Chemical Composition of Cell-wall Polysaccharide of Rough Mutants of *Salmonella typhimurium*

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SUMMARY

Quantitative analyses of monosaccharide constituents of the cell-wall polysaccharide of the smooth form and rough mutants of *Salmonella typhimurium* were made by gas-liquid chromatography of glycol acetates produced from acid hydrolysates of phenol + water extracts of cell-wall preparations. The presence, in varying amounts, of sugars characteristic of the S-specific repeating unit was detected in all rough mutants investigated. Definite conclusions about the core structure of the different rough mutants could not be drawn by using only the basis of the monosaccharide composition of the cell-wall polysaccharide. Ribose was found in the cell-wall polysaccharide of the smooth form in small amounts.

INTRODUCTION

The essential structural features of the cell-wall polysaccharide in *Salmonella* have been elucidated by immunochemical and chemical methods (Kauffmann, Lüderitz, Stierlin & Westphal, 1960; Kauffmann, Krüger, Lüderitz & Westphal, 1961; Beckmann, Subbaiah & Stocker, 1964; Osborn *et al.* 1964; Lüderitz *et al.* 1965; Nikaido, Naide & Mäkelä, 1966). The polysaccharide molecule is composed of a core to which long side chains containing repeating units are attached (Fig. 1). The repeating units contain the specific structures of the O antigen determinants. The chemical composition of some of these determinants has been completely clarified. (For a review see Lüderitz, Staub & Westphal, 1966.) Rare monosaccharides which have not been found in the core structure are often present in the repeating unit. The core region is believed to be similar in all salmonellas. A side chain containing glucose, galactose and *N*-acetylglucosamine is linked to an internal backbone composed of Lipid A, 2-keto-3-deoxyoctulosonic, ethanolamine, phosphate and heptose. The biosynthesis of this side chain appears to involve a sequential transfer of the monosaccharides in the form of nucleotide sugars to the backbone by transferase systems (Osborn, 1966). Studies of the core have been made possible through the use of rough mutants defective in different biosynthetic steps. The designation of these mutants is based on both the chemical composition of the cell-wall polysaccharide—

Ra, Rb, Rc, Rd, Re according to Lüderitz, Staub & Westphal (1966) and the serological specificity, RI–RII (Beckmann, Lüderitz & Westphal, 1964; Lüderitz, Beckmann & Westphal, 1964).

Studies on the monosaccharide composition, by using thin-layer chromatography, of the cell-wall polysaccharide of rough mutants of *Salmonella typhimurium*, selected by the use of phages, revealed the unexpected presence of mannose (Holme, 1965). The application of gas-liquid chromatography for the separation of monosaccharides as described by Sawardeker, Sloneker & Jeanes (1965) offered a more sensitive method for such studies. The aim of the investigations is to study the structure and function of the receptors for different bacteriophages. The present report deals with the chemical composition of the cell-wall polysaccharide of smooth and rough strains of *S. typhimurium* and *S. minnesota*.

Fig. 1. Main features of the proposed structure of the cell-wall lipopolysaccharide of *Salmonella typhimurium*. The abbreviations used are: *KDO*, 2-keto-3-deoxy-D-manno-octulosonic acid; *Hep*, L-glycero-D-manno-heptose; *Glu*, D-glucose; *Gal*, D-galactose; *GNAc*, N-acetyl-D-glucosamine; *Rha*, L-rhamnose; *Man*, D-mannose; *Abe*, abequose (3,6-dideoxy-D-xyllo-hexose).

METHODS

Bacterial strains. *Salmonella typhimurium* 395MS was the parent strain of the isolated rough mutants. The mutant strains derived from *S. typhimurium* LT2—TV 160, TV 161, TV 163, TV 166, TV 208, TV 225, TV 226 and TV 227—were obtained from Dr B. A. D. Stocker (Lister Institute of Preventive Medicine, London). The mutants TV 119 and TV 148 were obtained from Dr M. Raynaud (Institut Pasteur, Paris). *Salmonella minnesota* s99 and the mutants R60 and R345 derived from this strain were supplied by Dr O. Lüderitz (Max Planck-Institute für Immunbiologie, Freiburg).

Phage stocks. The *Salmonella* phages Felix 0–1 (Felix & Callow, 1943) and 4 (Lilleengen, 1948) were propagated by the soft agar layer method (Adams, 1959).

Isolation of mutants. For the isolation of phage-resistant mutants, the bacteria were plated with an appropriate dilution of the phage stocks using the soft top-layer agar technique (Adams, 1959). The initial bacterial number on each plate was approximately 10^8 . The input multiplicity of phage was 10. After incubation at 37° for 18 hr five colonies were picked from each plate and transferred to nutrient agar plates. Daily transfer was done until no free phage could be detected by spot test against the parent strain. No adsorption of the phages FO-1 or 4 to the isolated mutants could be demonstrated.

Another series of mutants was isolated by picking rough-looking colonies from broth cultures of the strain *Salmonella typhimurium* 395MS. The mutant 395MR0 was isolated from a 1-week-old nutrient broth culture. The mutants 395MR1-R4 were prepared as follows. An 18 hr culture of the parent strain was subjected to ultraviolet (u.v.) irradiation so as to obtain approximately 0.1 % survival. Ten 0.05 ml. portions from the u.v.-treated suspension were each inoculated into 10 ml. nutrient broth. From these cultures rough mutants were isolated after incubation at 37° for 2 weeks. Mutants with different antigenic patterns in the gel precipitin test were chosen for further study. The mutants used are listed in Table 1.

Virulence and protection tests. The methods used have been described previously (Holme & Edebo, 1961, Edebo & Holme, 1965). In the virulence tests 20 g. mice were used.

Preparation of cell walls. Bacteria were grown in a medium of the following composition (g.): glucose, 5; tryptone (Difco), 10; yeast extract (Difco), 5; K₂HPO₄, 7; KH₂PO₄, 3; NaCl, 0.5; in 1000 ml. distilled water.

Table 1. *Rough mutants of Salmonella typhimurium* 395MS

Method of isolation	Mutant no.			
Broth culture	395MR0			
U.v., broth culture	395MR1	395MR2	395MR3	395MR4
Phage FO-1	395MR5	395MR6	395MR10	
Phage 4	395MR7	395MR8	395MR9	

Cultivation was in 5 l. indented Erlenmeyer flasks; the culture volume in each flask was 2 l. The flasks were placed on a rotary shaker and incubated for 18 hr at 37°. The yield varied between 1.5 and 2.5 g. dry wt bacteria/l. culture. Cell walls were prepared by disintegrating the centrifuged organisms in the frozen state in the X-press (Edebo, 1960). Initial separation of walls from cell debris was effected by centrifugation at 10,000g for 20 min. in an IEC-PR2 refrigerated centrifuge at +4°. The cell walls collected as a loosely packed layer on the pellet surface; they were washed off by careful rinsing. The wall preparation was then washed twice in 0.1 M-phosphate buffer (pH 7), once in M-sodium chloride and once in distilled water. The washing procedure was done in the cold. The cell walls were then suspended in distilled water. No intact bacteria could be observed when the preparations were examined by phase microscopy.

Extraction of lipopolysaccharide. Lipopolysaccharide was extracted from the cell walls by the phenol + water procedure of Westphal, Lüderitz & Bister (1952). A scanning spectrophotometer (Beckmann Dk2) was used for the detection of ultraviolet absorbing substances in the preparations.

Estimation of hexosamines. Hexosamine was estimated according to Gatt & Berman (1966) on polysaccharides obtained by hydrolysis in acetic acid of lipopolysaccharides according to Sutherland, Lüderitz & Westphal (1965).

Gas-chromatographic analysis of the carbohydrate composition of cell-wall lipopolysaccharide hydrolysates. The analysis was based on the method described by Sawardeker *et al.* (1965). The dialysed cell-wall extracts contained between 0.6 and 4.6 mg. polysaccharide/ml. by the anthrone method and expressed as glucose (Whistler & Wolfson, 1962). For the analysis a volume containing approximately 2 mg. was taken. The extracts were hydrolysed in 0.5 N-sulphuric acid at 100° overnight.

The acid was removed by Dowex 3 (free base) and the solution concentrated under reduced pressure to about 1 ml. The mixtures of aldoses were reduced to the corresponding alditols with sodium borohydride overnight. Excess borohydride was destroyed by Dowex 50 W-X8 and boric acid removed as methyl borate by several distillations with methanol under reduced pressure. The syrups were dried by distillation under reduced pressure with benzene, and then fully acetylated with acetic anhydride + pyridine (1:1 v/v) at 100° for 10–15 min. Excess of acetic anhydride was removed by

Fig. 2. Chromatogram of alditol acetates of reference sugars. Column: 3 % ECNSS-M on Gas-Chrom Q. Injector temperature 270°, column temperature 170°, detector temperature 240°. Flow: 30 ml. N₂/min. 1, Tyvelose (3,6-dideoxy-D-*arabino*-hexose); 2, abequose (3,6-dideoxy-D-*xylo*-hexose); 3, rhamnose; 4, fucose; 5, ribose; 6, arabinose; 7, xylose; 8, mannose; 9, galactose; 10, glucose; 11, L-glycero-D-manno-heptose.

Fig. 3. Chromatogram of alditol acetates of sugars from *Salmonella typhimurium* TV 225 cell-wall polysaccharide. Column: 3 % ECNSS-M on Gas-Chrom Q. Injector temperature 270°, column temperature 180°, detector temperature 240°. Flow: 32 ml. N₂/min. 1, Abequose; 2, rhamnose; 3, mannose; 4, galactose; 5, glucose; 6, heptose.

adding water and the resulting solution concentrated to dryness. The resulting mixture of fully acetylated alditol acetates was dissolved in chloroform and analysed by gas-liquid chromatography. The solutions (1 μ l.) were injected on to an ECNSS (polyester-silicone) glass column fitted in a Perkin-Elmer Model 801 gas chromatograph. The injection temperature was 265–270°, column temperature 170–175°, detector temperature 235–240° and gas flow (nitrogen) 30–32 ml./min. The retention times of the various acetates were identical to those of authentic polyacetates of each of the various glycitols. The glycitols were further identified by adding reference materials

to the mixtures. Complete separation of the glycitol acetates was obtained (Figs. 2, 3). The response of the flame-ionization detector to all the glycitol acetates was found to be linear and was determined for each one. The relative peak areas were determined for the mixtures from the various lipopolysaccharides and molecular ratios for the constituent sugars calculated from these, after suitable correction for the different response of the detector to the various glycitol acetates. The method was tested by subjecting known mixtures of the various sugars to the above procedure. The ratios found differed from those calculated by not more than ± 3 mole %. Losses of the most volatile of the glycitol acetates, that derived from abequose, during the working-up of the acetylation mixture, were found to be negligible.

RESULTS

Growth characteristics of the mutants

All the mutants developed rough colonies after incubation for 48 hr on nutrient agar. On galactose Endo agar all strains except R9 showed positive fermentation reaction. The mutant R9 developed smooth colonies on this medium. This mutant was also unable to grow on minimal medium with galactose as the sole carbon source. All the mutants grew on minimal media with glucose as the sole carbon source.

Biological characteristics

The parent strain was highly virulent for mice. All mice in a group of 10 died within 7 days after inoculation of approximately 50 viable bacteria/mouse. For virulence tests of the rough mutant strains, doses of $2-8 \times 10^5$ viable bacteria were inoculated into groups of 10 mice. All strains of *Salmonella typhimurium* except R4 listed in Table 1 were tested. Strain R4 was lost after the chemical analyses had been made, and no virulence tests could be done. No deaths were recorded.

Protection tests were made using suspension of heat-killed cells of the smooth form and the mutants R0, R5, R6, R7, R8, R9 and R10 as immunizing agents. PD50 of the smooth form was 6×10^{-5} mg. (dry weight). No protection was obtained in groups of mice treated with 0.3 mg. of cells of the rough mutants.

Chemical composition of cell-wall polysaccharides

The data from the gas chromatographic analysis of the polysaccharide hydrolysates and the separate determination of glucosamine are given in Table 2. The quantities of the individual sugars are given in mole % of the detected sugars to facilitate the comparison of ratios between polysaccharides from different strains.

Various peaks obtained from contaminating materials were always observed, particularly in the region of the peaks for pentitol and deoxyhexitol acetates. The alditols in this region were therefore always particularly carefully identified by adding reference glycitols, thereby obtaining 'mixed' chromatograms. The assignments were further confirmed by employing a mass spectrometer connected to the gas chromatograph. The mass spectra of abequitol, rhamnitol, pentitols and hexitols were clearly different. The peaks of the glycitol acetates obtained from the hydrolysates gave mass spectra identical to those of the corresponding reference substances.

In the region of the hexitol peaks, including that given by mannitol acetate, inter-

fering non-carbohydrate material was absent. Between the peaks of glucitol and heptitol an unidentified peak was regularly found. The presence of mannose in some of the hydrolysates was confirmed by paper and thin-layer chromatography. The detection of rhamnose and abequose was facilitated by the use of gas chromatography, the sensitivity of which is much greater than that of paper chromatography.

Table 2. *Chemical composition of cell-wall polysaccharides from Salmonella typhimurium 395MS and rough mutants, rough mutants of S. typhimurium LT2 (TV strains) and S. minnesota S99 and rough mutants R60 and R345*

Figures for all monosaccharides except glucosamine represent mole % of detected sugars. Glucosamine is given in % of polysaccharide by the anthrone method. ND = not detected.

Mutant	Hep	Glu	Gal	Man	Rha	Abe	GN % of polysacch.	Proposed chemical designation
395MS	< 1	30	22	21	18	9	3.6	XIV
395MR0	32	31	33	3	1	< 1	16.7	Ra
395MR1	40	21	31	4	3	1	1.4	Rb
395MR2	38	22	37	2	1	< 1	2.6	Ra
395MR3	46	22	25	4	2	1	5.0	Ra
395MR4	30	30	31	5	3	1	5.0	Ra
395MR5	23	19	26	17	11	4	1.8	Rb
395MR6	27	19	28	12	8	6	1.0	Rb
395MR7	43	27	19	7	3	1	2.1	Rb
395MR8	50	22	20	5	2	< 1	0.8	Rb
395MR9	41	46	5	4	2	2	0.7	Rc
395MR10	67	3	9	14	5	2	1.3	Rd
TV160	32	22	32	7	5	2	1.1	Rb
TV161	34	19	32	9	4	2	0.6	Rb
TV163	27	26	28	9	7	3	3.3	Ra
TV208	7	9	34	24	17	9	5.2	Ra
TV225	30	26	31	7	4	2	2.7	Ra
S99	23	23	54	ND	ND	ND	—	II
R60	26	36	38	ND	ND	ND	—	Ra
R345	30	20	50	ND	ND	ND	—	Rb

Hep = heptose, Glu = glucose, Gal = galactose, Man = mannose, Rha = rhamnose, Abe = abequose, GN = glucosamine.

The parent strain, 395MS, showed an extremely low value of heptose, which indicates that the core region is only a minor component of the S polysaccharide. The same tendency was apparent for strain TV208. One mutant, R10, with the proposed chemotype Rd, displayed a heptose value of 67. The remaining mutants showed values between 23 and 50 mole %. Glucose was significantly lower in the mutants R10 and TV208 than in the other strains. Galactose was low in strains R9 and R10. The galactose-non-fermentor R9 is of chemotype Rc.

The glucosamine values for the proposed Ra mutants fell between 2.6 and 16.7. Mutants belonging to chemotypes Rb, Rc and Rd had glucosamine values between 0.6 and 2.1. The quantities of glucosamine in the *Salmonella minnesota* strains were not determined, since the specific structure contains galactosamine, and the method of analysis did not distinguish between these hexosamines. Ribose was found in small amounts, about 1 %, in the cell-wall polysaccharide of the smooth form of *S. typhimurium* but not in the smooth form of *S. minnesota*. It was also present in small amounts in some of the mutants representing all chemotypes. The ribose values

were not incorporated in Table 1 because of the uncertainty of the origin of this sugar, when it was present in very small amounts.

The S-specific sugars mannose, rhamnose and abequose were found in appreciable amounts in all the rough mutants. The strains TV208, R5 and R6 contained these sugars in especially high quantities. Mannose was always present in greater amounts than rhamnose and abequose.

DISCUSSION

Various methods for the analysis of the monosaccharide composition of lipopolysaccharides have been used. Analysis by paper chromatography is complicated by the number of components present. This makes the use of several solvent systems necessary. The use of enzymic methods on the other hand makes the combination of these various analyses into relative ratios for the monosaccharides somewhat uncertain.

The method used in the present work is essentially that described by Sawardeker *et al.* (1965) in which hydrolysates containing the monosaccharide mixture are converted into the corresponding glycol acetates. This mixture is readily separated and analysed by gas chromatography. The method is rapid, relatively simple and has the advantage of giving a simultaneous determination of the components. In the procedure used here the glucosamine is, however, removed from the mixture.

The presence of mannose, rhamnose and abequose in all the rough mutants of *Salmonella typhimurium* raised the question whether the strains were unstable and frequently reverted to the wild type. The conclusion that this was not the case was drawn from the following facts. (1) The mutants from *S. typhimurium* 395MS have been kept in the laboratory for 5 years without any signs of reversion, only rough colonies being observed when the strains were plated on nutrient agar. (2) The antigenic pattern in gel precipitin tests has been constant during this time (unpublished). (3) Vaccines prepared from the mutant strains did not confer protection in mice against challenge with viable *S. typhimurium* 395MS. (4) The mutants behaved as typical rough strains in serological tests and phage typing (Lindberg & Holme, 1968). (5) All mutants were avirulent. The dose used in these tests was approximately 5×10^5 viable bacteria. Since the smooth bacteria are highly virulent ($LD_{50} < 50$), revertants would have been detected in a proportion of less than 1 in 10^4 bacteria. Such a low proportion of smooth bacteria could not have any influence on the results of the chemical and immunological investigations.

Several explanations can be given for the presence of mannose, rhamnose and abequose in the rough mutants. The enzymic block which can lead to roughness may result in defective synthesis of one or more of the component monosaccharides, or it may result in a defective transfer of the sugars to the lipopolysaccharide core. A partial synthesis of the S-specific polysaccharide would result if the enzymic block was not complete or if secondary synthetic pathways could be used in the synthesis. Gemski & Stocker (1967) attributed the capacity of some rough mutants to maintain the propagation of the smooth specific phage P22 and the evocation of P22 transductants from such strains to incomplete deficiencies (leaky mutations). Kalckar, Laursen & Rapin (1966) attributed the presence of small amounts of galactose in an UDP-galactose-4-epimerase negative mutant of *Escherichia coli* K12 to the possibility that galactose was synthesised by an alternative pathway. The small epimerase

activity detected was considered insufficient to explain the amount of galactose detected in the lipopolysaccharide.

The parent smooth strain and some of the rough mutants of *Salmonella typhimurium* displayed small amounts of ribose. The presence of 1-4% ribose in the lipopolysaccharide of RII-mutants of different *Salmonella* was reported by Lüderitz *et al.* (1966). Lipopolysaccharides from *Salmonella* T1 strains, which lack the S-specific side chains, contain large amounts of ribose and galactose. Genetic analysis showed that T1 strains have active genes (*rft I* gene) for producing T1 specificity which are lacking or inactive in the smooth strain (Sarvas, 1968). Recombination experiments between T1 strains and well-defined rough mutants indicated that the *rft I* gene was only expressed when the complete core was present. Ribose should then be found only in mutants of chemotype Ra and in smooth strains if the *rft I* gene is not completely repressed. The determination of this sugar is, however, complicated by the possibility of cytoplasmic contamination of the lipopolysaccharide preparations. The lipopolysaccharide extracted from cell walls of the smooth strain *S. typhimurium* 395MS did not show any absorption at 260 m μ which indicates that the ribose (approximately 1%) detected in this extract was not derived from contaminating ribonucleic acid. No ribose was found in the extracts of *S. minnesota* R60, which is of chemotype Ra. This finding needs further investigation, since it was reported by Lüderitz *et al.* (1966) that this mutant incorporates small amounts of ribose in its lipopolysaccharide.

Ribitol was found by Lilly (1962) in extracts from isolated cell walls from *Escherichia coli* 26-26. Since the gas chromatographic method was based on the separation of glycitols, no differentiation could be made between ribose and ribitol. By using paper chromatography it could be shown that the hydrolysates of cell walls from *Salmonella typhimurium* 395MS contained small amounts of ribose as compared to galactose, mannose and rhamnose.

It was not possible to designate the mutants to the groups Ra, Rb, etc., using only the basis of the chemical composition of the cell-wall polysaccharide (Table 2). The presence of haptenic polysaccharide containing S-specific determinants in supernatant fluids from phenol + water extracts from whole bacteria (L1-fraction of Beckmann, Subbaiah & Stocker, 1964), which was easily detected by the gel precipitin reaction (unpublished), could not be used as a criterion for the Rb mutants, since two of the known Ra mutants, TV163 and 225, both displayed this antigen. The proposed chemical grouping of Table 2 was therefore based on phage patterns and immunological investigations (see Lindberg & Holme, 1968), in addition to cultural and chemical data. A difference in the glucosamine content between the Ra mutants, on the one hand, and Rb, Rc and Rd mutants, on the other, was recorded, but the amounts detected did not make a sharp distinction possible. The presence of large amounts of glucosamine in Lipid A complicates an accurate determination of this amino sugar in the polysaccharide. After the removal of the lipid by acid hydrolysis, small amounts of contaminating glucosamine might remain in the preparation. Part of the glucosamine in some of the mutants was, however, probably associated with the presence of the S-specific sugars.

The fact that the rough mutants selected for resistance against the phages FO-1 or 4 were of chemotype Rb, Rc and Rd, supports the assumption that the receptor sites for these phages are associated with the Ra structure (Lindberg, 1967). Mutants

selected for rough colony type appear to be predominantly of the chemotype Ra.

The mutant R9, which was a galactose-non-fermenter, showed characteristics of being deficient in the enzyme UDP-gal-4-epimerase. The presence of S-specific sugars in this mutant raised the question whether the culture medium used contained galactose. When analysed by the gas chromatographic method described above no traces of galactose, mannose, ribose, rhamnose or abequose could be detected in the tryptone or the yeast extract. Strain R10, which was the only mutant showing a low glucose content of its cell-wall polysaccharide, was believed to be deficient in the glucosyl-I-transferase system (Osborn *et al.* 1964), since it showed normal growth characteristics on a glucose-salts medium.

A further class of mutants of *Salmonella* was described by Naide *et al.* (1965). These mutants were termed semi-rough and displayed characteristics intermediate between the smooth and the rough forms with respect to cultural and serological behaviour. The lipopolysaccharide of these strains all contained the S-specific sugars but in smaller amounts than found in the wild-type strain. The results of phage typing and serological investigations presented by Lindberg & Holme (1968) disclose the possibility that the mutants used in the present investigation belong to the semi-rough group.

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