified by hydrazinolysis (lane 2) LPS from the anion-exchange of starting material from the same sample (lane 3) and similarly purified LPS from a commercial *Salmonella enteritidis* preparation (lane 4).

In the first method, we used anion-exchange chromatography in the presence of the nonionic detergent, Triton X100 (TX100), for partial purification, followed by removal of residual LPS using a polymyxin B affinity column. SDOC PAGE shows this method to be largely effective at purifying the SCPS (Fig. 1A). The resulting material stained positive for carbohydrate by silver staining and migrated, as expected, near the interface of the separating and stacking gels. The ladder-like pattern of bands noticed in the starting fraction (Fig. 1A, lane 2) is characteristic of LPS. Though most of the LPS elutes only at a high concentration of salt (250–750 mM) during ion exchange, a small amount is present in the purified SCPS, even after polymyxin treatment (Fig. 1A, lane 3).

Our second method begins by treatment of SCPS with anhydrous hydrazine. This removes both N- and O-linked fatty acids,[29] but has no effect on the negatively charged Kdo residues of the LPS inner core, so that LPS saccharides will be small and charged, while deacylated SCPS will be large and uncharged. At this point, either anion-exchange chromatography or ultrafiltration can be used to purify the deacylated SCPS. Figure 1B shows the results of anion-exchange chromatography after hydrazinolysis of the SCPS. The ladder-like pattern of bands observed in both the standard LPS (Fig. 1B, lane 1) and starting material (Fig. 1B, lane 2) is completely absent in the hydrazine-treated material (Fig. 1B, lane 3). For this reason, we used only this material for our glycosyl composition, linkage and NMR studies.

SDOC PAGE on a 4% acrylamide gel was used to both determine the MW of the hydrazine treated SCPS and examine LPS (either purified from the same preparation of material as the SCPS, or from commercial sources) for ultrahigh-MW bands. Hydrazine treated SCPS (Fig. 1C, lane 2) co-migrates with the FITC dextran standard of MW $= 2 \times 10^6$ Da (Fig. 1C, lane 1), whereas virtually all *S. enteritidis* LPS, either from our preparations or from commercial sources, migrates near the solvent front (Fig. 1C, lanes 3 and 4).

## 2.2. Glycosyl composition analysis

Glycosyl composition analysis of the hydrazine-treated salmonella capsular polysaccharide is shown in Table 1. Note that rhamnose, mannose, and galactose are found in roughly the same proportions, while glucose is considerably less prevalent. In addition, several peaks eluting early in the chromatogram are noted, which did not match any of our monosaccharide standards, but matched both the retention time and mass spectrum of peaks detected in LPS from *S. enteritidis*, which contains the dideoxyhexose, tyvelose (Fig. 2). Therefore, it was concluded that the SCPS contains rhamnose, mannose, glucose, galactose, and tyvelose.

**Table 1.** Glycosyl composition of salmonella capsular polysaccharide

| Glycosyl residue | Mole % |
|---|---|
| Tyvelose | 18 |
| Rhamnose | 22 |
| Mannose | 24 |
| Galactose | 28 |
| Glucose | 9.1 |

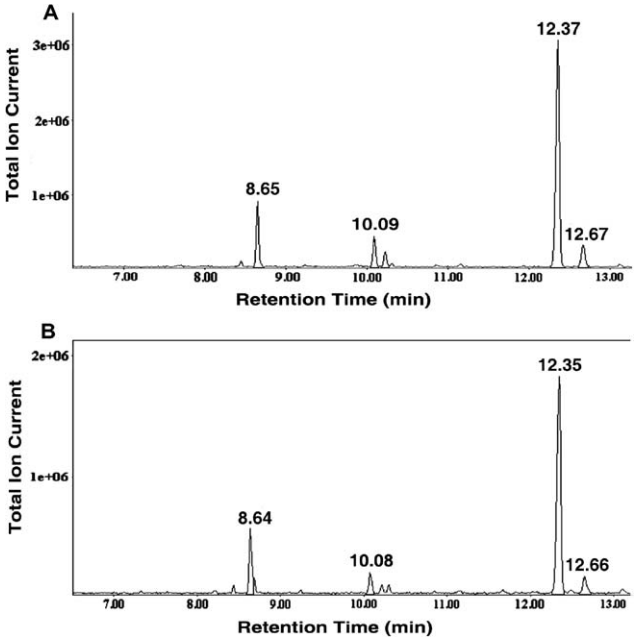


**Figure 2.** Identification of a dideoxyhexose in salmonella capsular polysaccharide. GC–MS analysis of $ME_4Si$-treated methyl glycosides from salmonella capsular polysaccharide (panel A) and *Salmonella enteritidis* LPS (panel B). Peaks at 12.3 and 12.6 min are rhamnose. The LPS from *Salmonella enteritidis* is known to contain the dideoxyhexose tyvelose in its O antigen and peaks at 8.6 and 10.1 min in panel B match the expected mass spectrum of this compound.

## 2.3. Fatty acid analysis

Our previous study suggests that SCPS is difficult to dissociate from the bacterial cell surface and that it may be capsular.[28] Further, solutions of purified, nonhydrazinolyzed SCPS are turbid, as would be expected for a lipidated molecule. In order to examine the possibility that the molecule may be lipid-anchored, we analyzed the fatty-acid composition of anion-exchange/polymyxin purified SCPS. Results of this analysis are shown in Table 2. Dodecanoic, tetradecanoic, and hexadecanoic fatty acids were detected, as well as 3-hydroxytetradecanoic acid. These could be accounted for as residual contamination by LPS. In addition, we see octadecanoic acid and unsaturated fatty acids such as hexadecenoic acid and octadecadienoic acid, which are not normally seen in the LPS of enteric bacteria. The observed levels of fatty acid are consistent with what we would expect

**Table 2.** Comparison of fatty acid constituents of salmonella LPS and CPS

| Fatty acid | Mole % | |
|---|---|---|
| | LPS | CPS |
| Dodecanoic acid | 22 | 10 |
| Tetradecanoic acid | 14 | 12 |
| Hexadecanoic acid | 32 | 30 |
| 3-Hydroxytetradecanoic acid | 32 | 26 |
| Octadecanoic acid | Trace | 13 |
| Hexadecenoic acid | n.d.[a] | 7.0 |
| Octadecadienoic acid | n.d. | 1.0 |

[a] n.d. = not detected.

for the lipid anchor of a glycolipid that is significantly larger than LPS.

### 2.4. Glycosyl linkage analysis

The glycosyl linkage analysis of purified deacylated SCPS is shown in Table 3. This analysis indicates that mannose is present primarily as a 2,3-linked residue, while galactose is largely 3-linked and rhamnose mostly 4-linked. A 3,4-linked galactopyranose was also observed corresponding to approximately 30% of the area of 3-linked galactopyranose. Two peaks of approximately equal area are observed early in the chromatogram, as would be expected for a dideoxyhexose such as tyvelose.

Gas chromatography/chemical ionization mass spectrometry of the partially methylated alditol acetate preparation of SCPS shows the expected masses for the three major peaks (see Fig. 3). The peak at 8.5 min has a signal at $m/z$ 339, consistent with the mass expected for the ammonium ion of a 4-linked rhamnose. Likewise, the peaks at 10.3 and 11.2 min have masses at $m/z$ 369 and 397, respectively, consistent with a 3-linked galactopyranose and 2,3-linked mannopyranose. The peak at 6.8 min (A) has a signal at $m/z = 281$, which is consistent with a dimethylated diacetylated dideoxyhexose. This derivative is expected for terminal dideoxyhexoses. Likewise, the peak at 7.5 min (B) shows a signal at $m/z$ 309, consistent with the ammonium ion of a monomethylated, triacetylated dideoxyhexose. This is the derivative we would expect from either the 2- or 4-linked dideoxyhexose residue. The corresponding peak in the GC/EIMS has peaks at $m/z$ 118 and 173 as would be expected for the 4-linked (but not the 2-linked) residue. The absence of signals at $m/z$ 145 and 146, which should be seen in the electron-impact ionization mass spectrum of the 2-linked residue (the only other linkage possible here), leads us to support the conclusion that tyvelose is 1,4-linked.

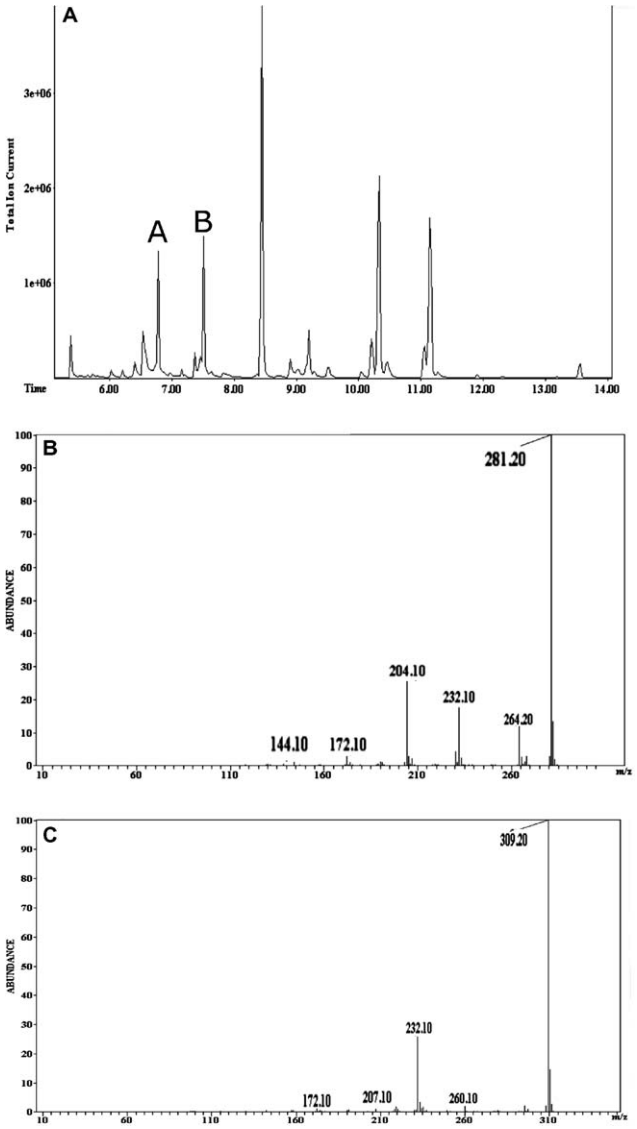


**Figure 3.** GC–CIMS of partially methylated alditol acetates. (A) Analysis of salmonella capsular polysaccharide partially methylated alditol acetates shows two peaks associated with tyvelose, one at 6.8 min (labeled B) and one at 7.5 min (C). The CIMS of peak A is shown in panel B with the maximum mass at $m/z$ 281 indicative of a dimethylated diacetylated (terminal) dideoxyhexose. Panel C shows CIMS of peak B with mass at $m/z$ 309 indicative of a monomethylated triacetylated (linked) dideoxyhexose.

**Table 3.** Glycosyl-linkage analysis of salmonella CPS

| Glycosyl residue | Area[a] (%) |
|---|---|
| Terminal tyvelose | 3.9 |
| 1,4-Linked tyvelose | 4.2 |
| 4-Linked rhamnopyranose | 17 |
| Terminal glucopyranose | 1.1 |
| 3,4-Linked rhamnopyranose | 3.4 |
| 3-Linked mannopyranose | 1.4 |
| 3-Linked galactopyranose | 22 |
| 4-Linked glucopyranose | 4.2 |
| 2,3-Linked mannopyranose | 29 |
| 3,4-Linked galactopyranose | 7.1 |
| 3,4-Linked glucopyranose | 2.0 |

[a] For clarity, peaks under 1% of total are not shown.

### 2.5. NMR spectroscopy

NMR spectroscopy was used to determine the monosaccharide sequence and anomeric configuration of the polysaccharide. The 1D proton NMR spectrum displayed four major anomeric signals, a complex ring region, two signals at 2.06 and 1.82 ppm, respectively, and two singlets corresponding to H-6 of 6-deoxysugars. Inspection of the COSY and TOCSY spectra revealed that the two signals at 2.06 and 1.82 ppm were the two protons at position 3 of a 3,6-dideoxyhexopyranose, which was identified as 3,6-dideoxy-D-*arabino*-hexopyranose by glycosyl composition analysis in comparison with *S. enteritidis* LPS (vide supra).

The interpretation of the 2D NMR spectra was hampered by significant signal overlap, but the combination of gCOSY, TOCSY, and gHSQC spectra allowed the partial assignment of the signals belonging to the four major monosaccharide residues (Table 4). The characteristic downfield shift of the glycosylated carbon peaks helped in the assignment of the NMR signals and confirmed the results obtained from linkage analysis. Thus, mannose is 2,3-linked; galactose, 3-linked; rhamnose, 4-linked; and tyvelose is consistent with being at least partially terminal. The four anomeric signals were all singlets indicating small $J_{1,2}$ coupling constants (Fig. 4).

Unfortunately, the small coupling shows only the α-anomeric configuration of the galactosyl residue, whereas proton–proton coupling constants do not allow determination of the anomeric configuration of the mannosyl, rhamnosyl, and tyvelosyl residues. However, proton chemical shifts indicate α-configuration for each of these residues. In order to confirm the anomeric configuration of the carbohydrate residues in the SCPS repeating unit, we determined the one-bond C–H coupling constants by application of a 1D HMQC experiment without carbon decoupling during acquisition. The C–H coupling constants of all four anomeric C–H pairs were around 170 Hz (Table 4), confirming that they are all in the α-configuration.

The 2D NOESY spectrum showed cross peaks between H-1 of tyvelose and H-3 of mannose, between H-1 of rhamnose and H-3 of galactose, between H-1 of galactose and H-2 of mannose, and between H-1 of mannose and H-4 of rhamnose. These correlations allowed unambiguous determination of the monosaccharide sequence of SCPS (Fig. 5).

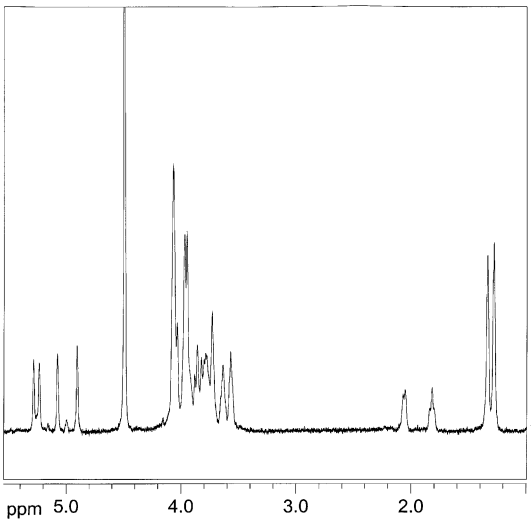


**Figure 4.** $^{1}$H NMR spectrum of salmonella capsular polysaccharide.

The relative absolute configurations of the mannosyl, galactosyl, and rhamnosyl residues were determined by comparing the observed $^{13}$C NMR glycosylation shifts with those compiled by Lipkind et al.[30] Thus, glycosylation by rhamnose caused a large positive α-effect on C-3 of galactose (+8.0$\Delta$ ppm) and a small negative β-effect on its C-2 (−0.1$\Delta$ ppm). This pattern, and also the large positive α-effect on the anomeric carbon of rhamnose (+7.9$\Delta$ ppm) indicate different absolute configurations of galactose and rhamnose. Likewise, the relatively large negative β-effect on C-3 (−0.6$\Delta$ ppm) and the large positive α-effect on C-4 (+9.4$\Delta$ ppm) of rhamnose, caused by its glycosylation at position 4 with mannose, revealed a different absolute configuration of rhamnose and mannose. The carbon chemical shifts and their assignments are in close agreement with the published NMR data of two tetrasaccharides, α-D-Gal*p*-(1→2)-[α-Tyv*p*-(1→3)]–α-D-Man*p*-(1→4)-α-L-Rha*p*-1-$(CH_2)_8CO_2Me$ and α-Tyv*p*-(1→3)–α-D-Man*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-1-*p*-$NO_2C_6H_4$, corresponding to two representa-

**Table 4.** $^{1}$H and $^{13}$C chemical shifts, anomeric $^{1}J_{CH}$ coupling constants, and interresidue NOEs of SCPS

| | | Chemical shift ($\delta$ ppm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Glycosyl residue | | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 | H-1 Inter-NOEs |
| α-Tyv*p*-(1→ | $^{1}$H | 4.90 | 4.07 | 2.06/1.82 | 3.64 | 3.78 | 1.29 | 3.97 (Man H-3) |
| | $^{13}$C | 102.7 (170 Hz) | 70.7 | 34.7 | 68.3 | 71.9 | 18.3 | |
| →4)-α-L-Rha*p*-(1→ | $^{1}$H | 5.08 | 4.07 | 3.97 | 3.57 | 3.92 | 1.34 | 3.95 (Gal H-3) |
| | $^{13}$C | 103.1 (172 Hz) | 71.9 | 70.7 | 82.9 | 69.5 | 18.5 | |
| →3)-α-D-Gal*p*-(1→ | $^{1}$H | 5.24 | 3.95 | 3.95 | 4.05 | 4.05 | 3.75/3.71 | 4.03 (Man H-2) |
| | $^{13}$C | 102.3 (167 Hz) | 69.5 | 78.4 | n.d. | n.d. | 62.2 | 4.06 (Man H-4) |
| →2)-α-D-Man*p*-(1→ | | | | | | | | |
| 3 | $^{1}$H | 5.28 | 4.03 | 3.97 | 4.06 | 3.97 | 3.86/3.81 | 3.57 (Rha H-4) |
| ↑ | $^{13}$C | 101.1 (171 Hz) | 80.0 | 75.2 | 68.7 | n.d. | 61.8 | |

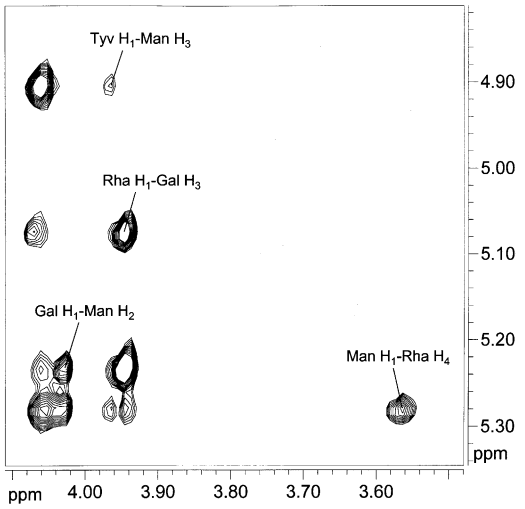


**Figure 5.** Part of a NOESY spectrum of salmonella capsular polysaccharide.

tions of the *S. enteritidis* LPS repeating unit.[31] This close agreement and the relative absolute configurations of the backbone sugar residues we have determined indicate that the SCPS is either identical to the LPS in its absolute configuration or all its residues have the inverse configuration. Given that the inverse configuration has never been observed in nature, we favor the former interpretation, and conclude that the SCPS contains tyvelose, D-mannose, L-rhamnose, and D-galactose, rather than ascarylose, L-mannose, D-rhamnose, and L-galactose.

## 3. Discussion

At first glance, Group 1 capsular polysaccharides would appear to be better classified as lipopolysaccharides. They sometimes have the same lipid A and core oligosaccharide anchor as LPS and in some cases even have a similar repeating oligosaccharide.[23,24] The main difference between the molecules is that CPS is of higher molecular weight (allowing the molecule to form a capsule), not dependent on LPS core biosynthesis for expression at the cell surface, and reliant on a separate biosynthetic and transport apparatus.

The structure for the CPS shown in Figure 6 is very similar to that of the O antigen of this organism,[32,33] but this is hardly unusual. For example, the CPS of *E. coli* O9:K38 is identical to *E. coli* O antigen O178,[34] while *V. ordalii* capsular and O-chain polysaccharides share exactly the same structure.[24] *Vibrio cholerae* O139 has a CPS which, like ours, migrates more slowly in its electrophoresis than its LPS and is serologically identical to the O antigen.[35] Identical structures for LPS O antigen and CPS have also been noted in *Aeromonas salmonicida*,[36] *Acetobacter methanolicus*,[37] and *Vibrio anguillarum*.[38]

The LPS of *S. enteritidis* belongs to serogroup D1 and was first described by Hellerqvist et al.[32] This first report did not identify the anomeric configuration of the mannosyl residue. Subsequently, the same group[39] established the mannosyl residue in D1 polysaccharides to be in the α-configuration by subjecting the peracetylated polysaccharides to chromic acid, which readily oxidizes β-glycosides, but to which α-glycosides are stable. Fukada et al.[40] independently revisited the issue of the mannosyl configuration. They partially hydrolyzed the D1 O antigen and found that the resulting Man-Rha disaccharides are stable to β-mannosidase, but are cleaved by α-mannosidase. Thus, the mannose is α-linked in LPS, and the CPS is identical to the LPS with respect to the anomeric configuration of its mannosyl residue.

However, the CPS is chemically distinct from LPS in several ways. First, we are unable to detect sugar residues associated with the LPS core region in the purified material. Second, salmonella capsular polysaccharide can be separated from LPS saccharides by ion-exchange chromatography, suggesting that it has a lower net charge than LPS. Third, according to the linkage analysis

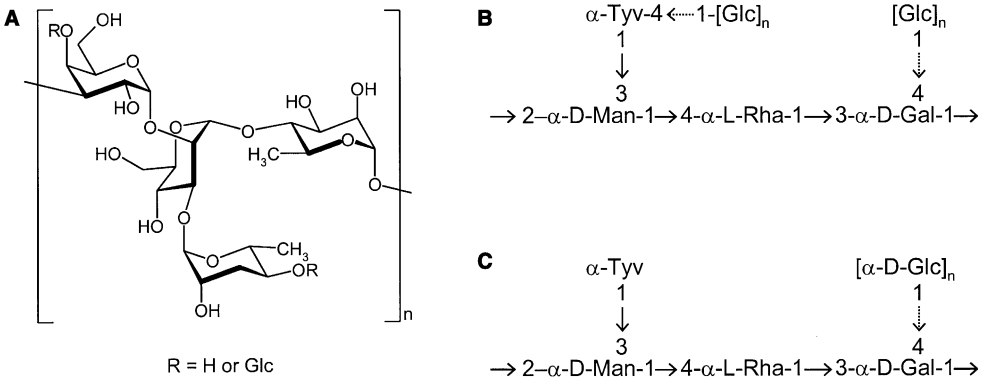


**Figure 6.** The salmonella capsular polysaccharide repeating unit polysaccharide structure. (A) Configuration and (B) linear drawing of the repeating oligosaccharide unit. (C) The linear structure of the O-Ag repeating unit from *Salmonella enteritidis*.[33]

data, the CPS is partially substituted with a glucose-containing side chain on tyvelose, while LPS has this modification only on galactose.[33] We did not detect glucose in the NMR spectrum, but this is not surprising given the much lower sensitivity of this technique compared to GC–MS. Further, we notice several non-terminal glucose species in the glycosyl-linkage analysis, which would be consistent with substitution by a chain of glucose residues. This altered substitution pattern may account for the large difference in immunoreactivity observed between the CPS and LPS.[28] Lastly, the size of this molecule is considerably larger than what one would expect for an enteric O antigen, migrating well above the LPS high molecular weight species in SDOC PAGE and close FITC dextran standard of MW $2 \times 10^6$ Da. Even if we assume that the CPS has a lipid A and core region that we are unable to detect, it would account for only about 5000 Da. This would leave a saccharide of over 3000 repeating units in size. A typical enteric high molecular weight O antigen consists of approximately 70 repeating units.[23] This size difference may be adequate to confer immunological distinction to the O-Ag capsule due to conformationally dependent epitopes on the O-Ag capsule. This is the case for other HMW polysaccharides that have been shown to have conformationally dependent epitopes due to extended helices formed by large polysaccharides and their modifications.[41] Thus, although the LPS O antigen and CPS of this organism share a similar repeating unit, the CPS differs in size, charge, substitution patterns and immunoreactivity from the O-chain saccharide.[28]

The nature of the lipid anchor in the SCPS can be difficult to identify due to the large size of the saccharide relative to the putative lipid anchor. Though we have detected fatty acids consistent with a lipid A anchor, we also note low levels of LPS in the nonhydrazinolyzed sample we use for this analysis. We have also observed longer chain, unsaturated fatty acids that are not associated with LPS from this organism. Enteric bacteria are known to add long-chain unsaturated fatty acids to their membrane phospholipids.[42] This makes it tempting to speculate that the lipid anchor for SCPS is a phospholipid(s), although we cannot rule out a lipid A of altered structure at this point.

One unresolved question is why this bacterium would use a similar repeating oligosaccharide in its CPS and its LPS. It may be more appropriate to ask the reverse question: Why would bacteria go to the additional biosynthetic effort of creating a completely different saccharide? In many cases, there are quite good reasons to do this. In some cases, the capsule is capable of masking LPS from antigenic responses of the host, making it beneficial to have a different, less immunogenic structure.[43–46] In other cases, the capsule may prevent nonspecific host factors such as complement or factor H from reaching the cell surface.[47] This, too, requires specialized structures such as neuraminic acids[48] or other negatively charged saccharides.[49,50] Additionally, in the human adapted *Salmonella enterica* serotype Typhi, Vi CPS has recently been noted to reduce expression of the neutrophil chemoattractant IL 8 in macrophages and epithelial cells,[51] and can mediate its effects through direct interaction with cell-surface receptors on the host.[52] Alternatively, the bacterium may obtain the genes for capsule biosynthesis from an unrelated strain of bacterium.[16] Our prior work suggests that none of these seem to be the case with *S. enteritidis* CPS. The genes involved are conserved throughout *Salmonellae* and the primary function of the capsule appears to be to protect the bacterium from desiccation,[28] ensuring the environmental persistence that is used to perpetuate the cyclic lifestyle for *Salmonella*. This being the case, only minor alterations to the O antigen may be necessary. It is possible that the addition of glucose-containing side chains onto tyvelose may assist in forming a protective film around the bacterium during desiccation.

## 4. Experimental

### 4.1. Initial CPS isolation

Cells scraped off agar surfaces were resuspended in 1% phenol, mixed vigorously by vortexing and incubated at room temperature for 30 min. Cellular debris was pelleted by centrifugation (16,000*g*, 4 °C, 5 h). The aqueous phase of the supernatant was removed, and 4 vol of ice-cold acetone was added on ice while constantly stirring with a glass rod for at least 10 min. The precipitated material was spooled, washed with acetone and air-dried at room temperature overnight. The material was solubilized in $dH_2O$ with heat and gentle agitation, dialyzed (MWCO 6–8000 Da; Spectrum®) overnight in $dH_2O$ and lyophilized. Approximately one plate of cells ($d = 140$ mm; Fischer) yielded 4 mg of crudely purified SCPS. The SCPS (10 mg/1 mL) was separated on a Superose 6 column (Pharmacia) that had been equilibrated in phosphate-buffered saline, pH 7.4 (PBS). The column was washed with PBS at a flow rate of 0.4 mL/min and 50 1-mL fractions were collected. Fractions with a high $A_{220}$ were collected and analyzed by immunoblotting, as well as by silver and protein staining.

### 4.2. Hydrazinolysis

SCPS was subjected to hydrazinolysis to remove both N- and O-linked fatty acids using the method of Patel et al.[53] Briefly, the sample was incubated in anhyd hydrazine at 95 °C for 4 h and then dried under a gentle stream of air.

### 4.3. Anion-exchange chromatography

Hydrazine-treated material was resuspended in water and loaded on acetate-equilibrated Accel® QMA ion-exchange resin. The material was eluted with 3 mL of $dH_2O$, then 4 mL of 0.2 M NaOAc. The eluate was precipitated with 5–10 vol of ice-cold acetone and centrifuged.

Nonhydrazine-treated material was suspended in 15 mM NaOAc, pH 5.5, containing 1% Triton X 100 (TX100) and loaded onto acetate-equilibrated Accel® QMA ion-exchange resin. The material was eluted with a stepwise gradient of NaOAc starting at 100 mM, followed by 250, 750, and 1500 mM NaOAc, in the presence of 1% TX100. TX100 was removed by overnight incubation with MeOH washed Bio-Rad SM2 Bio-Beads®. NaOAc was removed by dialysis against three changes of $dH_2O$ followed by lyophilization.

### 4.4. Polymyxin B chromatography

The material isolated by anion-exchange chromatography was loaded onto a 12.5 cm × 2.5 cm polymyxin affinity column in 50 mM $NH_4HCO_3$, then eluted with 50 mL of the same solvent, lyophilized and resuspended in approximately 1 mL of $dH_2O$. The sample was then dialyzed 3× in $dH_2O$.

### 4.5. SDOC PAGE

Electrophoresis was performed on 8 × 10 cm 18% T 2.7% C mini-gels using a Tris/glycine (1:4.8 w/w) 0.25% sodium deoxycholate running buffer at 30 mA current and 400 V potential (or 7.5 mA current 100 V potential for the 4% gel). Alcian blue silver staining was used to stain for total carbohydrates, and silver staining without alcian blue was used for LPS specific staining. For alcian blue silver staining, the gels were fixed in 40% EtOH, 5% HOAc, and 0.005% alcian blue for 30 min and then reincubated in the same solution overnight. Gels were then rinsed in water and oxidized in 0.7% $NaIO_4$ for 10 min. After this, they were rinsed 5× in water and incubated 10 min in 10% Bio-Rad silver reagent concentrate, rerinsed in water, and developed in 3.2% Bio-Rad developer until satisfactory staining was observed. Staining was stopped with 5% HOAc.

### 4.6. Glycosyl composition

Methyl glycosides were first prepared by methanolysis in 1 M HCl in MeOH at 80 °C (16 h), followed by N-reacetylation with pyridine and $(MeCO)_2O$ in MeOH in a 1:1:2 vol/vol ratio for 20 min at 25 °C. The samples were then per-O-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80 °C (0.5 h). These procedures were carried out as previously described.[54] GC–MS analysis of the $Me_3Si$-derivatized methyl glycosides was performed on an HP 5890 GC interfaced to a 5970 MSD, using a All Tech EC-1 fused silica capillary column (30 m × 0.25 mm ID). Chemical-ionization GC–MS was performed on an Agilent 6890N GC interfaced to a 5973 MSD using an Agilent DB-1 0.25 mm × 30 m capillary column. In both cases, a temperature program starting with a 2 min hold at 80 °C, followed by a 20 °C/min gradient to 160 °C, 2 min hold, 2 °C/min gradient to 200 °C, 10 °C/min gradient to 250 °C and 11 min hold was used.

### 4.7. Glycosyl linkage

The sample was permethylated, depolymerized, reduced, and acetylated, and the resultant partially methylated alditol acetates (PMAAs) were analyzed by GC–MS as described by York et al.[55] Initially, an aliquot of sample was permethylated by the method of Ciukanu and Kerek,[56] which involves treatment with sodium hydroxide and iodomethane in dry $Me_2SO$. Briefly, 0.7 mL of a suspension of approximately 1.0 M NaOH in $Me_2SO$ was added to a sample predissolved in $Me_2SO$ and allowed to incubate for 10 min prior to the addition of 0.1 mL of MeI, which was also allowed to incubate for 10 min. The permethylation was repeated twice (with the last methylation using 0.2 mL of MeI for 40 min) in order to aid complete methylation of the polymer. Following extraction into the organic phase of a 1:1 $CH_2Cl_2$–$H_2O$ extraction, the permethylated material was hydrolyzed using 2 M $CF_3CO_2H$ (2 h in a sealed tube at 121 °C), reduced with $NaBD_4$ (10 mg/mL of $NaBD_4$ in 1 M $NH_4OH$ overnight, followed by neutralization with HOAc and drydown in MeOH), and acetylated using 0.23 mL concd $CF_3CO_2H$ and 0.25 mL $(MeCO)_2O$ at 50 °C for 10 min. After extraction into the organic phase of a $CH_2Cl_2$–$Na_2CO_3$ extraction, the resulting PMAAs were analyzed on a Hewlett–Packard 5890 GC interfaced to a 5970 MSD (mass-selective detector, electron-impact ionization mode) using a 30-m Supelco 2330 bonded-phase fused silica capillary column. Chemical-ionization GC–MS was performed on an Agilent 6890N GC interfaced to a 5973 MSD using an Agilent DB-1 0.25 mm × 30 m capillary column. The temperature program started at 80 °C with 2-min hold, followed by a 30 °C/min gradient to 170 °C, 4 °C/min gradient to 235 °C and finished with a 20 min hold at 235 °C.

### 4.8. NMR spectroscopy

NMR experiments were carried out on a Varian Inova-600 MHz spectrometer using a 5-mm triple resonance probe, which was kept at a temperature of 60 °C. Standard pulse sequences from Varian were used to acquire 1D Proton, 2D gradient-enhanced COSY,

gradient-enhanced HSQC, TOCSY, and NOESY spectra. The TOCSY spin-lock time was 80 ms, and the NOESY mixing time was 200 ms. Chemical shifts are reported in ppm downfield from internal DSS, but were actually measured relative to internal acetone ($\delta_H$ 2.225 ppm, $\delta_C$ 31.07 ppm) or free acetate ($\delta_H$ 1.908 ppm, $\delta_C$ 24.99 ppm at pH 6–8). One-bond C–H coupling constants were measured on a Varian Inova-800 MHz spectrometer at 35 °C by application of a 1D HMQC pulse sequence without carbon decoupling during acquisition.

## References


1. Austin, J. W.; Sanders, G.; Kay, W. W.; Collinson, S. K. *FEMS Microbiol. Lett.* **1998**, *162*, 295–301.
2. Pang, T.; Bhutta, Z. A.; Finlay, B. B.; Altwegg, M. *Trends Microbiol.* **1995**, *3*, 253–255.
3. Kennedy, M.; Villar, R.; Vugia, D. J.; Rabatsky-Ehr, T.; Farley, M. M.; Pass, M.; Smith, K.; Smith, P.; Cieslak, P. R.; Imhoff, B.; Griffin, P. M. *Clin. Infect. Dis.* **2004**, *38*, S142–S148.
4. Romling, U.; Sierralta, W. D.; Eriksson, K.; Normark, S. *Mol. Microbiol.* **1998**, *28*, 249–264.
5. Anriany, Y. A.; Weiner, R. M.; Johnson, J. A.; De Rezende, C. E.; Joseph, S. W. *Appl. Environ. Microbiol.* **2001**, *67*, 4048–4056.
6. Guard-Petter, J.; Keller, L. H.; Rahman, M. M.; Carlson, R. W.; Silvers, S. *Epidemiol. Infect.* **1996**, *117*, 219–231.
7. White, A. P.; Gibson, D. L.; Kim, W.; Kay, W. W.; Surette, M. G. *J. Bacteriol.* **2006**, *188*, 3219–3227.
8. Collinson, S. K.; Doig, P. C.; Doran, J. L.; Clouthier, S.; Trust, T. J.; Kay, W. W. *J. Bacteriol.* **1993**, *175*, 12–18.
9. Zogaj, X.; Nimtz, M.; Rohde, M.; Bokranz, W.; Romling, U. *Mol. Microbiol.* **2001**, *39*, 1452–1463.
10. White, A. P.; Gibson, D. L.; Collinson, S. K.; Banser, P. A.; Kay, W. W. *J. Bacteriol.* **2003**, *185*, 5398–5407.
11. Solano, C.; Garcia, B.; Valle, J.; Berasain, C.; Ghingo, J. M.; Gamazo, C.; Lasa, I. *Mol. Microbiol.* **2002**, *43*.
12. Ryu, J. H.; Beuchat, L. R. *Appl. Environ. Microbiol.* **2005**, *71*.
13. Romling, U.; Gomelsky, M.; Galperin, M. Y. *Mol. Microbiol.* **2005**, *57*, 629–639.
14. Parsek, M. R.; Fuqua, C. *J. Bacteriol.* **2004**, *186*, 4427–4440.
15. Roberts, I. S. *Ann. Rev. Microbiol.* **1996**, *50*, 285–315.
16. Whitfield, C.; Valvano, M. A. *Adv. Microb. Physiol.* **1993**, *35*, 135–246.
17. Whitfield, C.; Roberts, I. S. *Mol. Microbiol.* **1999**, *31*, 1307–1319.
18. Jann, B.; Jann, K. Capsules of *Escherichia coli*. In *Escherichia coli: Mechanisms of Virulence*; Sussman, M., Ed.; Cambridge University Press: Cambridge, 1997; pp 113–143.
19. Clarke, B. R.; Pearce, R.; Roberts, I. S. *J. Bacteriol.* **1999**, *181*, 2279–2285.
20. Jann, B.; Jann, K. *Curr. Top. Microbiol. Immunol.* **1990**, *150*, 19–42.
21. MacLachlan, P. R.; Keenleyside, W. J.; Dodgson, C.; Whitfield, C. *J. Bacteriol.* **1993**, *175*, 7515–7522.
22. Goldman, R. C.; White, D. J.; Oskov, F.; Orskov, I.; Rick, P. D.; Lewis, M. S.; Bhattacharjee, A. K.; Leive, L. *J. Bacteriol.* **1982**, *151*, 1210–1221.
23. Peterson, A. A.; McGroarty, E. J. *J. Bacteriol.* **1985**, *162*, 738–745.
24. Sadovskaya, I.; Brisson, J.-R.; Khieu, N. H.; Mutharia, L. M.; Altman, E. *Eur. J. Biochem* **1998**, *253*, 319–327.
25. Heyns, K.; Kiessling, G. *Carbohydr. Res.* **1967**, *3*, 340–353.
26. Kossaczka, Z.; Bystricky, S.; Bryla, D.; Shiloach, J.; Robbins, J. B.; Szu, S. C. *Infect. Immun.* **1997**, *65*, 2088–2093.
27. de Rezende, C. E.; Anriany, Y.; Carr, L. E.; Joseph, S. W.; Weiner, R. M. *Appl. Environ. Microbiol.* **2005**, *71*, 7345–7351.
28. Gibson, D. L.; White, A. P.; Snyder, D. S.; Azadi, P.; Kay, W. W. *J. Bac.* **2006**, accepted for publication (JB00809-06).
29. Patel, T. P.; Paraekh, R. B. *Methods Enzymol.* **1994**, *230*, 57–66.
30. Lipkind, G. M.; Shashkov, A. S.; Knirel, Y. A.; Vinogradov, E. V.; Kochetkov, N. K. *Carbohydr. Res.* **1988**, *175*, 59–75.
31. Bock, K.; Meldal, M.; Bundle, D. R.; Iversen, T.; Pinto, B. M.; Garegg, P. J.; Kvanstrom, I.; Norberg, T.; Lindberg, A. A.; Svenson, S. B. *Carbohydr. Res.* **1984**, *130*, 35–53.
32. Hellerqvist, C. G.; Lindberg, B.; Svensson, S.; Holme, T.; Lindberg, A. A. *Acta Chem. Scand.* **1969**, *23*, 1588–1596.
33. Rahman, M. M.; Guard-Petter, J.; Carlson, R. W. *J. Bacteriol.* **1997**, *179*, 2126–2131.
34. Ali, T.; Urbina, F.; Weintraub, A.; Widmalm, G. *Carbohydr. Res.* **2005**, *340*, 2010–2014.
35. Waldor, M. K.; Colwell, R.; Mekalanos, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11388–11392.
36. Wang, Z.; Larocque, S.; Vinogradov, E.; Brisson, J.-R.; Dacanay, A.; Greenwell, M.; Brown, L. L.; Li, J.; Altman, E. *Eur. J. Biochem.* **2004**, *271*, 4507–4516.
37. Grimmecke, H. D.; Knirel, Y. A.; Shashkov, A. S.; Kissel, B.; Lauk, W.; Voges, M. *Carbohydr. Res.* **1994**, *153*, 277–286.
38. Sadovskaya, I.; Brisson, J.-R.; Altman, E.; Mutharia, L. M. *Carbohydr. Res.* **1996**, *283*, 111–127.
39. Hellerqvist, C. G.; Hoffmann, J.; Lindberg, B.; Pilotti, A.; Lindberg, A. A. *Acta Chem. Scand.* **1971**, *25*, 1512–1513.
40. Fukuda, S.; Egami, F.; Hammerling, G.; Luderitz, O.; Bagdian, G.; Staub, A. M. *Eur. J. Biochem.* **1971**, *20*, 438–441.
41. Brisson, J. R.; Uhrinova, S.; Woods, R. J.; van der Zwan, M.; Jarrell, H. C.; Paoletti, L. C.; Kasper, D. L.; Jennings, H. J. *Biochemistry* **1997**, *36*, 3278–3292.
42. Tomlins, R. I.; Watkins, T. R.; Gray, R. J. H. *Appl. Environ. Microbiol.* **1982**, *44*, 1110–1117.
43. Bhattacharjee, A. K.; Jennings, H. J.; Kenny, C. P.; Martin, A.; Smith, I. C. *J. Biol. Chem.* **1975**, *250*, 1926–1932.
44. Vann, W. F.; Schmidt, M. A.; Jann, B.; Jann, K. *Eur. J. Biochem.* **1981**, *116*, 359–364.
45. Roberts, I. S.; Saunders, F. K.; Boulnois, G. J. *Biochem. Soc. Trans.* **1989**, *17*, 462–464.
46. Wyle, F. A.; Artenstein, M. S.; Brandt, B. L.; Tramont, E. C.; Kasper, D. L.; Altieri, P. L.; Berman, S. L.; Lowenthal, J. P. *J. Infect. Dis.* **1972**, *126*, 514–521.
47. Howard, C. J.; Glynn, A. A. *Immunology* **1971**, *20*, 767–777.
48. Michalek, M. T.; Mold, C.; Bremer, E. G. *J. Immunol.* **1988**, *140*, 1588–1594.
49. Brown, E. J.; Joiner, K. A.; Gaither, T. A.; Hammer, C. H.; Frank, M. M. *J. Immunol.* **1983**, *131*, 409–415.
50. Moxon, E. R.; Kroll, J. S. *Curr. Top. Microbiol. Immunol.* **1990**, *150*, 65–85.

51. Raffatellu, M.; Chessa, D.; Wilson, R. P.; Dusold, R.; Rubino, S.; Baumler, A. J. *Infect. Immun.* **2005**, *73*, 3367–3374.
52. Sharma, A.; Qadri, A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17492–17497.
53. Patel, T. O.; Bruce, J.; Merry, A.; Bigge, C.; Wormald, M.; Jacques, A.; Parekh, R. *Biochem. Soc. Trans.* **1993**, *32*, 679–693.
54. Wozniak, D. J.; Wyckoff, T. J. O.; Starkey, M.; Keyser, R.; Azadi, P.; O'Toole, G.; Parsek, M. R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7907–7912.
55. York, W. S.; Darvill, A. G.; McNeil, M.; Stevenson, T. T.; Albersheim, P. *Methods Enzymol.* **1985**, *118*, 3–40.
56. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.