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Characterization of *Shigella flexneri* Sequences Encoding Congo Red Binding (*crb*): Conservation of Multiple *crb* Sequences and Role of IS*1* in Loss of the Crb$^+$ Phenotype

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The ability to bind Congo red (Crb$^+$) is associated with virulence of *Shigella flexneri* and is encoded by a large, 220-kilobase plasmid. We cloned fragments of this plasmid to isolate the sequences encoding Congo red binding, to determine the degree of conservation of these sequences among *S. flexneri* strains, and to study the molecular basis for loss of the Crb$^+$ phenotype. At least two separate *Bam*HI fragments cloned into plasmid vectors encode Congo red binding in *E. coli* or *S. flexneri*. One Crb$^+$ clone, pTKS2, contains a copy of IS*1* adjacent to the *crb* sequences. IS*1* appears to be responsible for deletions leading to loss of Congo red binding in this clone. In addition, this clone was found to integrate into the chromosome at relatively high frequency. Integration resulted in loss of the Crb$^+$ phenotype. A second clone, pTKS15, which has only limited homology to pTKS2, also encodes Congo red binding. The Crb$^+$ phenotype of transformants carrying pTKS15 was detected at 37°C but not at 30°C, and thus it resembles Congo red binding in wild-type *S. flexneri*. *Hin*dIII digests of plasmid DNA from 10 different *S. flexneri* strains were hybridized to both of these Crb$^+$ clones and to an IS*1* probe. More than one fragment hybridized to pTKS2 or pTKS15. In general, the sizes of these fragments were the same in *S. flexneri* strains of different serotypes, indicating conservation of these sequences. Three of five copies of IS*1* were also found on the large *S. flexneri* plasmids. Two of the copies were on fragments of the same size in each strain. Analysis of Crb$^-$ derivatives of the 10 strains indicated that, although IS*1* may be closely linked to *crb* sequences on the 220-kilobase plasmid, it is not responsible for the majority of deletions of this plasmid associated with loss of Congo red binding.

Members of the genus *Shigella* are invasive intestinal pathogens responsible for bacillary dysentery, a disease affecting the large intestine (8). Genetic analysis of *S. flexneri* has revealed that pathogenicity is multifactorial and requires a number of genetic determinants. One of the required chromosomal loci is the *kcpA* locus, which is responsible for the ability of *S. flexneri* to evoke keratoconjunctivitis in experimental animals (6). Another locus involved in virulence is located in the histidine region of the chromosome and encodes O antigen production (5). Genes in the xylose-rhamnose region facilitate bacterial survival in the intestinal mucosa (7), and genes in the arginine-mannitol regions are necessary for a positive Sereny test (23). In addition to these chromosomal genes, a 220-kilobase (kb) plasmid which encodes several polypeptides is essential for the virulence of *S. flexneri* (10, 23). Loss of the plasmid or deletions of plasmid sequences results in loss of virulence (18).

Expression of virulence in *S. flexneri* has been shown to be regulated by temperature. Invasion of HeLa cells is detected at 37°C but not at 30°C (17). Recent studies have shown that the polypeptides encoded by the 220-kb plasmid are also produced at 37°C but not at 30°C, and these polypeptides may be responsible for the temperature dependence of invasion (10).

Another phenotype associated with pathogenicity of wild-type *Shigella* spp. is their ability to bind the dye Congo red from agar medium (19), thus producing red (Crb$^+$) colonies. Spontaneous mutants which are unable to bind the dye and produce white (Crb$^-$) colonies can be readily isolated from the wild-type strains. Mutation from Crb$^+$ to Crb$^-$ occurs at a frequency of approximately 10^{-4} in *S. flexneri* (3) and is often associated with loss or deletions of the 220-kb plasmid (18). However, occasional Crb$^-$ isolates appear to have plasmids identical to the wild-type plasmid as determined by restriction endonuclease digestion (3). The Crb$^-$ mutants are avirulent for chick embryos (19) and are unable to invade epithelial cells (18). It is not clear whether the ability to bind Congo red represents a specific virulence factor or whether the gene encoding this trait is tightly linked to a gene required for virulence. Congo red binding is also thermoregulated, and the organisms bind the dye at 37°C but not at 30°C (17). Therefore, the gene(s) encoding the ability to bind the dye may be coordinately regulated with other plasmid-encoded virulence factors. Recently, the genes of *S. flexneri* encoding Congo red binding were cloned (3). *Escherichia coli* and Crb$^-$ *S. flexneri* harboring the cloned genes were able to bind the dye from agar medium (Crb$^+$). However, the cloned genes did not fully restore virulence of Crb$^-$ *S. flexneri* for chick embryos (3), indicating that additional plasmid sequences are needed to restore the virulence phenotype. In recent studies by Maurelli et al. (16), additional sequences encoding virulence factors were cloned from the 220-kb plasmid. Avirulent *S. flexneri* transformed with these sequences were able to invade epithelial cells but were negative for the Sereny test and unable to bind Congo red (16).

The presence of an insertion sequence, IS*1*, has been detected on the 220-kb plasmid (3). The cloned genes which encode Congo red binding were found to contain a copy of IS*1*, and the insertion sequence was found to map in the same region as the Congo red gene(s). Since IS*1* can cause deletions of adjacent sequences (21), we wished to determine

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TABLE 1. List of bacterial strains and plasmids used

Strain or plasmid	Marker	Source or reference[a]
S. flexneri		
8-2031	Crb$^+$, serotype 1b	TDH
1029	Crb$^+$, serotype 2a	TDH
2457	Crb$^+$, serotype 2a	S. B. Formal, WRAIR
3958	Crb$^+$, serotype 2a	TDH
SA100	Crb$^+$, serotype 2a	12
4293	Crb$^+$, serotype 2a	TDH
4428	Crb$^+$, serotype 3a	TDH
1372	Crb$^+$, serotype 4a	TDH
229272	Crb$^+$, serotype 5	TDH
9-2102	Crb$^+$, serotype 5	TDH
E. coli		
AB1515	*trpE leu-6 proC thi-1 purE lacY mtl-1 xyl tonA* λ^- F$^-$	C. F. Earhart, UT
RM1058	*thi$^-$ leu$^-$ thr$^-$ lacY* Rif^r Nal^r *recA$^+$ tonA supE* F$^-$ λ^-	R. Meyer, UT
HB101	*proA leu$^-$ lacY ara-14 galK12 xyl-5 mtl-1 recA12 hsdR hsdM supE44* Str^r	13
CGSC5678	Hfr *thr-31 carB8 relA metB1*	CGSC
pTKS2	Crb$^+$ 9-kb *Bam*HI fragment of 220-kb plasmid of *S. flexneri* in pAT153	3
pTKS15	Crb$^+$ 3.0-kb *Bam*HI fragment from 220-kb plasmid of *S. flexneri* in pACYC184	This study
pAT153	Ap^r Tc^r cloning vector	13
pACYC184	Tc^r Cm^r cloning vector	13

[a] TDH, Texas Department of Health; WRAIR, Walter Reed Army Institute of Research; UT, University of Texas; CGSC, *E. coli* Genetic Stock Center.

whether IS*1* was responsible for the deletions of the 220-kb plasmid associated with loss of the Crb$^+$ phenotype.

In this study, the cloned sequences encoding Congo red binding were examined. We characterized Crb$^-$ derivatives of the clone to determine the molecular mechanism for loss of Congo red binding and the possible role of IS*1* in this process. Additional sequences encoding Congo red binding were also cloned, and the degree of conservation of these sequences among strains of *S. flexneri* was determined.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Plasmid pTKS2 (3) contains a 9-kb *Bam*HI fragment of the 220-kb plasmid of *S. flexneri* 8-2031 cloned into pAT153, a high-copy-number derivative of pBR322 (13). Plasmid pTKS15 contains a 3.0-kb *Bam*HI fragment of the 220-kb plasmid cloned into pACYC184 (13). Strains were stored frozen at −80°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 20% glycerol.

Media and chemicals. Trypticase soy broth with 1.5% agar and 0.01% Congo red was used to determine Congo red binding (19). The minimal medium used for conjugation experiments was N$^-$C$^-$ medium (1) with 10 mM NH_4Cl, required amino acids, and 0.4% of the appropriate sugar as a carbon source. Carbohydrate utilization was also detected on MacConkey agar base (Difco Laboratories, Detroit, Mich.) with 1.0% xylose or mannitol. The antibiotics added where indicated were carbenicillin (500 μg/ml), streptomycin (200 μg/ml), nalidixic acid (40 μg/ml), and chloramphenicol (10 μg/ml). Carbenicillin was used in place of ampicillin because less background growth was observed. Luria broth was used for all other cultures. All chemicals used were reagent grade.

Bacterial conjugations. Overnight cultures of the donor (*E. coli* CGSC5678) and recipient (*E. coli* RM1058 or AB1515) were diluted in Luria broth and grown to mid-log phase. Equal volumes (1 ml) were mixed in a 125-ml flask and incubated at 37°C. For standard matings, a sample was withdrawn after 2 h. In the case of interrupted matings, samples were withdrawn at various times and plated on selective medium.

Southern hybridization. Chromosomal DNA was prepared by the procedure of Marmur (15). Plasmid DNA was prepared by the procedure of Kado and Liu (11). DNA was cut with restriction endonucleases (New England BioLabs, Inc., Beverly, Mass.) and electrophoresed through 0.9% agarose gels. DNA fragments were transferred to GeneScreen membranes (New England Nuclear Corp., Boston, Mass.) by the method of Southern (24). Southern blots were hybridized at 68°C and washed with 0.1 × SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for stringent hybridization conditions and 2 × SSC for reduced stringency (13). The IS*1* hybridization probe was prepared by *Hin*dIII digestion of VAλ3 (4), separation of the fragments on 0.9% agarose, and elution of the 10.2-kb fragment as described previously (12). The 9.0-kb *Bam*HI, 2.8-kb *Eco*RI, and 1.1-kb *Eco*RV probes from pTKS2 and the 3.0-kb *Bam*HI probe from pTKS15 were also prepared by electroelution (12). The eluted fragments were labeled with [^{32}P]dCTP (New England Nuclear) by nick translation (14).

RESULTS

Loss of Congo red binding by cloned *crb* genes. *E. coli* strains harboring the recombinant plasmid encoding Congo red binding, pTKS2, were found to give rise to Crb$^-$ mutants at approximately the same frequency (10^{-4}) observed in the *S. flexneri* strain containing the 220-kb plasmid (3). To determine whether the loss of the Crb$^+$ phenotype in *E. coli* RM1058(pTKS2) was accompanied by molecular changes similar to the ones reported for the 220-kb plasmid (3, 18), we performed the following experiments. Single colony isolates were grown at 37°C in Luria broth with carbenicillin, diluted, and plated on Congo red agar containing the antibiotic. Fifty Crb$^+$ colonies and 40 Crb$^-$ mutants were picked, and their plasmids were analyzed by agarose gel electrophoresis. Two of the 40 Crb$^-$ isolates contained plasmids which appeared smaller than pTKS2. Restriction enzyme digestion of these two plasmids revealed that deletions had occurred in the region previously shown to encode Congo red binding (3) (Fig. 1). A copy of IS*1* has been identified in this region (3), and IS*1* is known to mediate deletions of adjacent sequences while leaving the copy of IS*1* intact (21). Therefore, the deletions were analyzed by Southern hybridization with an IS*1* probe. Although the precise endpoints of the deletions and the IS*1* sequence were not determined, the hybridization indicated that the deletions were adjacent to the IS*1* sequence of pTKS2 (Fig. 1; data not shown), suggesting that IS*1* was responsible for the deletions. In contrast, of 50 Crb$^+$ isolates tested, none had detectable alterations in the recombinant plasmid.

The majority (38/40) of the Crb$^-$ isolates of RM1058 (pTKS2) had apparently lost the plasmid while retaining the antibiotic resistance marker encoded by the cloning vector. No plasmid DNA was detected after agarose gel electro-

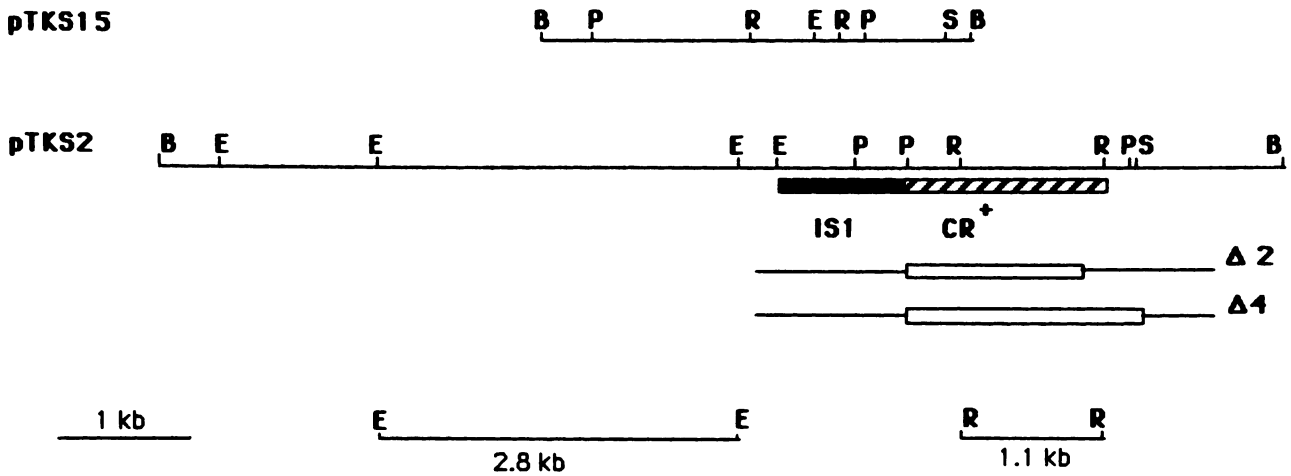

FIG. 1. Restriction maps of cloned fragment in pTKS15 and pTKS2. Vector sequences are not shown. The restriction enzymes used were *Bam*HI (B), *Eco*RI (E), *Eco*RV (R), *Pst*I (P), and *Sal*I (S). Location of the insertion sequence IS*1* and sequences encoding Congo red binding on pTKS2 are indicated by filled boxes. Open boxes indicate the deleted sequences in two Crb$^-$ mutants of pTKS2. The 2.8-kb *Eco*RI and 1.1-kb *Eco*RV fragments used as hybridization probes are shown at the bottom.

phoresis of cleared lysates (Fig. 2). Retention of antibiotic resistance suggested that the plasmid had integrated into the chromosome. If integration was responsible for loss of the Crb$^+$ phenotype, then excision of the plasmid from the chromosome should lead to the reappearance of the recombinant plasmid and a Crb$^+$ phenotype. Single colonies of RM1058 (Crb$^-$ Cb^r) thought to contain the plasmid in integrated form were passaged in broth at 37°C. The cultures were diluted and plated on Congo red agar plus carbenicillin after each subculturing. After seven passages, two Crb$^+$ colonies were detected, and these appeared to contain a plasmid. Since pTKS2 isolated from RM1058 usually ran as a smear, restriction enzyme analysis was used to determine that these Crb$^+$ revertants contained a plasmid identical to pTKS2 (Fig. 2).

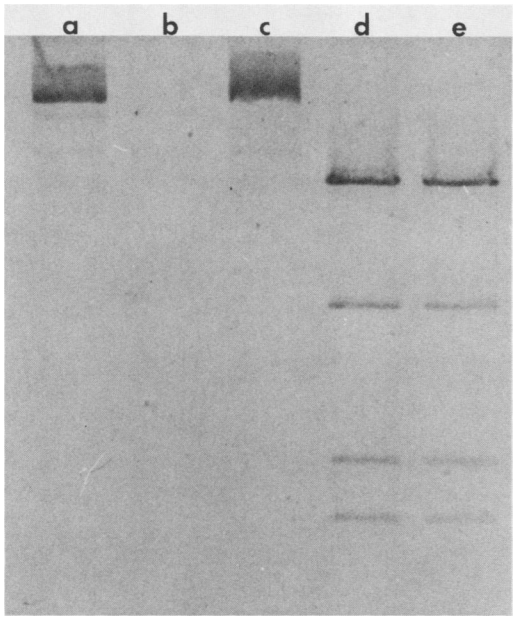

FIG. 2. Loss of detectable plasmid associated with loss of Congo red binding. Agarose gel electrophoresis of plasmid DNA isolated from (lanes): a, RM1058(pTKS2), which is Crb$^+$; b, Crb$^-$ Cb^r derivative of RM1058(pTKS2); c, a Crb$^+$ revertant of the isolate in lane b. Lanes d and e contained *Eco*RI digests of plasmids isolated from lanes a and c, respectively. The smallest *Eco*RI fragment of pTKS2 (0.3 kb) was not seen on this gel.

Conjugation experiments were performed to confirm that plasmid integration into the chromosome had occurred. *E. coli* CGSC5678 (Hfr) was transformed with pTKS2, and a Crb$^+$ transformant was isolated. The Crb$^+$ colony was grown in broth and plated on Congo red agar. Several well-isolated Crb$^-$ colonies were selected. Plasmid analysis indicated that these Crb$^-$ isolates had no detectable plasmid present although they had retained the carbenicillin resistance encoded by pAT153. Using RM1058 as the recipient strain, mating experiments were performed with the Crb$^+$ isolate of CGSC5678 and with two of the Crb$^-$ mutants. Recombinants were selected on Congo red agar plus carbenicillin and nalidixic acid. No transfer of the carbenicillin resistance marker was observed in the cross between the Crb$^+$ isolate of CGSC5678 and RM1058. However, in the crosses between each of the Crb$^-$ isolates of CGSC5678 and RM1058, transfer of carbenicillin resistance occurred at a frequency of 5×10^{-4}. These results indicated that carbenicillin resistance was transferred by the Crb$^-$ mutants as a chromosomal marker. To determine the relative map position of the integrated plasmid in the *E. coli* chromosome, we performed interrupted liquid matings between the Crb$^-$ derivative of CGSC5678 and the streptomycin-resistant multiple auxotroph AB1515. Recombinants were selected on Congo red agar plus streptomycin and carbenicillin. One hundred Sm^r Cb^r colonies were screened for linkage of the carbenicillin resistance marker to the markers of the donor strain. No linkage was observed between carbenicillin resistance and leucine, proline, adenine, or lactose, but 85% of the transconjugants were Mtl$^+$ and 87% were Xyl$^+$. This indicated that plasmid integration occurred close to the *xyl* and *mtl* regions of the chromosome, which map at 80 and 81 min, respectively.

To determine whether integration was a *recA*-dependent process, we transformed a *recA* strain of *E. coli*, HB101, with pTKS2. Crb$^+$ transformants were isolated, but no Crb$^-$ Cb^r colonies lacking the plasmid were obtained in five separate trials. It is likely that integration of the recombinant plasmid is *recA* dependent and occurs by recombination between homologous sequences on the plasmid and in the

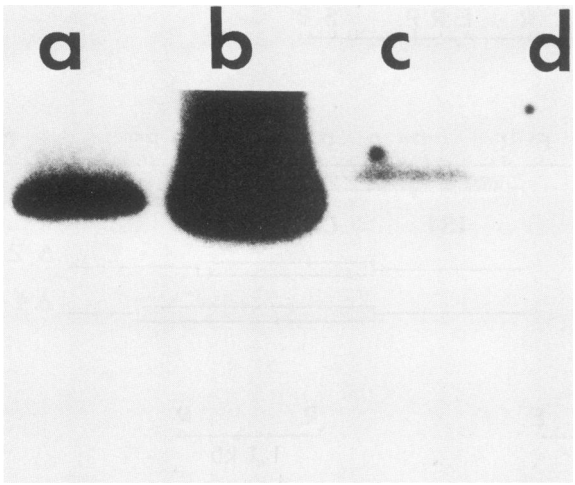

FIG. 3. Detection of pTKS2 sequences in Crb$^-$ mutants lacking detectable plasmid DNA. Total DNAs isolated from (lanes): b, RM1058(pTKS2), which is Crb$^+$; c, the Crb$^-$ Cb^r derivative of RM1058(pTKS2); and d, untransformed RM1058 were cut with *Eco*RV and hybridized with the 2.8-kb *Eco*RI fragment of pTKS2. *Eco*RV-digested pTKS2 plasmid DNA was included in lane a for comparison.

mtl-xyl region of the chromosome. To determine which sequences of the hybrid plasmid were homologous to the chromosomal sequences, we hybridized *Eco*RV-digested total DNAs of untransformed *E. coli* CGSC5678, the Crb$^+$ transformant, and the Crb$^-$ derivative to four different probes (Fig. 3; data not shown). The probes used were the cloning vector pAT153, the entire *Bam*HI insert, a 2.8-kb internal *Eco*RI fragment, and a 1.1-kb *Eco*RV fragment of the Crb$^+$ coding region of pTKS2 (Fig. 1). In each case, the Crb$^+$ transformant chromosome hybridized with the probes in the same way pTKS2 did, indicating that plasmid contamination of the chromosome preparation was responsible for the hybridization signal. In the Crb$^-$ transformant, the size of the *Eco*RV fragment hybridizing with the 2.8-kb *Eco*RI probe was larger, indicating that integration of the plasmid had occurred (Fig. 3). No homology was detected between the chromosome of the untransformed RM1058 and pAT153, suggesting that the vector was not responsible for integration. The internal *Eco*RI fragment and the *Eco*RV fragment also failed to hybridize to the untransformed chromosome, even under conditions of reduced stringency. However, when the entire *Bam*HI fragment was used as a probe, five bands were detected and the pattern appeared the same as when an IS*1* probe was used (data not shown). Thus, the copy of IS*1* in pTKS2 is a source of homology between the plasmid and chromosome and may represent the site of recombination.

The observation that plasmid integration into the chromosome was accompanied by loss of the Crb$^+$ phenotype suggested that copy number was critical in the expression of the cloned Congo red genes. The 9-kb insert of pTKS2 was cloned into the unique *Bam*HI site of pACYC184, a lower-copy-number vector (13). Approximately 1,000 transformed colonies of RM1058 were screened, and none was found to be Crb$^+$. Plasmid and restriction enzyme analyses indicated that the 9-kb *Bam*HI fragment had been cloned into pACYC184 in both orientations (data not shown), yet the Crb$^+$ phenotype was not expressed. These findings suggested that the cloned *crb* genes could be defective in the sense that they lack certain additional regulatory regions necessary for expression of the genes in low copy number. Cloning the genes in a high-copy-number vector could have compensated for the lack of the additional sequences and allowed expression. It was also possible that additional sequences elsewhere in the plasmid encode Congo red binding.

Cloning additional sequences for Congo red binding. To determine whether other sequences from the 220-kb plasmid were able to encode Congo red binding, *Bam*HI restriction enzyme fragments of the purified wild-type plasmid of strain 8-2031 were cloned into pACYC184. Six RM1058 transformants were found to be Crb$^+$. All six contained plasmids with an identical 3.0-kb *Bam*HI fragment. A restriction map of one of these plasmids, pTKS15, is shown in Fig. 1. The 3.0-kb fragment of pTKS15 did not hybridize with pTKS2 under stringent conditions, but some hybridization was seen when the stringency was reduced (data not shown). This homology was between pTKS15 and sequences to the left of IS*1* in pTKS2 rather than between the Crb$^+$ coding regions. This indicated that only limited homology exists between the 3.0-kb *Bam*HI fragment and the fragment of pTKS2, although both confer Congo red binding. Cells harboring pTKS15 differed from those containing pTKS2 in that pTKS15 produced Crb$^+$ colonies at 37°C but Crb$^-$ colonies at 30°C. This indicated that sequences on the 3.0-kb *Bam*HI

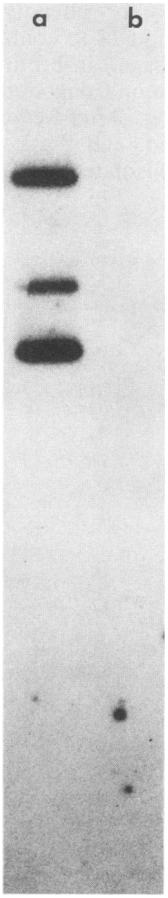

FIG. 4. Plasmid DNAs isolated from strain 8-2031 (lanes: a; Crb$^+$ and b, Crb$^-$) were cut with *Hin*dIII and separated by agarose gel electrophoresis. A Southern blot of the gel was hybridized with the 3.0-kb *Bam*HI fragment cloned into pTKS15.

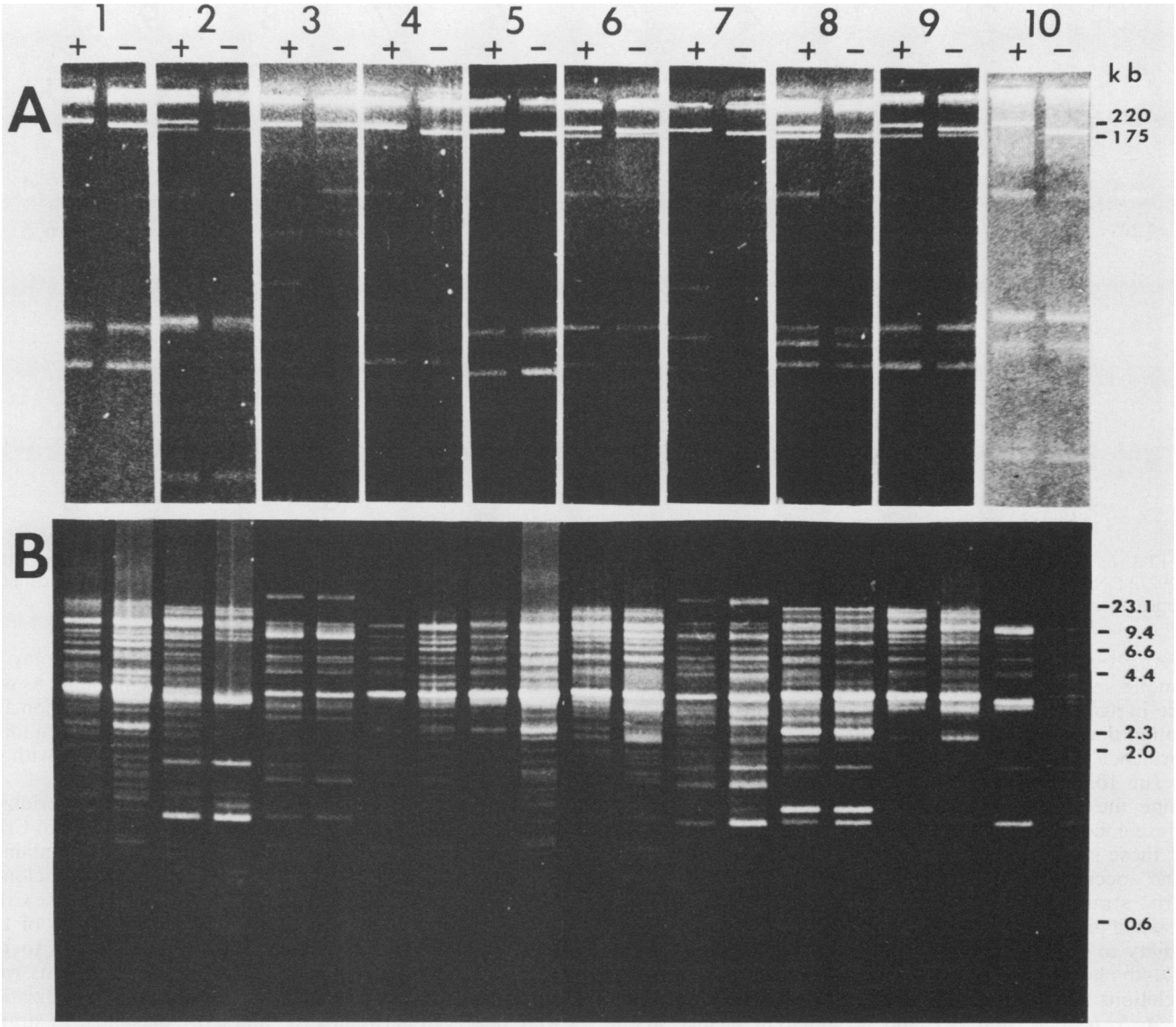

FIG. 5. Plasmids of Crb$^+$ and Crb$^-$ *S. flexneri* isolates. Agarose gel electrophoresis of plasmids from Crb$^+$ strains and their Crb$^-$ derivatives are shown in panel A (lanes): 1, 2457; 2, 8-2031; 3, 4428; 4, 4293; 5, SA100; 6, 1372; 7, 9-2102; 8, 1029; 9, 3958; 10, 229272. Panel B contains *Hin*dIII digests of the plasmids shown in A. The numbers on the right indicate sizes (in kb) of wild-type plasmids in panel A and *Hin*dIII fragments of λ in panel B.

fragment of pTKS15 were subject to the same type of thermoregulation as the wild-type plasmid.

The 3.0-kb *Bam*HI fragment of pTKS15 was hybridized to total plasmid DNA from strain 8-2031 (Fig. 4). The plasmids were digested with *Hin*dIII, which does not cut within this *Bam*HI fragment. Three *Hin*dIII fragments of 2.9, 3.9, and 9.4 kb hybridized with this probe. No hybridization was detected in a Crb$^-$ derivative which had lost the large plasmid (Fig. 4, lane b), indicating that all of the *Hin*dIII fragments were on the 220-kb plasmid. These three fragments may indicate multiple copies of the gene or the presence of a repetitive sequence, such as an insertion sequence.

Conservation of sequences encoding Congo red binding. Studies by Sansonetti et al. (22) have indicated that, although plasmids from independent isolates of *S. flexneri* appear to have evolved from the same ancestor, they nevertheless exhibit considerable genetic variation. Sequences of primary importance to the organism should, therefore, have been extensively conserved on the plasmid, whereas those of secondary importance are more likely to have been altered or discarded. To determine the degree of conservation of the sequences encoding Congo red binding among the 220-kb plasmids from different isolates of *S. flexneri*, we grew 10 strains of various serotypes (Table 1) on Congo red agar and chose well-isolated Crb$^+$ colonies for further study. Plasmid DNA was isolated from the fully grown liquid cultures of these 10 strains. In four of those strains, a 175-kb plasmid, in addition to the 220-kb plasmid, was seen on agarose gels, whereas the remaining six lacked this cryptic plasmid (Fig. 5A). All of the strains had one or more small plasmids. Total plasmid DNA was cut with the restriction enzymes. *Hin*dIII (Fig. 5B), *Eco*RI, and *Pst*I (data not shown). Considerable differences in the restriction patterns

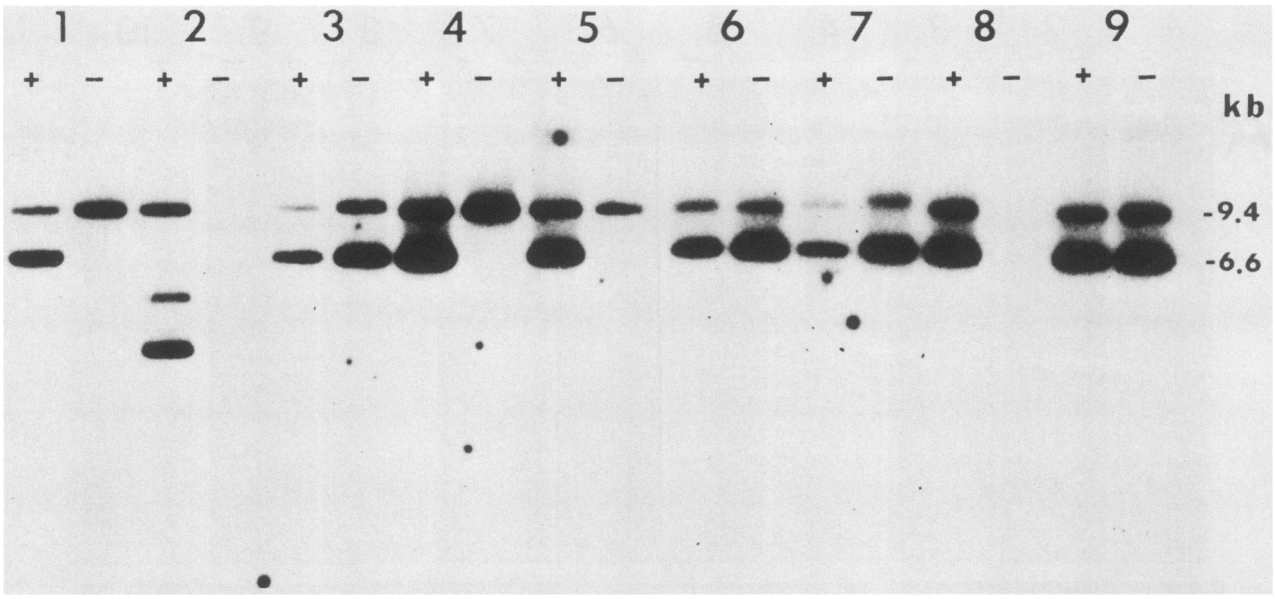

FIG. 6. Southern blot of the gel shown in Fig. 5B hybridized with the 3.0-kb *Bam*HI fragment of pTKS15. Lane 10, containing strain 229272 DNA, was omitted from this blot. The symbols + and − indicate Crb$^+$ and Crb$^-$, respectively. The numbers on the right indicate the approximate sizes of major fragments hybridizing to this probe.

were observed among strains of various serotypes, as well as among strains belonging to the same serotype. These data are in agreement with those of Sansonetti et al. (22), indicating that considerable genetic diversity exists among the virulence plasmids from independent *S. flexneri* isolates.

The 10 strains were analyzed by hybridization to determine the presence of plasmid sequences homologous to those encoding Congo red binding and to determine the fate of these sequences when conversion of Crb$^+$ *S. flexneri* to Crb$^-$ occurs. *Hin*dIII-digested plasmid preparations of the Crb$^+$ strains were electrophoresed alongside digests of the plasmid preparations of Crb$^-$ mutants (Fig. 5B). Loss of the ability to bind Congo red was accompanied by loss of the 220-kb plasmid in 2 of the 10 strains (Fig. 5A, lanes 2 and 8), deletions in the 220-kb plasmid in 7 of the 10 (Fig. 5A, lanes 1, 4, 5, 6, 7, 9, and 10), and no apparent change in the remaining isolate (Fig. 5A, lane 3). Additional Crb$^-$ mutants of each of these strains were characterized and, whereas strains may have shown a propensity for either plasmid loss or deletion, there was no consistent pattern of molecular alteration. In any given strain, the Crb$^-$ phenotype may be associated with loss or deletion of the 220-kb plasmid, as was reported by Maurelli et al. (18). The sizes of the deletion and the particular restriction fragments deleted also varied (data not shown). More rarely, Crb$^-$ isolates were found with no detectable difference in restriction pattern.

Southern blots of these *Hin*dIII digests were hybridized with the 3.0-kb *Bam*HI insert of plasmid pTKS15. All Crb$^+$ strains tested, with the exception of strain 8-2031, contained two fragments of 9.4 and 6.6 kb hybridizing to the cloned Congo red binding sequences (Fig. 6). In some of those Crb$^-$ isolates with deletions in the 220-kb plasmid, loss of the smaller of the two fragments (6.6 kb) hybridizing to the probe was observed. However, in some of the strains with deletions in the 220-kb plasmids no changes in hybridization were observed between Crb$^+$ and Crb$^-$ plasmids. In strains which had lost the plasmid, all of the fragments hybridizing with the probe were missing.

Southern blots of duplicate gels were hybridized with a 1.1-kb *Eco*RV fragment of pTKS2 (Fig. 7). This fragment

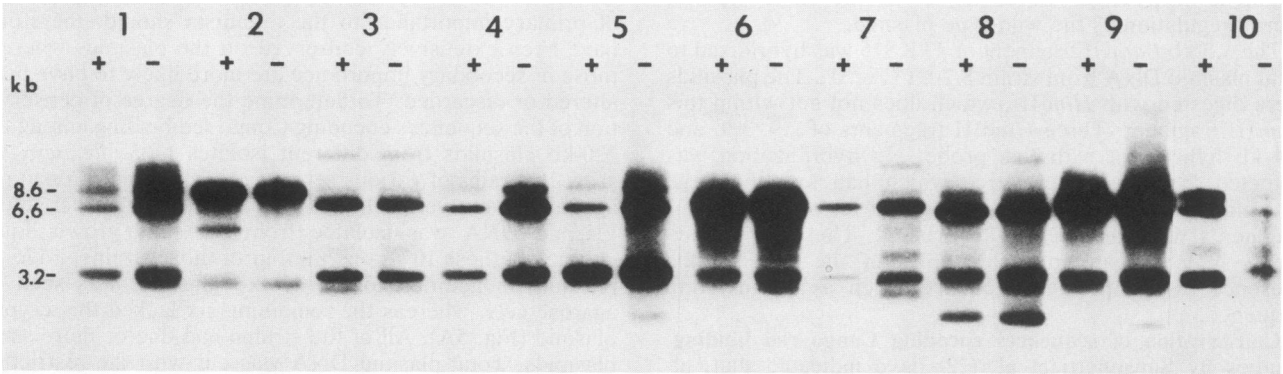

FIG. 7. Southern blot of the gel shown in Fig. 5B hybridized with the 1.1-kb *Eco*RV fragment of pTKS2. The symbols + and − indicate Crb$^+$ and Crb$^-$, respectively. The numbers on the left indicate the approximate sizes of major fragments hybridizing to this probe.

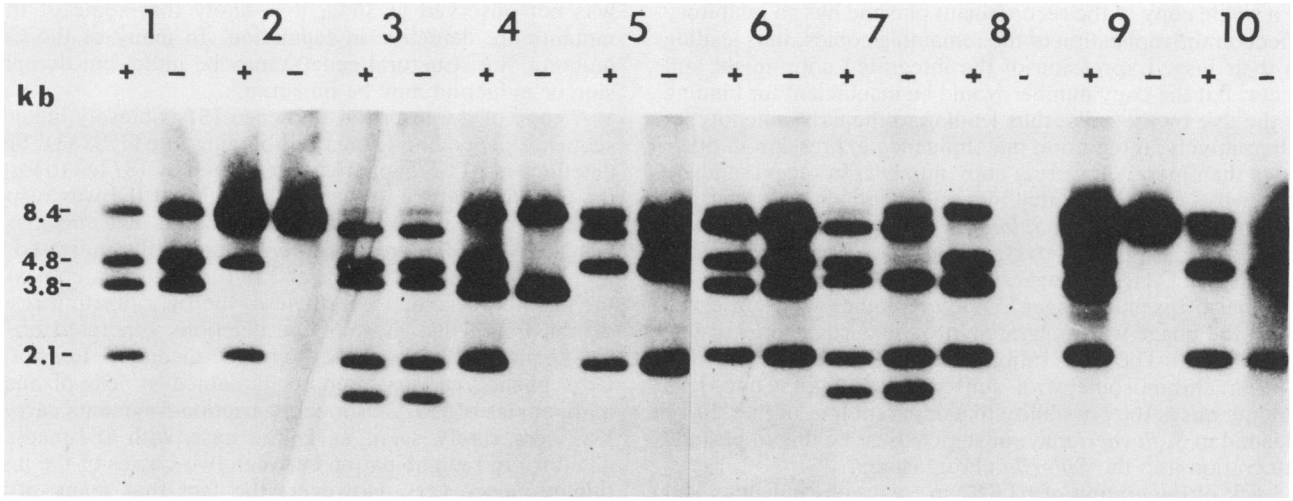

FIG. 8. Southern blot of the gel shown in Fig. 5B hybridized with the *Hin*dIII fragment of VAλ3 containing a copy of IS*1*. The symbols + and − indicate Crb$^+$ and Crb$^-$, respectively. The numbers on the left indicate the approximate sizes of major fragments hybridizing to this probe.

maps within the region of pTKS2 which encodes Congo red binding and contains none of the IS*1* (Fig. 1). At least two fragments were detected with this probe, one of which, a 3.2-kb fragment, was conserved among the strains. In the strain from which this fragment was cloned, 8-2031, loss of the large plasmid resulted in loss of only one of the three bands hybridizing to this probe (Fig. 7, lane 2). The remaining two fragments were from the 175-kb plasmid. Thus, there is homology between the virulence plasmids and the cryptic 175-kb plasmid.

Presence of IS*1* on plasmids of Crb$^+$ and Crb$^-$ isolates. Copies of insertion sequence IS*1* have been detected on the 220- and 175-kb plasmids (3). A copy of the insertion sequence was also found on pTKS2 and appears to be responsible for deletions of the recombinant plasmid leading to the Crb$^-$ phenotype. Therefore, the possibility was considered that a copy or copies of IS*1* are responsible for the types of molecular rearrangements observed in the 220-kb plasmid leading to the Crb$^-$ phenotype. To determine the role of IS*1* in the deletions seen in the 220-kb plasmid, we cut plasmid DNAs isolated from the 10 Crb$^+$ strains and their Crb$^-$ mutants with *Hin*dIII and hybridized them under stringent conditions to an IS*1* probe. The ^{32}P-labeled probe used was a fragment of VAλ3, which contains a copy of IS*1* derived from R100 (4). Plasmids from all of the Crb$^+$ strains tested contained three to five copies of IS*1* (Fig. 8). Fragments of 4.8 and 2.1 kb were found in all of the strains tested. Loss of the Crb$^+$ phenotype was often accompanied by loss of one or more copies of the insertion sequence. However, in only one case (Fig. 8, lane 7) was loss of a fragment containing IS*1* accompanied by the appearance of a different-size fragment hybridizing to the probe, as is expected with most IS*1*-mediated deletions. Therefore, it seems that the majority of deletions generated in the 220-kb plasmids when the Crb$^+$ phenotype is lost are not IS*1* mediated. Loss of IS*1* is likely due to the fact that the insertion sequence was closely linked to the sequences encoding Congo red binding.

DISCUSSION

Although loss of ability to bind Congo red is associated with loss of virulence in *Shigella* spp., little is known about the nature of the binding. We cloned the sequences encoding Congo red binding from the 220-kb wild-type plasmid to facilitate characterizing the gene product, its role in virulence, and the DNA alterations leading to loss of the Crb$^+$ phenotype. One recombinant plasmid, pTKS2, contained a *Bam*HI fragment which restored Congo red binding to Crb$^-$ *S. flexneri* and *E. coli* but failed to restore virulence and lacked the thermoregulation characteristic of the wild-type plasmid (3). Since pTKS2 lacked some of the characteristics associated with Congo red binding by wild-type *S. flexneri*, additional fragments of the 220-kb plasmid were cloned. A 3.0-kb *Bam*HI fragment cloned into pACYC184 was also found to encode Congo red binding. This clone, designated pTKS15, permits Congo red binding at 37°C but not at 30°C, thus resembling Congo red binding by virulent *S. flexneri*. Some homology was detected between this clone and pTKS2, but only under hybridization conditions of reduced stringency. This homology appeared to be due to DNA adjacent to the *crb* sequences.

It is not clear why two relatively unrelated cloned fragments can produce the same Crb$^+$ phenotype. It is not yet known whether these clones code for similar proteins or whether they might be two different components of the Congo red binding system. One difference between these two plasmids is that expression of the Crb$^+$ phenotype by pTKS2 was apparently dependent on copy number. Cloning into lower-copy-number vectors or integration into the chromosome eliminated Congo red binding. It is possible that pTKS2 does not contain all of the sequences necessary for Congo red binding, such as regulatory elements, or Congo red binding in this case may be an artifact of overproduction of a protein encoded by the sequence.

Integration of pTKS2 into the *E. coli* chromosome occurs at high frequency and leads to complete loss of the Crb$^+$ phenotype. The cloning vector pAT153 is a high-copy-number vector (35 to 40 copies per cell) (13), and integration of one of the recombinant plasmids into the chromosome should leave enough copies of the plasmid in the cytosol of the cell for expression of the Congo red genes. However, when integration occurs no recombinant plasmids appear in agarose gels, indicating that only the integrated copy of the recombinant plasmid remains. It is possible that integration

of a single copy of the recombinant plasmid has an inhibitory effect on the replication of the remaining copies, thus leading to their loss. Expression of the integrated copy might still occur, but the copy number would be insufficient for binding of the dye by the cells, thus leading to the Crb^- phenotype. Alternatively, integration may influence expression in other ways than merely lowering copy number. In recent studies, Bochner (2) has shown that integration influences Congo red binding by P22-infected *Salmonella typhimurium*. Cells containing a P22 phage defective for lysogeny produced red colonies on Congo red agar when the phage replicated in the cytoplasm (pseudolysogen). White colonies were observed when the phage was integrated into the chromosome or lost from the cell. The observation that pTKS2 integrates into the *E. coli* chromosome with subsequent loss of Congo red binding raises the possibility that apparent loss of the 220-kb plasmid in *S. flexneri* may, in some cases, be due to plasmid integration into the *Shigella* chromosome.

Lack of integration of pTKS2 in *recA* cells indicates that integration results from homologous recombination (20). Homology was detected between the chromosome and the cloned fragment, possibly because of the IS*1* adjacent to the *crb* sequences. Although several copies of IS*1* are found in the *E. coli* chromosome, none have been reported to reside in the *xyl-mtl* region, where integration of pTKS2 occurs. Recent reports indicate that sequences between the *xyl-mtl* regions of *S. flexneri* encode a yet to be defined virulence factor (9). The fact that a cloned fragment of the 220-kb plasmid also integrates in that region raises interesting questions about the genetic and physiological relationship of the cloned sequences and the sequences around the *xyl-mtl* region of the *S. flexneri* chromosome.

Southern hybridizations of *S. flexneri* plasmids with both Crb^+ clones were used to determine conservation of *crb* sequences and to investigate the molecular changes associated with loss of Congo red binding. Unexpectedly, more than one restriction fragment was found to hybridize to each probe. An internal *Eco*RV fragment from pTKS2 hybridized to both the 220- and 175-kb plasmids, indicating homology between these two plasmids. The pTKS15 fragment had no homology with the 175-kb cryptic plasmid but hybridized to two or three restriction fragments of the 220-kb plasmids. It is possible that regions of the 220-kb plasmid are duplicated and that there is more than one copy of these genes. The extent of homology between these fragments and whether or not these represent functional copies remain to be determined. The sizes of the *Hin*dIII fragments hybridizing to both probes were similar in all of the strains tested, indicating conservation of these sequences. However, the 220-kb plasmids were not identical in the various isolates because considerable variation was noted in the restriction enzyme fragment patterns. The results are in agreement with data of Sansonetti et al. (22), indicating that the *S. flexneri* plasmids are diverse but have some highly conserved regions.

Hybridization of the clones to plasmids isolated from Crb^- mutants of *S. flexneri* failed to reveal any consistent pattern of molecular changes associated with loss of the Crb^+ phenotype. As expected, loss of the large plasmid was accompanied by loss of copies of both sequences. Deletions of the 220-kb plasmids leading to loss of Congo red binding were associated with loss of one of the fragments hybridizing to pTKS15 in three of seven cases (Fig. 6, lanes 1, 4, and 5). However, no change in the hybridization pattern was observed in four of the deletion mutants or in the one mutant which had no detectable changes in the large plasmid. Since the Crb^+ phenotype is regulated in *S. flexneri* and expression was not observed at 30°C, it is likely that some of these mutants are defective in regulation. In many of the Crb^- mutants, the structural gene(s) may be intact but derepression or induction may be impaired.

A copy of the insertion sequence IS*1* is closely linked to sequences encoding Congo red binding in pTKS2 (3). Since deletions of pTKS2 apparently mediated by IS*1* led to loss of the Crb^+ phenotype, it was postulated that IS*1* was responsible for the high frequency of deletions causing loss of Congo red binding in *S. flexneri*. Our results indicated the presence of multiple copies of IS*1* (three to five copies) on the 220-kb plasmid. Nevertheless, the hybridization results demonstrated that most of the deletions generated on the 220-kb plasmid are not likely to be IS*1* mediated. Loss of the Crb^+ phenotype was often accompanied by loss of one or more copies of IS*1*, but novel restriction fragments carrying IS*1* were rarely seen, as is the case with IS*1*-mediated deletions or recombination between two copies of the insertion sequence (21). However, the fact that many of the deletions resulted in loss of one or more copies of IS*1* demonstrated that a close topological relationship exists between IS*1* and the gene(s) encoding Congo red binding. Other IS*1*-mediated DNA rearrangements such as inversions could lead to loss of Congo red binding in cases in which no apparent change in the size of the 220-kb plasmid is observed yet the ability of the cells to bind the dye is lost. Close association of IS*1* with the Congo red genes could mean that the insertion sequence has played a role in generation of the multiple copies of genes encoding Congo red binding. IS*1* could also provide a site for recombination between this plasmid and the chromosome or between this and other plasmids.

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