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Association of Plasmids with the Carbohydrate Composition of the O-antigen in *Shigella dysenteriae* type 1

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ABSTRACT

Six different prepared mutant strains of *Shigella dysenteriae* type 1 with various combinations of 'core' plasmids of 140, 6, and 2 Mdal, were cultivated separately and their lipopolysaccharide (LPS) samples were isolated, which on delipidation afforded polysaccharides and oligosaccharides. The LPS and prepared O-antigen polysaccharides were subjected to acid hydrolysis, and the constituent sugars were analysed by paper chromatography and gas liquid chromatography (GLC), using CP Sil 8 CB fused silica capillary column equipped with a flame ionization detector. The mutant strains, harbouring the 6 Mdal plasmid, contained rhamnose, galactose, and N-acetyl glucosamine in their O-antigen polysaccharides with a minor variation in the relative proportions of these sugar constituents as compared to those of the control strain. The sugar composition of the O-antigen polysaccharides prepared from the mutant strains, without the 6 Mdal plasmid but containing 2 or 140 Mdal plasmid, contained galactose and N-acetyl glucosamine but no rhamnose. However, mutant strains with none of the core plasmids contained traces of rhamnose and were devoid of N-acetyl glucosamine, but contained heptose. These results suggest that 6 Mdal plasmid is essentially required for the complete synthesis of O-antigen polysaccharides. However, the 140 and 2 Mdal plasmids might also have an association with synthesis of carbohydrate composition of the O-antigen in *S. dysenteriae* type 1.

Key words: O-antigenic polysaccharides; *Shigella dysenteriae* type 1.

INTRODUCTION

Thirteen different serotypes of *Shigella dysenteriae* have been described till now, among which *S. dysenteriae* type 1 causes the most severe form of bacterial dysentery in man. Structural investigations on the lipopolysaccharides isolated from 10 different serotypes of *S. dysenteriae* have been reported (1). The polysaccharide antigens (O-antigen) of these serotypes were composed of regular repeating units varying in size with widely different sugar compositions. *S. dysenteriae* serotypes 1, 5, 6, 9, and 10 contained tetrasaccharide, and the other serotypes (types 2,3,4,7 and 8) had pentasaccharide repeating units in their O-antigen polysaccharides. Several non-sugar substituents such as O-acetyl, carboxyethyl, and pyruvic acid acetal were also present in the repeating units of some of the serotypes. *S. dysenteriae* type 1 had four sugar repeating units consisting of rhamnose, galactose, and N-acetyl glucosamine.

Genetic analysis of *S. dysenteriae* type 1 has shown that a small 6 megadalton (Mda) plasmid (2) as well as chromosomal loci (3) are involved in the expression of its LPS. Recent investigations (4) showed that a significant number of strains of *S. dysenteriae* type 1 collected from different geographical locations contained three "core" plasmids of 140, 6, and 2 Mda. It has also been demonstrated that strains containing 6 Mda plasmid expressed complete O-antigen in the LPS, whereas mutant strains (5) containing either the 140 or 2 Mda plasmid, in the absence of 6 Mda plasmid produced smaller amounts of O-antigen in the LPS. When none of these three plasmids was present the strains produced no O-antigen in their LPS (5).

In the present investigation the carbohydrate compositions of LPS of the mutant strains, containing plasmids of various sizes, were compared with the LPS obtained from a virulent strain of *S. dysenteriae* type 1.

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MATERIALS AND METHODS

Bacterial strains. *Shigella dysenteriae* type 1 strain Z24623 was collected from a patient at the clinical research centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) in Dhaka, Bangladesh, and the mutant strains, namely PSD-1, PSD-20, PSD-30, PSD-40, PSD-50, and PSD-60, were isolated by repeated subculturing on MacConkey agar plates. Loss of virulence of the isolated strains were determined by the Congo-red binding assay, salt aggregation test (6), Sereny test (7), and modified ELISA method (8). Serological identification of the mutant strains was also made by slide agglutination test with *S. dysenteriae* type 1 antiserum (9).

Preparation of Lipopolysaccharides. Bacteria were inoculated on tripticase soy agar (TSA) plates and incubated at 37°C for 24 h, and the cells were harvested by washing with sterile saline (0.85 M). Cells were collected by centrifugation of the washings. LPS was extracted from the cells by phenol water extraction (10) and purified by ultracentrifugation at 105,000 X *g* for 4 h. Purified LPS was obtained by lyophilisation.

Delipidation of LPS samples. The lipopolysaccharide (100 mg) prepared from each strain was suspended in aqueous 1% acetic acid (150 ml) and the mixture was heated at 100°C with stirring for 3 h. After cooling, the mixture was centrifuged and the supernatant was extracted with diethyl ether (100 ml X 3). The aqueous layer was concentrated to about 10 ml and was applied to a Bio-gel P-10 column (40 X 2.5 cm). The column was eluted with water containing 0.05% trichlorotertiary butanol, and the column eluate was collected in fractions (5 ml) with the help of a fraction collector. Fractions were assayed with phenol-sulphuric acid (11) and the carbohydrate containing fractions were pooled, concentrated, and freeze-dried.

Sugar analysis of the LPS samples. The LPS samples (5 mg each) were hydrolysed separately with aqueous trifluoroacetic acid (2 M, 2 ml) at 120°C for 3 h. The hydrolysates were evaporated to dryness to remove the acid and were separately dissolved in water (0.5 ml) and analysed by paper chromatography on Whatman No. 1 paper using *n*-butanol: ethanol: water (40:11:19) solvent system and the paper chromatograms were developed by staining with *p*-anisidine phthalate, followed by heating.

The remaining portions of the hydrolysates were treated with sodium borohydride (20 mg) in aqueous ammonia (1 M, 2 ml) for 2 h at room temperature. Excess borohydride was neutralised with acetic acid, and the resulting solutions were evaporated to dryness by co-distillation with 10% acetic acid in methanol (1 ml X 3), followed by methanol (1 ml X 3). The dry residues were acetylated with pyridine and acetic anhydride (1:1, 1 ml) at 100°C for 1 h. The excess acetylating reagents were removed by co-distillation with toluene, and the dry residues were dissolved in chloroform and partitioned with water. The chloroform layer was collected and analysed by GLC (12), using an SE-54 fused silica capillary column (25 m X 0.2 mm i.d.) at 160-220°C, 2 min, 2°C min^{-1}. The peak areas were integrated by a LKB 2220 computing integrator.

Similar hydrolyses of all the polysaccharides and oligosaccharides were carried out and the constituent sugars were analysed by GLC as their alditol acetates as described above.

RESULTS

Delipidation followed by gel filtration (Table I) showed that the LPS of all the mutant strains except PSD-40 (without the 140, 6, and 2 Mda plasmid) contained O-antigen polysaccharide, whereas the latter gave only an oligomeric material.

Table I. Sugar analyses of the LPS samples of *Shigella dysenteriae* type 1 strains

Strains	Plasmid profile (Mda)	Relative mole percent of the sugars*				
		Rha	Glc	Gal	GlcNAC	Hep
Z-24623Φ	140,6,4,2	52	–	36	12	+
PSD-1	2	16	21	60	3	+
PSD-20	6	53	–	29	5	+
PSD-30	140,90	–	–	83	17	+
PSD-40	90	8	20	50	6	16
PSD-50	6,2	45	–	45	12	+
PSD-60	140,6	38	–	50	12	+

* Rha = rhamnose, Glc = glucose, Gal = galactose, GlcNAC = 2-acetamido-2-deoxy glucose, Hep = Heptose.
Φ Control strain
+ indicates a small amount not included in integration.

Sugar analysis of the LPS of the *S. dysenteriae* type 1 strain containing a different combination of plasmids (Table II), showed that rhamnose, galactose, and 2-acetamido-2-deoxyglucose (commonly known as *N*-acetyl glucosamine) were the main sugar constituents of all the LPS samples. In addition, all the LPS samples contained heptose in small amounts, except PSD-40, in which it was present to the extent of 16%. LPS from PSD-1 and PSD-40 showed the presence of 21% and 20% glucose, respectively. The hydrolysates were also analysed by paper chromatography and no acidic sugar (uronic acid) was detected.

Analysis of the PS of the O-antigen showed that the virulent strain (Z-24623) contained rhamnose, galactose and 2-acetamido-2-deoxyglucose. The polysaccharide material from the mutant strains PSD-20, PSD-50 and PSD-60 had the same sugar constituents (rhamnose, galactose, D-GlcNAC), but minor differences were noted in the relative percentage of the sugars (Table II) from those of the virulent strain. However, PSD-1 and PSD-30 had incomplete O-antigen and neither contained

rhamnose. In addition, PSD-1 contained glucose.

The LPS of PSD-40 had no O-antigen and the carbohydrate material eluted from the column was derived from the core having glucose, galactose, heptose, and trace amounts of rhamnose. it was devoid of glucose. However, glucose may be a component of the core of the LPS. On the other hand, the presence of glucose in the core oligosaccharide of PSD-40 is not unusual since glucose is known to be present in some cores.

Table II. Association of plasmid profile and sugar constituents of O-antigen polysaccharides/core carbohydrates of *Shigella dysenteriae* type 1 strains

Strain	Plasmid profile (Mda)	Relative mole percent of sugars in O-antigen (PS)					Nature of O-antigen
		Rha	Glc	Gal	GlcNAC	Hep	
Z 24623	140,6,4,2	55	–	36	9	+	Complete
PSD-1	2	–	23	56	21	+	Reduced
PSD-20	6	53	–	33	14	+	Complete
PSD-30	140,90	–	–	84	16	+	Reduced
PSD-40*	90	trace	29.4	41.2	–	29.4	No O-antigen
PSD-50	6,2	52	–	36	11	+	Complete
PSD-60	140,6	53	–	39	7	+	Complete

*Sugars derived from the core oligosaccharide.
+ = Present in small amount not included in integration.

DISCUSSION

The presence of an O-antigen polysaccharide in the *S. dysenteriae* type 1 strain containing the 140, 6, and 2 Mda plasmids either singly or in combination, and its absence in the PSD-40, which contained none of these plasmids, indicated that they might have some involvement in the synthesis of the O-antigen. This finding was consistent with the results obtained previously (5), when it was shown that mutants containing these plasmids contained either a complete or reduced number of regularly spaced LPS bands on SDS-PAGE. In PSD-40 only the core oligosaccharide without any O-antigen repeating unit was detected.

Sugar constituents of the O-antigen polysaccharide of the virulent strain Z-24623 correlates with that reported earlier (13). The 6 Mda plasmid has been shown (2) to be necessary for the complete synthesis of the O-antigen, and we have also found that in the presence of 6 Mda plasmid, PSD-20, PSD-50 and PSD-60 had the same sugar constituents as the virulent strain. However, the variation in the proportion of the constituent sugars in these mutant strains was not surprising since they did not contain the same combinations of the core plasmids.

Absence of the sugar rhamnose in PSD-1 and PSD-30 correlates with the results of the SDS-PAGE which also showed the reduced amount of high molecular-sized O-antigen repeating units. Thus, it again suggests that the 2 and 140 Mda plasmids might have some role in the expression of LPS of this serotype, as was shown earlier (5).

The presence of glucose in PSD-1 was unexpected, since this sugar was found neither in the LPS nor in O-antigen PS of the other strains, except the PSD-40 oligosaccharide. Possible contamination from the medium was ruled out since a control sugar analysis of the medium showed that Plasmid analysis of these two strains showed that PSD-40 contained none of the core plasmids, whereas PSD-1 contained only a small 2 Mda plasmid. This suggests that the presence of glucose may be an indication of a change in the biosynthesis of the LPS occurring due to the absence of the other two core plasmids. Thus, it is evident from the foregoing results and discussion that the prepared mutant strains with the variation of plasmids had incomplete or no O-antigen polysaccharide and thereby lost their virulence character. These results regarding the constituent sugars of LPS and O-antigen PS fractions can also be used for ascertaining the virulence/mutant character of *S. dysenteriae* type 1 strains.

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