Role of plasmids in virulence-associated attributes and in O-antigen expression in *Shigella dysenteriae* type 1 strains

K. HAIDER, A. K. AZAD, F. QADRI, S. NAHAR and I. CIZNAR

International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B), GPO Box 128, Dhaka 1000, Bangladesh

Summary. The association of plasmids with virulence characters and O-antigen expression was studied in two virulent and seven avirulent mutant strains of *Shigella dysenteriae* type 1. Deletion of a 12-Mda segment from a 140-Mda plasmid in two smooth avirulent mutants made the derivatives avirulent in the Sereny test and non-invasive in HeLa cells. The mutants were unable to bind Congo red, and did not express the virulence marker antigen. Mutants completely lacking the 140-Mda plasmid also showed similar avirulent characters. However, rough mutants retained the ability to bind Congo red. Our results indicate that the essential gene(s) for virulence may be located on the 140-Mda plasmid, a small deletion from which may lead to avirulence. This deletion did not affect the protein antigen expression nor change their antigenicity. Analysis of lipopolysaccharide (LPS) patterns showed that strains containing the 6-Mda plasmid produced the complete LPS and were smooth, whereas strains containing either the 140- or a 4- or 2-Mda plasmid, in the absence of the 6-Mda plasmid, produced smaller amounts of O antigen and were rough. Western-blot analysis and crossed immuno-electrophoresis gave similar results. The 140-, 4-, or 2-Mda plasmid, in the absence of the 6-Mda plasmid, may code for non-specific galactosyl transferase-like activity which can add, non-specifically and at a reduced level, the galactose residue (the first sugar in the O antigen repeat unit) to the LPS core. This permits the completion of the O-antigen side chain in the absence of the *rfp* gene (present in the 6-Mda plasmid) which encodes the specific galactosyl transferase involved in O-antigen biosynthesis. Thus, our findings indicate that the 6-Mda plasmid of *S. dysenteriae* type 1 has an important role in the synthesis of the O-antigen side chains of LPS. However, the 140-, 4- and 2-Mda plasmids may also be indirectly responsible for these properties, but this may be less pronounced in the absence of the 6-Mda plasmid.

Introduction

Recently we reported¹ the presence of four plasmids (140, 6, 4, and 2 Mda), in a significant number of *Shigella dysenteriae* type 1 strains from widely scattered geographic locations. Our findings indicated that these plasmids may play a role in certain virulence-associated characters in this serotype of *Shigella*. We isolated mutant strains that contained only one of the four plasmids.

Recently, there has been considerable interest in the role of plasmids in the biological properties of shigellae. Until 1980, no plasmids, other than resistance plasmids, were thought to play any role

in the virulence of dysentery bacilli. Studies over the past 8 years have demonstrated that the ability of shigellae^{2–4} and enteroinvasive *Escherichia coli* (EIEC)⁵ to invade epithelial cells is plasmid-mediated. Hale *et al.*⁶ demonstrated that synthesis of outer-membrane proteins was plasmid-mediated. Seven of these proteins appeared to be associated with the virulent phenotype in both *S. flexneri* and EIEC. Recent studies have shown that plasmids may also be involved in expression of lipopolysaccharide (LPS) in *Salmonella*⁷ and in *E. coli*.⁸ Similarly, the expression of LPS has been shown to be mediated by plasmids in several *Shigella* spp.^{9,10} and by chromosomal genes in others.^{10,11}

In this study we report the association between the presence of four plasmids in *S. dysenteriae* type

1 strains and (1) the expression of particular virulence characters—agglutination, colony morphology, Congo red binding, production of outer-membrane protein (OMP), toxin production, presence of virulence marker antigen, invasiveness—and (2) LPS expression.

Materials and methods

Bacterial strains

Two virulent and seven avirulent strains of *S. dysenteriae* type 1 were used in the study (table). The virulent strains were isolated from patients at the ICDDR, B, Dhaka treatment centre. The avirulent strains were derived from the virulent strains by repeated subculturing (approximately seven times) on MacConkey Agar (Difco). Loss of virulence was confirmed by the Congo red binding assay, the Sereny test, and HeLa-cell infectivity as described below. The roughness of the avirulent mutants was ascertained by autoagglutination in physiological saline. Non-pathogenic *E. coli* strain C-600 was obtained from the Centers for Disease Control (Atlanta, GA, USA) and enteroinvasive *E. coli* strain BH-16-1 was from the Armed Forces Research Institute of Medical Services, Bangkok, Thailand. All these strains were stored at -70°C in Trypticase Soy Broth (TSB; Gibco), with glycerine 15%.

Strains were subcultured on Trypticase Soy Agar (TSA) and MacConkey Agar and were identified serologically by slide agglutination tests with *S. dysenteriae* type 1 antiserum (Wellcome).¹²

Congo red binding assay and salt aggregation test

Strains were streaked on TSA with Yeast Extract (Gibco) 0.6% containing Congo red (Fisher Scientific Co., NJ, USA) 0.003%. Plates were incubated at 37°C for 18 h to differentiate between pigmented (Pcr^{+}) and non-pigmented (Pcr^{-}) colonies as described previously.^{13,14} The salt aggregation test (SAT) for hydropho-

bicity was performed with growth taken from TSA;¹⁴ aggregation was determined in ammonium sulphate solutions of various molarity.

Sereny test

For Sereny's test of virulence, 20 μl of a suspension of *S. dysenteriae* type 1 containing 3×10^8 cfu/ml was inoculated into the eye of adult guinea pigs.¹⁵ Strains that produced keratoconjunctivitis within 72 h were considered to be virulent.

Invasion of HeLa cells

Broth cultures (18 h) of all strains, adjusted to a concentration of 2×10^8 cfu/ml, were used to infect non-confluent HeLa cell monolayers for determination of invasive ability by the method of Hale *et al.*¹⁶

Analysis of plasmid DNA

Plasmid DNA was extracted from the strains by a modification of the method of Birnboim and Doly.¹⁷ The plasmids were separated by electrophoresis for 2 h at 30 mA in agarose 0.7%.¹⁸ Preparations of plasmid DNA from each virulent *S. dysenteriae* type 1 strain were electrophoresed, either singly or mixed with DNA from its avirulent isogenic pair to detect the deletion in the large (140 Mda) plasmid, for 4 h at 30 mA in agarose 0.7%. Reference marker plasmids of pDK-9 (140, 105, 2.7, and 2.0 Mda), R1 (62 Mda), RP4 (36 Mda) and Sa (23 Mda) were used to determine the M_r of the plasmid DNA of *S. dysenteriae* type 1 strains.

Modified ELISA for detecting virulent *Shigella* strains

Virulence marker antigen (VMA) was detected by the ELISA method as modified by Pal *et al.*¹⁹ Virulent and avirulent strains were distinguished by tests with an absorbed immune serum that contained ELISA-reactive

Table. Plasmid content and virulence characters of *S. dysenteriae* type 1 strains

Strain no.	Plasmid profile (Mda)	Colony morphology	SAT value	Congo red binding	Sereny test	VMA production	Shiga toxin
Z-24623	140, 6, 4, 2	Smooth	1.5	+	+	+	+
Z-24623-1	128, 6, 4, 2	Smooth	1.5	—	—	—	+
Z-26406	140, 6, 4, 2	Smooth	1.5	+	+	+	+
Z-26406-1	128, 6, 4, 2	Smooth	1.5	—	—	—	+
PSD-1	2	Semi-rough	0.25	+	—	—	+
PSD-10	4, 20	Semi-rough	aa	+	—	—	+
PSD-20	6	Smooth	1.0	—	—	—	+
PSD-30	140, 90	Semi-rough	aa	+	—	—	+
PSD-40	90	Rough	aa	+	—	—	+

aa, Autoagglutination in physiological saline, i.e., rough strain.

antibodies specific for a possibly unique antigen site (VMA) of the virulent strains.

Toxin assay

Overnight bacterial growth on heart-infusion agar slopes was washed with 1 ml of polymyxin B sulphate (Sigma) 1 mg/ml in 0.01 M phosphate-buffered saline, incubated for 15 min at 37°C and centrifuged in a microfuge for 10 min. The supernates were diluted 1 in 10 and 1 in 100 in cell-culture media and 0.1 ml amounts of the dilutions were inoculated into the wells of a microtitration plate containing a 24-h culture of HeLa cells.²⁰ The treated cell cultures were incubated overnight and examined microscopically for cell death, indicative of toxin production by the *Shigella* strain.

Outer-membrane protein (OMP) extraction

Shigella strains were grown in 100 ml of TSB containing yeast extract 0.3% at 37°C for 18 h. After centrifugation, the bacterial pellets were suspended in 80 ml of deionised water and incubated at room temperature for 1 h with slow shaking (60 rpm on a rotary shaker) according to the method described previously.²¹ After removal of bacterial cells by centrifugation at 16 000 *g* for 20 min, the supernate was again centrifuged at 100 000 *g* for 2 h. The clear supernate was dialysed against buffer, concentrated by dialysis against polyethylene glycol (PEG 15 000–20 000) and stored at –20°C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of OMPs

Isolated OMPs were analysed by SDS-PAGE²² with separating gels containing acrylamide 13.5% and stacking gels containing acrylamide 5%, and with electrophoresis at a constant current of 10 mA per gel overnight. Gels were stained with Coomassie Brilliant Blue R-250 0.2% for the demonstration of proteins.

Extraction and analysis of LPS

LPS was extracted from the strains by a modified phenol-water extraction procedure²³ and purified by ultracentrifugation at 105 000 *g* for 4 h. Purified preparations of LPS were analysed by SDS-PAGE in polyacrylamide 12% gels.²² LPS components separated in the gel were visualised by the silver staining technique of Hitchcock and Brown.²⁴

Preparation of antiserum

S. dysenteriae type 1 strain Z-24623 was grown in 100 ml of TSB for 18 h at 37°C. The bacterial cells were harvested by centrifugation at 8000 *g*, washed in PBS (pH 7.4), and suspended in 10 ml of distilled water. Whole-cell extracts were prepared by shaking the pellets with glass beads at high speed, on a rotary shaker, for 1 h at room temperature. The suspension was then sonicated

for three 1-min bursts, at intervals of 30 s, in an ultrasonic disintegrator (Fisher Sonic Dismembrator, Model 300, Fairlawn, NJ, USA). Two adult albino rabbits of 2 kg average weight were immunised with the preparation. The first dose, equivalent to 10⁶ cells, was given intravenously; after 1 week, four doses (10⁷, 5 × 10⁷, 10⁸, and 5 × 10⁸ cells) were given at 2-day intervals and a last dose of 10⁹ cells was given after another 1-week gap. The titre of the sera was determined and a further booster injection was given if required. Serum was collected one week after the last dose, pooled, divided into 1-ml volumes and stored at –20°C.

Immunoblotting

OMPs and LPS, after separation on SDS-polyacrylamide gels, were separately transferred to nitrocellulose membranes.²⁵ The polyclonal antisera, raised in rabbits against whole-cell extracts of the *S. dysenteriae* type 1 strain, were diluted 1 in 500 and incubated, separately, with OMPs and LPS on the nitrocellulose sheet for 2 h at 37°C. OMP and LPS antigens were detected with anti-*S. dysenteriae* type 1 (Z-24623) rabbit serum. Swine anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Dakopatts, Denmark) was used as secondary antibody. Alkaline phosphatase activity was detected with naphthol AS-MX phosphate (2 g/L) and Fast Red TR Salt (Sigma) (1 g/L) in 50 mM Tris-HCl buffer, pH 8.5.

Crossed immuno-electrophoresis

Crossed immuno-electrophoresis was performed by the method of Kroll.²⁶

Results

Plasmid profile, colony morphology and SAT values

The virulence characters of the strains are summarised in the table. The two virulent strains (Z-24623 and Z-26406) each contained four plasmids of 140, 6, 4, and 2 Mda (fig. 1). Their avirulent isogenic pairs each had a small deletion of *c.* 12-Mda from the 140-Mda plasmid (fig. 2). Strains containing either all four plasmids or the 6-Mda plasmid alone (PSD-20) were smooth in physical appearance, did not autoagglutinate in physiological saline, and had SAT values of 1.0 or 1.5 M. Strains without the 6-Mda plasmid were either semi-rough (PSD-1, 10, 30) or completely rough (PSD-40) in appearance and either autoagglutinated in physiological saline or showed greater hydrophobicity (SAT value 0.25 M for strain PSD-1).

Congo red binding and virulence characters

Smooth strains, possessing all plasmids, bound Congo red and produced distinct red colonies after

Fig. 1. Plasmid DNA patterns of *S. dysenteriae* type 1 strains electrophoresed for 2 h at 30 mA in agarose 0.7% gel: lanes A and K contain marker plasmids; B, virulent strain Z-24623; C-G, avirulent mutant strains; H, avirulent strain Z-24623-1; I, virulent strain Z-26406; J, avirulent strain Z-26406-1.

Fig. 2. Electrophoretic patterns of plasmid DNA from virulent and avirulent isogenic pairs of *S. dysenteriae* type 1 strains electrophoresed for 4 h at 30 mA in agarose 0.7% gel: lanes A and D, avirulent strains Z-24623-1 and Z-26406-1, respectively; B and F, virulent strains Z-24623 and Z-26406; C, plasmid DNA from strains Z-24623 and Z-24623-1; E, plasmid DNA from strains Z-26406 and Z-26406-1; G, marker plasmids R1, Sa, RP4; H, marker plasmid pDK-9.

growth on Congo red agar. However, smooth strains with partial or complete deletion of the 140-Mda plasmid had distinct white colonies. Rough strains with or without the 140-Mda plasmid also bound Congo red. Only the smooth red colonies contained the VMA, gave positive results in Sereny's test of virulence and were invasive for HeLa cells; the smooth white colonies and rough red colonies gave negative results in both tests. All strains, whether they were smooth or rough, red or white, Sereny positive or negative, produced Shiga toxin.

Fig. 3. OMPs extracted from avirulent strains of *S. dysenteriae* type 1 (lanes A–D) and from a virulent strain (lane E) separated by SDS-PAGE in a 13.5% gel and stained with Coomassie Brilliant Blue. Polypeptides are present in almost equal quantities in all strains except PSD-10 (lane B). Marker proteins are shown in lane F.

Analysis of OMPs

SDS-PAGE of OMPs showed the presence of several (≈ 30) polypeptides of 10–>100 Kda (fig. 3). The OMP profile of strain PSD-10 that lacked the “core plasmids”,¹ i.e., those of 140, 6 and 2 Mda, showed both qualitative and quantitative differences (fig. 3; lane B) from the virulent parent strain. Other mutants did not show such a clear difference. No change in the antigenic profile was observed (fig. 4) as a result of the small deletion of the 12-Mda segment from the 140-Mda plasmid or complete deletion of the 140-Mda plasmid, although there was a change in their virulence properties.

Analysis of LPS

SDS-PAGE of LPS showed O-side chain repeat units. These were present in all smooth strains that contained the 6-Mda plasmid (fig. 5; lanes A and B). However, smaller numbers of regularly spaced LPS bands were also observed (fig. 5; lanes C–E) in strains that did not contain the 6-Mda plasmid. These strains contained the 2-, 4- or 140-Mda plasmid. In contrast, in the rough strain PSD-40, which contained none of the four plasmids, only

Fig. 4. Immunoblot analysis of OMPs extracted from avirulent strains PSD-1 (A), 10 (B), 20 (C) and 30 (D) and from the virulent *S. dysenteriae* type 1 strain Z-24623 (E). The OMPs were transferred to nitrocellulose and detected with a polyclonal rabbit antiserum raised against strain Z-24623.

Fig. 5. SDS-PAGE of LPS from *S. dysenteriae* type 1 strains carrying different plasmids. LPS bands were visualised by staining with silver stain (Bio-Rad). Lanes A and B, complete synthesis of LPS by strains Z-24623 and PSD-20; C-E, reduced quantities of O-repeat unit of LPS in strains PSD-30, PSD-10 and PSD-1, respectively; F, PSD-40 showing presence of core polysaccharide but absence of O-side chains.

core polysaccharide without any O-side chain repeat units was detected (fig. 5; lane F).

In Western blots, a characteristic pattern of complete O-side chain of LPS was recognised by rabbit anti-*S. dysenteriae* Z-24623 sera in strains containing the 6-Mda plasmid (fig. 6). However, strains PSD-1, PSD-10 and PSD-30 showed O-side chain repeat units but in much reduced amount. None of these oligosaccharide bands was observed in strain PSD-40 (fig. 6).

Crossed immuno-electrophoresis

Analysis of LPS by crossed immuno-electrophoresis with rabbit anti-*S. dysenteriae* Z-24623 serum showed the presence of a band of low mobility in strains Z-24623, Z-24606 and PSD-20 which is characteristic of LPS.²⁷ However, LPS from other strains, except PSD-40, showed a relatively faint precipitin band. No band was detected with LPS from PSD-40.

Discussion

Genetic analysis of *Shigella* spp. has shown that plasmids,^{3,9} as well as chromosomal loci,^{10,11} play

Fig. 6. Immunoblot analysis of LPS from strains Z-24623 (A), PSD-20 (B), PSD-30 (C), PSD-10 (D), PSD-1 (E) and PSD-40 (F). LPS bands were determined by electroblotting to nitrocellulose and detected with a polyclonal antiserum raised against strain Z-24623.

an important role in virulence properties. Evidence gathered over the past few years has demonstrated that large plasmids of 120–140 Mda are involved in the invasive process⁴ in all shigellae. Our study has indicated that a small segment of the 140-Mda plasmid in *S. dysenteriae* type 1 is involved in the invasive process and in the production of VMA. Other plasmids, i.e., those of 6, 4, and 2 Mda, which are present in significant numbers of *S. dysenteriae* type 1 strains¹ may not play any direct role in invasion or in VMA production.

The role of Shiga toxin in the pathogenesis of dysentery is not yet completely understood. However, it is probably an essential component of virulence. Little information is available on the genetics of Shiga toxin production but the genes for this toxin in *S. dysenteriae* type 1 and the Shiga-like toxin of *S. flexneri* appear to be located on the chromosome. The production of toxin by the plasmid-less or plasmid-deficient mutants of *S. dysenteriae* type 1 in our study also indicates that plasmids might not have any direct role in toxin production.

Previous studies in this laboratory have shown that Congo red binding may reflect an arrangement of cell-surface components.¹⁴ The presence of the

O-side chain of LPS is essential if the Congo red binding assay is to be used as an indicator of virulence. To understand the contribution of the O-side chain of LPS to Congo red binding, we have compared the binding ability of avirulent rough mutants of *S. dysenteriae* type 1 with that of the avirulent smooth strains, in both cases with or without the 140-Mda plasmid. We have found that the rough strains, with or without the 140-Mda plasmid, do bind Congo red whereas the smooth strains do not. Experiments also indicated that the O-side chains represent an important factor determining hydrophobicity of the cell, as indicated by the SAT.¹⁴ This was substantiated by the observation that rough mutants lacking antigens had much greater hydrophobicity than the equivalent smooth strain and were auto-agglutinating. The genetic locus encoding Pcr⁺ phenotype has been cloned and expressed in *E. coli* and has been found to be present in the 140-Mda plasmid.^{28,29} Absence of O-antigen may cause more exposure of outer-membrane and thus, probably, make the rough strains more hydrophobic and allow binding to Congo red. The role of O-antigen in virulence is not fully understood, but smooth LPS may be important both for intra-luminal survival of shigellae and for protection of the bacteria in tissue from the bactericidal activity of serum.

We have found a significant difference between the OMP profile of one of the rough mutants (PSD-10, containing 4- and 20-Mda plasmids) and that of the parent strain. Some minor differences of the other OMPs obtained from the three rough mutants of *S. dysenteriae* type 1 were also seen, although the major polypeptides were similar. Analysis of the OMPs by Western blotting showed that the avirulent mutants, either rough or smooth strains, did not have decrease antigenicity.

OMPs in strains of *S. flexneri*, *S. sonnei* and *E. coli*⁶ have been shown to influence virulence properties. The loss of major polypeptides led to loss of the invasive potential of the bacteria. However, the results obtained with mutants of *S. dysenteriae* type 1, show that loss of virulence does not result in loss of antigenicity of the OMPs. It is possible that, in *S. dysenteriae* type 1, the gene essential for the invasive process and VMA production may not be essential for the synthesis of the major polypeptides.

Plasmid-mediated synthesis of LPS in the Enterobacteriaceae has been described before.⁸ The involvement of a small, 6-Mda plasmid in expression of LPS in *S. dysenteriae* type 1⁹ has been shown previously. We have demonstrated that, although the 6-Mda plasmid of *S. dysenteriae* type 1 is

essential for the synthesis of intact LPS, other plasmids, i.e., those of 140, 4 and 2 Mda, might have some role in the expression of LPS of this serotype. It has been shown that when the *rfb-rfp* cassette is expressed in strains of *Salmonella* and *E. coli*, reduced levels of O-antigen are expressed.³⁰⁻³² The most likely explanation for this observation is that there are several housekeeping functions involved in galactose metabolism and some of these are shared (with relaxed specificity) not only for expression of O-antigen but also for core biosynthesis. Similar functions can, non-specifically and at a reduced level, add the galactose residue to the LPS core. This permits the completion of the O-antigen side chain in the absence of the *rfp* gene which encodes the specific galactosyl-transferase involved in O-antigen biosynthesis. Therefore, on the basis of previously published information³⁰⁻³² and our data, the 140-, 4- and 2-Mda plasmids most likely encode a non-specific galactosyl transferase-like activity when the gene for O-antigen biosynthesis is absent, i.e., the 6-Mda plasmid or the *rfp* gene. The 6-, 4- and 2-Mda plasmids are unique in *S. dysenteriae* type 1 and are not breakdown products of the 140-Mda plasmid.¹

In our study we did not determine how these plasmid-encoded factors are involved in O-side chain expression. It has been demonstrated that chromosomal determinants are important for LPS expression. However, the complex mechanism regulating assembly of LPS in the outer membrane may be under the influence of plasmid-determined factors. Watanabe and Timmis⁹ demonstrated that a small, 6-Mda plasmid in *S. dysenteriae* type 1 is involved in expression of somatic antigen, although other chromosomal factors may be needed as well.

Diminished reactivity of rabbit anti-*S. dysenteriae* Z-24623 sera with the LPS from strains containing the 140-, 4- or 2-Mda plasmid was seen and this may be caused by the decrease in the number of O-side chains per core. Other factors may be involved as well and this deserves further investigation.

From our study, it appears that the 140-, 4- and 2-Mda plasmids of *S. dysenteriae* type 1 strains also may have some involvement in the synthesis of the O-antigen. The strains may be deficient in the gene coding for an enzyme responsible for adding the first sugar of the O-antigen repeat unit to the LPS core. It is also possible that genes may encode factors that regulate chromosomal determinants of the LPS. However, detailed biochemical and genetic studies are required to understand the mechanisms.

As observed by other investigators,^{2-5,9} we also

feel that the 6- and 140-Mda plasmids are most important for the virulence potential of *S. dysenteriae* type 1 strains. However, other plasmids, such as those of 4 and 2 Mda, may also have an important role to play in the pathogenicity of this serotype although the effect may be less pronounced.

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