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Congo Red Binding and Salt Aggregation as Indicators of Virulence in *Shigella* Species

FIRDAUSI QADRI,[1]* SHAIKH ABU HOSSAIN,[1] IVAN ČIŽNÁR,[1] KHALEDA HAIDER,[1] ÅSA LJUNGH,[2] TORKEL WADSTROM,[2] AND DAVID A. SACK[3]

International Centre for Diarrhoeal Disease Research, Bangladesh, GPO Box 128, Dhaka 1000, Bangladesh[1]; University of Lund, Lund, Sweden[2]; and Division of Geographic Medicine, School of Public Health, The Johns Hopkins University, Baltimore, Maryland 21205[3]

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Smooth strains of *Shigella dysenteriae* type 1, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei* which form pigmented colonies (Pcr^+) on Congo red agar were virulent in the Sereny test. Smooth variants unable to bind Congo red (Pcr^-) were avirulent. Measurements of dye uptake from solution showed that *S. dysenteriae* type 1 bound the most dye, followed in order of uptake by *S. flexneri*, *S. boydii*, and *S. sonnei*. Using the salt aggregation test (SAT) to determine cell surface hydrophobicity, we found the same order of species. The SAT could not, however, detect differences in surface properties between Pcr^+ and Pcr^- pairs of isogenic smooth strains. Enteroinvasive *Escherichia coli* strains used in the study showed SAT and Congo red-binding properties which were similar to those of the *S. flexneri* strains. A direct correlation was found between pigment-binding ability and the presence of the large 140-megadalton plasmid in *S. flexneri*, enteroinvasive *E. coli*, and *S. boydii* but not in *S. dysenteriae* type 1 or *S. sonnei* strains. Congo red interacted with outer membranes and outer membrane proteins of *S. dysenteriae* type 1 but not with lipopolysaccharides. However, rough mutants of *Shigella* species deficient in lipopolysaccharides bound Congo red and formed pigmented colonies, showing that dye binding as a virulence assay may be misinterpreted in such cases. There was complete correlation of the Pcr^+ phenotype with virulence in the smooth strains in this study, suggesting that Congo red binding can be utilized as a quick and reliable alternative to the Sereny test.

Adherence and binding of enteropathogenic organisms to cells of the gastrointestinal tract are believed to be important processes in the pathogenesis of diarrheal diseases (18, 32). For this reason, the outer envelopes of these bacteria are now being intensively studied. In certain pathogens, virulent strains have been found to be more hydrophobic than the avirulent strains (18, 33, 36). Among important tests used to study the surface properties of bacteria, Congo red binding (38) and aggregation in salt solutions (22) are commonly used as markers of hydrophobicity. In several reports, Congo red binding has been linked directly to virulence and pathogenicity, for example, in *Shigella flexneri* (25), *Yersinia pestis* (38), *Yersinia enterocolitica* (2), *Aeromonas salmonicida* (15), and *Neisseria meningitidis* and *Vibrio cholerae* (31). Studies of the salt aggregation test (SAT) with different species of the family *Enterobacteriaceae* (9) as well as with strains of *Staphylococcus* species (33) have yielded similar results. A marked cell surface hydrophobicity has been found to be related to an increased capacity for *Escherichia coli*, *Salmonella* species, and strains of *S. flexneri* to adhere to host cells in experimental animals (23, 36).

Bacillary dysentery is associated with four species of *Shigella*: *S. dysenteriae*, *S. sonnei*, *S. flexneri*, and *S. boydii*, as well as with strains of enteroinvasive *E. coli* (3). The mechanism of pathogenicity of these organisms is not well understood, although a number of chromosomal as well as plasmid-mediated factors have been shown to be required for the expression of virulence (10, 13, 19, 35). Genetic studies suggest that in strains of *S. flexneri*, a 140-megadalton (MDa) plasmid is required for invasiveness. It has also been shown that this plasmid contains genes which are essential for Congo red binding (25). Although the biochemical and physical mechanisms involved in determining the virulence of *Shigella* strains remain unclear, it is evident that alterations on the surfaces of cells arising either from a deficiency of proteins (1, 14, 24) or from changes in the structure of the O antigen (11, 19, 40) may lead to a loss of the affinity of a strain for mucosal epithelial cells.

The aim of this study was to examine the relationship between the virulence of different *Shigella* species and their surface hydrophobicity determined by their ability to bind Congo red or aggregate in salt solution.

MATERIALS AND METHODS

Bacterial strains and media. The strains used for Congo red binding and the SAT are listed in Table 1. Nonpathogenic *E. coli* 36000 and *E. coli* strains used as standards for molecular mass estimations of plasmids were obtained from the Centers for Disease Control (Atlanta, Ga.). Enteroinvasive *E. coli* L-115-A3 and BH-23-1 were obtained from the Armed Forces Research Institute of Medical Services (Bangkok, Thailand). All other bacterial strains were isolated from patients at the treatment center of the International Centre for Diarrhoeal Disease Research, Bangladesh (Dhaka) and were stored at −70°C in tryptic soy (TS) broth (GIBCO Diagnostics, Madison, Wis.) containing 15% glycerol. To provide organisms for the experiment, we subcultured all strains on MacConkey agar (Difco Laboratories, Detroit, Mich.) or TS agar containing 0.6% yeast extract (GIBCO Diagnostics). A virulent strain of *S. dysenteriae* type 1 (no. 26406) was used to identify components of the cell surface responsible for binding Congo red.

Congo red binding. TS broth containing 0.6% yeast extract with 1.5% agar and 0.003% Congo red (Fisher Scientific Co., Fair Lawn, N.J.) was used to study the pigment-binding ability of strains (25). The plates were incubated at 37°C for

* Corresponding author.

TABLE 1. Congo red binding and salt aggregation characteristics of strains studied

Species (serotype)[a]	Strain	SAT [M $(NH_4)_2SO_4$][b]	Congo red binding ($\mu g/10^{10}$ cells)[c]
Shigella dysenteriae type 1	24623	1.5	15.0
Shigella dysenteriae type 1	8447	1.5	15.0
Shigella dysenteriae type 1	5613	1.5	15.0
Shigella dysenteriae type 1	7112	1.5	15.0
Shigella dysenteriae type 1	3351	1.5	18.0
Shigella dysenteriae type 1	3933	1.5	18.0
Shigella dysenteriae type 1	7249	1.5	18.5
Shigella dysenteriae type 1	12027	1.5	18.5
Shigella dysenteriae type 1	12694	1.5	19.0
Shigella dysenteriae type 1	26406	1.5	19.0
Shigella dysenteriae type 1	13757	1.5	20.5
Shigella dysenteriae type 1	8682	1.5	21.0
Shigella dysenteriae type 1	26508	1.5	21.0
Shigella dysenteriae type 1	19858	1.5	22.0
Shigella dysenteriae type 1	13305	1.5	23.5
Shigella dysenteriae type 1	7828	1.5	26.0
Shigella dysenteriae type 1	7920	1.5	26.0
Shigella dysenteriae type 1	14450	1.5	31.0
Shigella dysenteriae type 1	9357	1.5	39.0
Shigella dysenteriae type 1	16770	1.5	39.0
Shigella dysenteriae type 1	25812	1.5	39.5
Shigella dysenteriae type 1	R-40-17	1.5	42.0
Shigella flexneri type Y	26817	2.0	6.0
Shigella flexneri type 2a	611	2.0	7.5
Shigella flexneri type 1a	15987	2.0	7.5
Shigella flexneri type 3b	9471	2.0	7.5
Shigella flexneri type 1b	613	2.0	8.0
Shigella flexneri type 2a	A-18	2.0	8.0
Shigella flexneri type 2a	4668	2.0	10.0
Shigella flexneri type 2b	Z-1224	3.0	10.0
Shigella flexneri type 2a	10265	2.0	12.0
Shigella flexneri type 2a	13244	2.0	13.0
Shigella flexneri type 3a	A-6	1.0	13.0
Shigella flexneri type 2a	4986	2.0	14.5
Shigella flexneri type 2a	A-17	2.0	15.0
Shigella flexneri type 1a	1095	2.0	15.0
Shigella flexneri type 2a	31484	2.0	15.0
Shigella flexneri type 2a	20226	2.0	30.5
Shigella boydii type 1-6	33744	2.5	2.0
Shigella boydii type 7-11	10538	2.5	2.0
Shigella boydii type 7-11	3601	2.5	2.0
Shigella boydii type 1-6	27312	2.5	6.0
Shigella boydii type 7-11	13601	2.5	6.5
Shigella boydii type 7-11	13143	2.5	9.0
Shigella boydii type 12-15	7879	1.5	9.0
Shigella boydii type 12-15	10426	1.5	9.0
Shigella boydii type 12-15	11340	1.5	9.0
Shigella boydii type 12-15	10854	1.5	10.0
Shigella boydii type 7-11	10307	2.5	12.0
Shigella boydii type 1-6	19783	2.5	13.0
Shigella boydii type 7-11	16446	2.5	15.5
Shigella boydii type 7-11	13618	2.5	20.0
Shigella sonnei form 1 (smooth)	6312	3.0	0.0
Shigella sonnei form 1 (smooth)	6570	3.0	2.0
Shigella sonnei form 1 (smooth)	6577	3.0	4.0
Shigella sonnei form 1 (smooth)	6142	3.0	8.0
Shigella sonnei form 1 (smooth)	6363	3.0	8.0

Continued on following page

18 h to differentiate between pigmented (Pcr^+) and nonpigmented (Pcr^-) colonies. Autoagglutination in normal saline was routinely carried out to detect rough strains.

To study Congo red binding from solution, we grew strains in TS broth containing 0.6% yeast extract at 37°C for 18 h. The cells were collected by centrifugation at 6,000 × *g*, washed in phosphate-buffered saline, and recentrifuged. Cells were incubated for 10 min at room temperature in 3 ml of phosphate-buffered saline containing 50 μg of Congo red. A preliminary experiment with various amounts of cells, ranging from 1×10^9 to 1.2×10^{10} cells per ml, indicated that a concentration of 1×10^{10} cells per ml was optimum for these studies. Cells were removed by centrifugation at 27,000 × *g* for 30 min, and the amount of Congo red remaining in the supernatant was determined by measuring the A_{480} (15). Cell-free solutions of Congo red were used as

TABLE 1—*Continued*

Species (serotype)[a]	Strain	SAT [M $(NH_4)_2SO_4$][b]	Congo red binding ($\mu g/10^{10}$ cells)[c]
Shigella sonnei form 1 (smooth)	13020	3.0	9.0
Shigella sonnei form 2 (rough)	16643	aa	26.0
Shigella sonnei form 2 (rough)	14260	aa	27.0
Shigella sonnei form 2 (rough)	19784	aa	32.0
Shigella sonnei form 2 (rough)	13188	aa	33.0
Shigella sonnei form 2 (rough)	13030	aa	33.5
Shigella sonnei form 2 (rough)	14007	aa	34.4
Shigella sonnei form 2 (rough)	27684	aa	41.0
Escherichia coli (invasive)	L-115-A3	2.0	8.0
Escherichia coli (invasive)	BH-23-1	2.0	8.5
Escherichia coli (invasive)	4608	2.0	15.0
Escherichia coli (nonpathogenic)	36000	3.0	3.5

[a] *S. boydii* strains were broadly classified in serogroup 1-6, 7-11, or 12-15.
[b] M $(NH_4)_2SO_4$, Concentration required for aggregation. aa, Autoagglutination in physiological saline.
[c] Data represent an average of three values determined on the same day. *P* values for Congo red binding were as follows: *S. dysenteriae* > other three *Shigella* species, $P < 0.001$; *S. flexneri* > *S. boydii*, $P > 0.01$; *S. flexneri* > *S. sonnei*, $P < 0.05$; *S. boydii* > *S. sonnei*, $P > 0.01$.

controls. Student's *t* test was applied to calculate differences in Congo red binding between *Shigella* species.

To examine Congo red binding to pretreated cells, equal quantities of pelleted cells obtained from a TS broth culture of *S. dysenteriae* type 1 (no. 26406) were treated with (i) 50 mM Tris hydrochloride buffer (pH 7.8), (ii) 50 mM Tris hydrochloride buffer (pH 7.8) containing 50 mM EDTA, or (iii) physiological saline and stirred for 1 h at room temperature at low speed (60 rpm on a rotary shaker). Cells were separated by centrifugation and tested for their ability to bind Congo red. Cells not treated with buffer, EDTA, or physiological saline were used as controls.

Outer membranes, spheroplasts (17), and lipopolysaccharide (LPS) (41) obtained from strain no. 26406 of *S. dysenteriae* type 1 were tested for Congo red binding. The cell components were incubated at room temperature for 10 min in 3 ml of 10 mM Tris hydrochloride (pH 7.8) containing 50 μg of Congo red. The dye-bound complex was pelleted by centrifugation at 100,000 × *g* for 1 h. Unbound dye was estimated as described above.

Congo red binding to bovine serum albumin (BSA) (fraction V; Armour Pharmaceutical Co., Chicago, Ill.) and to water-extracted outer membrane proteins (28) was measured by incubating proteins with 40 μg of Congo red in 500 μl of 10 mM Tris hydrochloride (pH 7.8). After the mixture incubated for 10 min at room temperature, 2 ml of saturated ammonium sulfate was added. The mixture was chilled at 4°C for 1 h to precipitate the dye-protein complex and then centrifuged at 8,000 × *g* for 30 min. Unabsorbed dye in the supernatant was measured at 480 nm. Protein-free solutions were used as controls.

SAT. Fresh colonies of strains obtained after growth on TS agar for 18 h at 37°C were used to determine cell surface hydrophobicity (33). Solutions of ammonium sulfate in 0.02 M sodium phosphate buffer (pH 6.8) in concentrations ranging from 0.5 to 3 M were used to test aggregation of the strains. Autoagglutination in physiological saline was routinely done first to detect rough variants. Agglutination of bacteria in 0.1% acriflavine solution in physiological saline was also used for such purposes.

Plasmid isolation and analysis. Plasmid DNA was extracted (4) and separated by electrophoresis on 0.7% agarose gels (26). The control *E. coli* strains used to provide standard plasmids for molecular mass estimations were Pdk-9 (140 and 105 MDa), R_1 (62 MDa), RP_4 (36 MDa), and Sa (23 MDa). *E. coli* V517 was used as standard for determining the molecular masses of plasmids in the range of 35 to 1.5 MDa.

Assay for virulence (the Sereny test). Each strain was tested for virulence in the eye of a guinea pig (37). Pcr^+ and Pcr^- colonies obtained after 18 h of growth were subcultured and then layered onto TS agar containing 0.6% yeast extract. Cells were collected with a swab stick and diluted in sterile vials with normal saline. Suspensions were adjusted to approximately 3×10^8 cells per ml, and 20 μl of this suspension was dropped into one eye of an adult albino guinea pig. Strains which produced keratoconjunctivitis within 72 h were considered to be virulent.

RESULTS

Congo red binding experiments. After 18 h of incubation at 37°C on Congo red agar, pigmented Pcr^+ strains formed colonies which were small in size with a dark red center and a lighter outer zone. In contrast, Pcr^- strains formed relatively larger colonies which appeared white. This property was observed for all *Shigella* species as well as for enteroinvasive *E. coli*. However, if the plates were incubated for a longer period (24 to 48 h), all colonies showed diffuse pigmentation, making it difficult to distinguish between Pcr^+ and Pcr^- strains.

When the temperature of incubation was reduced to 30°C, only white nonpigmented colonies were seen. However, when these colonies were replated on Congo red agar and incubated at 37°C, the effect was reversed and pigmented colonies reappeared. The nonpathogenic *E. coli* 36000 produced white colonies after a similar period of incubation at 37°C on Congo red agar. This strain was used as a negative control. Rough variants of all *Shigella* species and enteroinvasive and nonpathogenic *E. coli* bound Congo red to form pigmented colonies, although the colonies had irregular margins.

Strains of *S. dysenteriae* type 1 bound dye from the solution in the range between 15 and 42 $\mu g/10^{10}$ cells (Table 1). In *S. flexneri*, 94% (15 strains) of the strains studied bound 15 μg of dye or less per 10^{10} cells. Similar results were obtained for *S. boydii*. All smooth strains of *S. sonnei* bound 9 μg of dye or less, while the rough mutants bound between 26 and 41 μg. The binding of dye from solution by the enteroinvasive *E. coli* strains (three strains) was similar to that of *S. flexneri*, i.e., in the range between 15 and 8 μg,

TABLE 2. Effect of pretreatment of cells on Congo red binding[a]

Pretreatment	Congo red binding ($\mu g/10^{10}$ cells)	
	S. dysenteriae type 1 26406	*E. coli* 36000
Untreated cells	20.0	3.5
Washed with physiological saline (0.9%)	19.0	4.0
Washed with 10 mM Tris hydrochloride (pH 7.8)	24.0	4.0
Washed with EDTA (5 mM) in buffer	40.0	36.0
Spheroplasts prepared by lysozyme-EDTA extraction	42.5	ND[b]

[a] Congo red binding was done as described in the text. Information on the two strains is given in table 1. Data represent an average of three values.
[b] ND, Not done.

while nonpathogenic *E. coli* 36000 bound 3.5 μg of dye. Differences in Congo red binding between *S. dysenteriae* type 1 and the other three *Shigella* species were statistically significant, as were the values between *S. flexneri* and *S. sonnei*. Comparison among the other species showed no such differences.

Modification of Congo red binding by pretreatment of cells. There was no change in Congo red binding properties when *S. dysenteriae* type 1 cells were washed with physiological saline or with Tris hydrochloride buffer (pH 7.8) (Table 2). However, after treatment with EDTA, binding of the dye by the cells increased from 24 to 40 $\mu g/10^{10}$ cells. After treatment, the nonpathogenic *E. coli* 36000 was also found to bind at least 10 times more Congo red. Similarly, freshly prepared spheroplasts obtained by the lysozyme-EDTA treatment also bound higher quantities than untreated bacteria.

Congo red binding to isolated cell components. Of the three cell components of *S. dysenteriae* type 1 (Table 3), the outer membrane proteins bound the most dye (34.5 μg/200 μg of protein). The outer membrane preparation bound one-third of the amount bound by outer membrane proteins (10.9 μg/200 μg of protein), while LPS did not bind any dye. BSA had the highest binding activity of all tested preparations.

SAT. Cell surface hydrophobicity estimated by the SAT showed that all 22 strains of *S. dysenteriae* type 1 aggregated in 1.5 M $(NH_4)_2SO_4$ (Table 1). All strains of *S. flexneri* type 2a aggregated at 2.0 M. Similar SAT values were obtained with *S. flexneri* types 1a, 3b, and Y. A strain of *S. flexneri* type 3a was more hydrophobic, aggregating at 1.0 M $(NH_4)_2SO_4$ concentration, while an *S. flexneri* type 2b strain was the least hydrophobic, showing an SAT value of 3.0 M. The hydrophobicities of *S. flexneri*, *S. sonnei*, and *S. boydii* (serotypes 1-6 and 7-11) strains assessed by the SAT were lower than that of *S. dysenteriae* type 1. Only *S. boydii* of serotype 12-15 showed a SAT value of 1.5 M, i.e., equal to the hydrophobicity of *S. dysenteriae* type 1. All *S. sonnei*

TABLE 3. Congo red binding to isolated components in an *S. dysenteriae* type 1 strain[a]

Material (μg)	Amt of Congo red bound (μg)
Outer membrane (200)	10.92 ± 0.06
Water-extracted outer membrane proteins (200)	34.50 ± 0.41
LPS (2,000)	None
BSA (50)	38.00 ± 0.82

[a] Proteins and outer membranes were quantitated on the basis of protein estimation (5), and LPS was quantitated by 2-keto-3-deoxyoctanoate content (30). Means and standard deviations of three determinations are given.

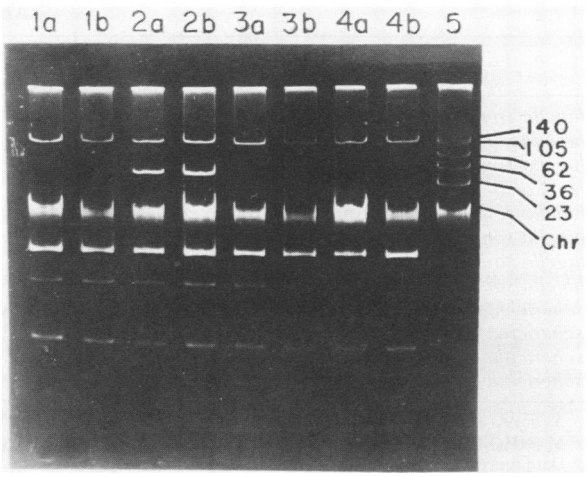

FIG. 1. Electrophoretic patterns of plasmid DNA obtained for pairs of *S. dysenteriae* type 1 strains, both nonpigmented (Pcr^-) and pigmented (Pcr^+). Lanes 1a, 2a, 3a, and 4a, Pcr^- isolates of strains 26406, 3351, 16770, and Z-24623, respectively. Lanes 1b, 2b, 3b, and 4b, The respective Pcr^+ counterparts. Lane 5 contains standards for plasmid DNA as described in the text. Sizes of plasmids (megadaltons) are indicated in the right. Chr, Chromosome.

form 1 (smooth) strains aggregated in 3.0 M solution. SAT values for the enteroinvasive *E. coli* strains were 2.0 M $(NH_4)_2SO_4$ concentration, while for nonpathogenic *E. coli* the SAT value was 3.0 M.

The Sereny test. The Sereny test showed that in all cases the smooth Pcr^+ strains were positive for virulence while the Pcr^- isolates were negative. The rough Pcr^+ forms of *Shigella* or *E. coli* strains were also negative in the Sereny test.

Plasmid analysis. Plasmid profiles of the Pcr^+ and Pcr^- strains of *S. dysenteriae* type 1 analyzed by agarose gel electrophoresis were identical, and both contained the large 140-MDa plasmid (Fig. 1). In *S. flexneri*, the 140-MDa plasmid was either absent or smaller in size in the Pcr^- strains. Such changes were also observed for Pcr^- strains of *S. boydii* and enteroinvasive *E. coli*, but not for *S. sonnei* (Fig. 2).

DISCUSSION

We observed that for smooth forms of *Shigella* species and enteroinvasive *E. coli*, the Congo red binding test can be related to virulence. This test has been used to differentiate between virulent and avirulent strains of a variety of gram-negative bacteria (15, 25, 31) and also as a criterion of hydrophobicity (36). Congo red and other dyes (2, 12, 16) have also been used as indicators of ligand-binding sites, either on the cell surface or on soluble proteins. All Pcr^+ smooth strains of *Shigella* species and enteroinvasive *E. coli* used in our study were virulent by the Sereny test, while those that were Pcr^- were always avirulent. This indicates that Congo red agar may be used as a quick and inexpensive alternative to the Sereny test to determine virulence in these strains. However, for the Congo red agar test, carefully controlled experimental conditions such as freshness of cultures, incubation time, and temperature are critical.

Congo red uptake from solution by the different *Shigella* species showed a distinct decline of binding in the order *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. This is in general agreement with the virulence of *Shigella* species and the severity of the disease these pathogens may cause.

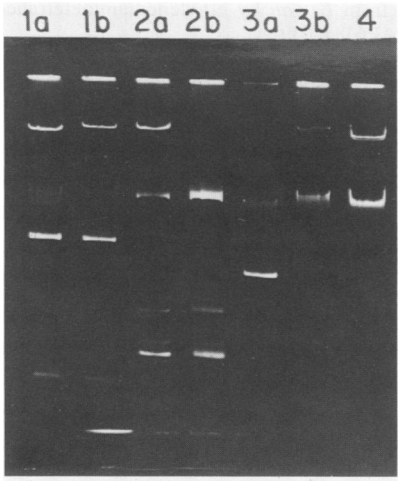

FIG. 2. Electrophoretic patterns of plasmid DNA from Pcr$^-$ and Pcr$^+$ pairs of strains. Lanes 1a, 2a, and 3a are plasmid patterns obtained from Pcr$^-$ strains of *S. boydii* 33744, *S. sonnei* 6577, and enteroinvasive *E. coli* 4608, respectively. Lanes 1b, 2b, and 3b are profiles of their respective Pcr$^+$ types. Lane 4 contains standards for plasmid DNA as shown in Fig. 1.

Similarly, a comparison of different *Shigella* species by the SAT showed that hydrophobicity decreased in the same order as for Congo red binding. All *S. dysenteriae* type 1 and a few *S. boydii* strains were relatively more hydrophobic, belonging to the group 3 range (22). The rest were poorly hydrophobic, with SAT values of 2 M and above. A comparison of these results with those obtained for other bacterial pathogens (9, 22, 33) suggests that the overall surface hydrophobicity measured by the SAT is relatively low in *Shigella* species. In some strains of *E. coli*, the cell surface hydrophobicity is related to the presence of fimbriae (9, 22, 39). Little information is available on the presence of these appendages in *Shigella* species (8, 27). It is therefore difficult to hypothesize whether differing cell surface properties can be related to fimbriae in these bacteria. An evaluation of the SAT values showed that although differences were found within strains of a species of *Shigella*, the SAT value was relatively constant for a particular serotype. This indicates that the SAT values may in some way be dependent on the arrangement of the O antigen. This point was further strengthened by our observation that the SAT values of strains increased if the cultures were old. Old cultures have a tendency to develop roughness, and although a smooth-to-rough transition can be detected by agglutination in physiological saline, we found that intermediate stages of roughness can be detected by the SAT. However, SAT values for the Pcr$^+$ and Pcr$^-$ pairs of isogenic strains were always identical. This suggests that the transition from the Pcr$^+$ to the Pcr$^-$ form results from a minor change of the surface conformation or a change in the composition of components present on the surface which cannot be differentiated by the SAT. For these reasons, we believe that Congo red binding on agar is more sensitive than the SAT for determining the virulence of *Shigella* species.

To determine the relative contribution of cell surface components of *Shigella* species to the expression of hydrophobicity and to understand Congo red uptake, we tested both modified cells and isolated cell components for their reaction with the dye. Treatment of *S. dysenteriae* type 1 and nonpathogenic *E. coli* with EDTA increased Congo red binding. EDTA is known to release LPS from the outer membrane of gram-negative bacteria (21). We assume that a decrease in the concentration of LPS increased the hydrophobicity of the cells, resulting in more Congo red binding. Purified LPS obtained from a strain of *S. dysenteriae* type 1 did not bind any dye. Isolated outer membrane proteins as well as BSA bound Congo red in relatively high amounts, indicating that proteins are the main cell components involved in dye interactions. The binding of dye by BSA is not surprising since serum proteins contain ligand-binding regions for transporting metabolites (12). The binding sites of proteins contain clefts, depressions, and specific stereochemical features which are particularly suitable for such interactions.

Rough mutants bound Congo red very well. The reason why rough mutants bind the dye may be explained in a number of ways. First, the loss of the hydrophilic oligosaccharide units from O side chains of LPS during the smooth-rough transition makes the cell surface more hydrophobic and the contact of the dye is increased with the lipid A and core of the amphipathic LPS molecules. Second, in the absence of steric hindrance from the O side chains, proteins on the bacterial surface are more exposed and accessible to the dye so that binding is favored. It has been observed (29) that rough mutants of *S. flexneri* can invade HeLa cells but are incapable of causing keratoconjunctivitis in the eyes of guinea pigs. On the other hand, smooth, avirulent colonial mutants of *S. flexneri* (20) were negative in both tests. Congo red binding by rough mutants and by smooth, virulent *Shigella* strains may reflect the invasion of mammalian cells in the sequence of events which starts pathogenesis. Smooth Pcr$^-$ mutants are possibly lacking in an essential component required to enter mammalial epithelial cells, while rough mutants can invade host cells although they are nonpathogenic. The Sereny test can only detect total pathogenicity, but to differentiate between invasion and the later steps of pathogenicity, the HeLa cell assay is more appropriate.

We found that the plasmid profiles of Pcr$^+$ and Pcr$^-$ strains of *S. dysenteriae* type 1 and *S. sonnei* were similar although restriction enzyme analysis was not done to confirm it. In contrast to this, in *S. flexneri*, *S. boydii*, and enteroinvasive *E. coli*, a change in plasmid profile was associated with loss of dye-binding activity. These results are in agreement with evidence which suggests that genes on the 140-MDa plasmid are required in *S. flexneri* for the expression of virulence and for Congo red binding ability (6, 7, 25). However, in some *S. flexneri* strains, it has also been shown that although the loss of virulence and of the dye-binding property often corresponds to the loss or decrease in the size of the 140-MDa plasmid, sometimes no changes can be observed. In such cases, it has been suggested that loss of the ability to bind Congo red results from the inversion of insertion sequence IS*1* on plasmid DNA so that although the size of the plasmid remains unchanged, the Pcr$^+$ phenotype is lost. This explanation may also be valid for the *S. dysenteriae* type 1 and *S. sonnei* pairs of Pcr$^+$ and Pcr$^-$ strains which have unaltered plasmid profiles. Virulence in *Shigella* species depends on a large number of factors, and genes present both on chromosomes and on plasmids may be necessary (10, 35). Hence, the loss of the Pcr$^+$ phenotype in some cases may arise from changes in chromosomal DNA.

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