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Cloning the Gene for Congo Red Binding in *Shigella flexneri*

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The ability to bind the dye Congo red from agar medium is associated with virulence of *Shigella* species. DNA sequences conferring this property have been cloned from a large, 140-kilobase plasmid of *Shigella flexneri* into a plasmid vector. This recombinant plasmid does not fully restore virulence to *S. flexneri* isolates which have lost the large plasmid. This indicates that other genes present on the 140-kilobase plasmid must also be required for virulence of *S. flexneri*. The cloned fragment contains a copy of the insertion sequence IS*1* closely linked to the gene for Congo red binding.

Shigella species are invasive pathogens which cause bacillary dysentery in humans (4). Several regions of the *Shigella* chromosome are associated with virulence (3), and a large plasmid has been shown to confer invasive properties on these bacteria (16). Expression of virulence factors encoded by this plasmid has been shown to be temperature dependent, and virulence is detected at 37°C but not at 30°C (11). One characteristic common to wild-type, virulent *Shigella* spp. is the ability to bind the dye Congo red from agar medium (14). Mutants which had lost the ability to bind the dye, CR^- mutants, were readily obtained and were found to be avirulent for chick embryos (14) or lacked the ability to invade epithelial cells (12). A correlation was found between Congo red binding and the presence of a 140-kilobase (kb) plasmid (12). It was not clear whether the factor responsible for Congo red binding is a virulence factor or whether Congo red binding and virulence are unrelated but encoded on the same plasmid. In this study, the DNA sequences encoding the ability to bind Congo red were cloned from *Shigella flexneri* and tested for their ability to restore virulence to CR^- *S. flexneri* strains.

MATERIALS AND METHODS

Bacterial strains and plasmids. Wild-type *S. flexneri* strains used in this study were 8-2031, serotype 1b, and SF100, serotype 5. Both strains were obtained from the Texas Department of Health. *Escherichia coli* 1058 was used as the recipient for transformation with hybrid plasmids. The cloning vector was pAT153, a high-copy-number derivative of pBR322 (9).

Media and chemicals. Tryptic soy broth (Difco Laboratories) with 1.5% agar and 0.01% Congo red was used to determine Congo red binding (14). Luria broth was used for all other cultures. All chemicals used were reagent grade.

Plasmid isolation and cloning. *S. flexneri* plasmids were isolated by the procedure of Hansen and Olsen (5) or Kado and Liu (6). The large plasmids were purified on a sequential cesium chloride-ethidium bromide density gradient and a 40 to 65% sucrose gradient. *S. flexneri* plasmids and vector DNA were cut with *Eco*RI or *Bam*HI restriction endonucleases (New England Biolabs), combined and ethanol precipitated, and ligated with T4 ligase (New England Biolabs). *E. coli* 1058 was transformed with the ligated DNA by the procedure of Mandel and Higa (8) and plated on Congo red agar containing 500 μg of carbenicillin per ml (Sigma Chemical Co.). Hybrid plasmids were isolated by the procedure of Kado and Liu (6) from transformed *E. coli* strains which were CR^+. The recombinant plasmids were transferred to CR^- strains of *S. flexneri* by transformation (8).

Virulence testing. Serial dilutions of *S. flexneri* carrying the hybrid plasmids were inoculated allantoically into 11-day-old chicken embryos. At least five embryos were inoculated with each dose, and the inoculated embryos were incubated at 37°C for 24 h. Values for 50% lethal doses were calculated by the method of Reed and Muensch (15).

Detection of IS*1* sequences. Wild-type or recombinant plasmids were cut with restriction endonucleases (New England Biolabs), and the fragments were separated by electrophoresis through 0.9% agarose. DNA fragments were transferred to nitrocellulose (Schleicher & Schuell, Inc.) by the method of Southern (17) and were hybridized by the procedure of Maniatis et al. (9). The IS*1* hybridization probe was prepared by *Hin*dIII digestion of VAλ3 (2), separation of the fragments on 0.9% agarose, and elution of the 10.2-kb fragment containing IS*1* as described previously (7). The eluted fragment was labeled with [^{32}P]dCTP (New England Nuclear Corp.) by nick translation (10). Filters were hybridized at 68°C and washed at 68°C with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

RESULTS

Strain 8-2031, which forms red colonies on Congo red agar, was tested to determine the rate of loss of Congo red binding. A single CR^+ colony was inoculated into Luria broth and grown at 37°C. The fully grown culture was diluted and plated on Congo red agar. The frequency of white colonies (CR^-) was approximately 10^{-4}. Addition of 2 μg of ethidium bromide per ml, a plasmid curing agent, to the broth culture increased the frequency of CR^- to 10^{-1}. None of the CR^- isolates were found to revert to CR^+ at a detectable frequency ($<10^{-8}$). These data and the results of Maurelli et al. (12) suggest a plasmid location for the gene(s) for Congo red binding.

Plasmids were isolated from the CR^+ parent and CR^- mutants. In most of the CR^- isolates, the 140-kb plasmid was missing, although some isolates (8/22) appeared to have a plasmid indistinguishable from the wild type on agarose gels (Fig. 1). The wild-type and mutant plasmids that appeared to be the same size were cut with *Eco*RI, *Bam*HI, or *Pst*I, and the fragments were compared by agarose gel electrophoresis. The sizes of the fragments appeared to be identical in all cases (data not shown), although very small deletions might not have been detected. To determine whether the 140-kb plasmid encoded Congo red binding,

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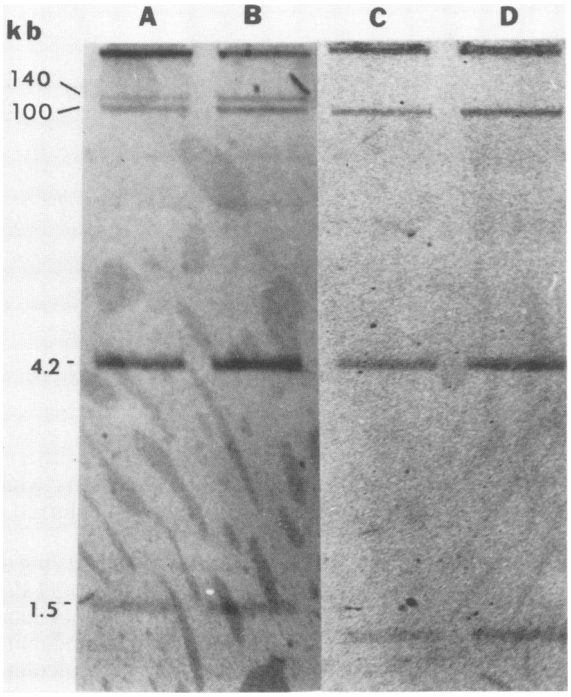

FIG. 1. Plasmids from CR^+ and CR^- *S. flexneri*. Plasmids were isolated from *S. flexneri*, electrophoresed through 0.9% agarose, and stained with ethidium bromide. (A) CR^+ parent strain. (B, C, and D) Individual CR^- isolates. Sizes of plasmids are indicated at the left.

*Bam*HI or *Eco*RI restriction enzyme fragments of the purified wild-type plasmid were cloned into a vector (pAT153), and recombinants were tested for their ability to confer Congo red binding on both *E. coli* 1058 and a CR^- mutant of *S. flexneri* 8-2031. Approximately 10,000 transformed colonies of *E. coli* 1058 were screened, and 12 of the 10,000 were found to be CR^+. Of the 12, 9 contained a *Bam*HI fragment, and the other 3 contained an *Eco*RI fragment. Two of the plasmids, designated pTKS2 and pTKS4 were chosen for further study. Both of these plasmids contained *Bam*HI fragments of the 140-kb plasmid inserted into pAT153. One transformant (pTKS3) which was CR^- and contained a *Bam*HI fragment distinct from that in pTKS2 and pTKS4 was included in some of these studies as a negative control.

The two recombinant CR^+ plasmids were digested with a variety of restriction enzymes, and a partial restriction map of pTKS2 is shown in Fig. 2. pTKS2 contained a single 9-kb *Bam*HI fragment inserted into pAT153, and pTKS4 contained two *Bam*HI fragments (9 and 5 kb) in addition to the vector (data not shown). These plasmids contained common *Eco*RV fragments (Fig. 3, panel II, lanes A and C). A CR^- isolate of *S. flexneri* was transformed with either pTKS2 or pTKS4, and all of the transformants were CR^+. This indicates that the 9-kb *Bam*HI fragment common to both plasmids confers the ability to bind Congo red.

Subclones of the plasmids were constructed to determine more precisely the location of the sequences encoding Congo red binding. Subclones which contained the 4.5-kb *Bam*HI-*Eco*RI fragment (Fig. 2) were found to confer the ability to bind Congo red on recipient cells. Therefore, the CR^+ sequences map within this region of the cloned DNA.

Both *E. coli* and *S. flexneri* transformed with pTKS2 and pTKS4 lost the ability to bind the dye when the plasmid was lost from the cells. Colonies were grown overnight in the absence of the selective antibiotic, plated on Luria agar, and screened for ampicillin sensitivity. Ampicillin sensitive colonies were uniformly white on Congo red medium. Loss of the plasmid was confirmed by agarose gel electrophoresis of cleared lysates (data not shown). This confirms that the recombinant plasmid was encoding the binding of Congo red.

To determine whether the gene(s) for Congo red binding was required for virulence, an *S. flexneri* isolate which had lost the 140-kb plasmid was transformed with pTKS2 and tested for ability to invade chicken embryos. CR^- mutants of *Shigella* sp. had previously been shown to be relatively avirulent when injected allantoically, and CR^+ wild-type isolates were highly invasive and lethal (14). The CR^- isolate transformed with the recombinant plasmid was slightly more virulent (30-fold decrease in the 50% lethal dose) than the CR^- parent, but it was still relatively avirulent compared to the wild-type CR^+ strain (Table 1). This suggests that sequences of the large plasmid, in addition to the gene for Congo red binding, are required to restore virulence to cells which have lost the plasmid.

Studies by Maurelli et al. (11) have shown that expression of virulence factors associated with the 140-kb plasmid of *S. flexneri* is temperature dependent. Virulence was expressed at 37°C but not at 30°C. The recombinant plasmids were tested to determine whether Congo red binding was regulated by temperature. The wild-type strains SF100 and 8-2031 produced red colonies at 37°C but failed to bind the dye and yielded only white colonies at 30°C. In contrast, CR^- mutants of these strains transformed with either pTKS2 or pTKS4 bound the dye at both temperatures. However, colonies of the transformants were pink rather than red at 30°C, indicating that the amount of dye bound at 30°C was less than that bound at 37°C.

Strains carrying the cloned CR^+ sequences were also tested to determine the frequency of CR^+ to CR^- mutation. Both *E. coli* and *S. flexneri* containing the recombinant

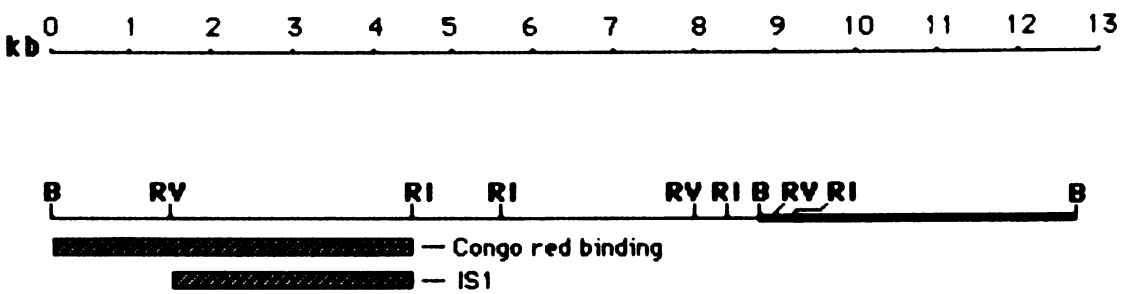

FIG. 2. Partial restriction map of pTKS2. Restriction endonucleases: B, *Bam*HI; RI, *Eco*RI; RV, *Eco*RV. Heavy line indicates vector (pAT153) sequences. Location of the sequences encoding Congo red binding and the region containing IS*1* are indicated by the boxes.

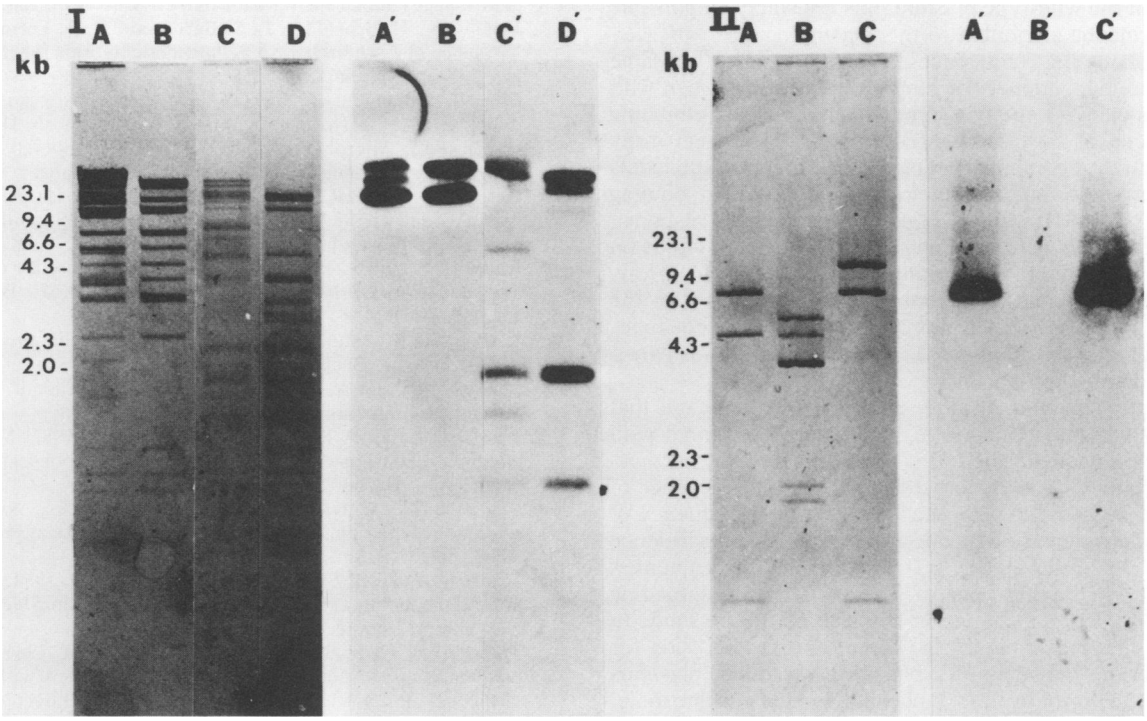

FIG. 3. Hybridization of *S. flexneri* plasmids and cloned fragments with IS*1*. Panel I, plasmids isolated from strain 8-2031 CR^+ (lanes A and C) and CR^- mutants lacking the 140-kb plasmid (lanes B and D) were cut with *Bam*HI (lanes A and B) or *Pst*I (lanes C and D). A Southern blot (17) of this gel was hybridized to a 10.2-kb fragment of VAλ3 containing IS*1* (2) (lanes A' through D'). Panel II, hybridization of cloned fragments of the 140-kb plasmid with the IS*1* probe. Plasmids pTKS2 (lane A), pTKS3 (lane B), and pTKS4 (lane C) were cut with *Eco*RV, electrophoresed through 0.9% agarose, and stained with ethidium bromide. A Southern blot of the gel was hybridized with IS*1* (lanes A' through C'). Both pTKS2 and pTKS4 code for Congo red binding (CR^+). pTKS3 lacks the fragment encoding Congo red binding and was included to ensure that IS*1* was binding to the cloned fragment and not the vector.

plasmid demonstrated the same frequency of CR^+ to CR^- mutation (10^{-4}) as the parental strain from which the cloned DNA was derived. This suggested that some element responsible for loss of Congo red binding had been cloned along with the sequences which code for this property. Since Maurelli et al. (12) detected a high frequency of deletions associated with the parent plasmid and since *S. flexneri* is known to have many copies of the insertion sequence IS*1* (13), it appeared likely that a copy of IS*1* might be closely associated with the gene(s) for Congo red binding. Deletions, or other DNA rearrangements generated by the insertion sequence, could result in loss of Congo red binding. *S. flexneri* plasmids and the cloned fragment were hybridized under stringent conditions to IS*1* to determine whether sequences homologous to IS*1* were present (Fig. 3). The ^{32}P-labeled probe used was a 10.2-kb *Hin*dIII fragment of VAλ3 which contains a copy of IS*1* derived from R100 (2). Both large plasmids (100 and 140 kb) from this strain had copies of IS*1*. Three copies of the sequence were present in the plasmid DNA from the CR^+ strain, whereas CR^- isolates which had lost the 140-kb plasmid had only two copies (Fig. 3, panel I). The DNA was also cut with *Pst*I, which cuts within IS*1* (13). Two additional *Pst*I fragments which hybridized to IS*1* were found in the strains containing the 140-kb plasmid (Fig. 3). This indicates a single copy of IS*1* on the 140-kb plasmid and two copies on the 100-kb plasmid. In addition, the cloned fragments encoding Congo red binding also contained IS*1* (Fig. 3, panel II). The location of the IS*1* in pTKS2 and pTKS4 has been mapped by digestion of the plasmids with a variety of restriction endonucleases and hybridization with the IS*1* probe (data not shown). The IS*1* was found to map in the same region that encoded Congo red binding (Fig. 2), indicating that the insertion sequence is closely linked to the gene for Congo red binding.

DISCUSSION

These results demonstrate that the ability to bind the dye Congo red is encoded by a large, 140-kb plasmid in *S. flexneri*. This plasmid had previously been shown to be involved in invasion, and loss or deletion of the plasmid was associated with loss of virulence (16), as well as loss of Congo red binding (12). The sequences encoding Congo red binding were cloned to facilitate characterization of the gene products associated with Congo red binding and virulence. Cells which have lost the 140-kb plasmid become CR^+ when transformed with the recombinant plasmid but show only a slight enhancement of virulence. Other sequences of the 140-kb plasmid must be required to fully restore virulence to the CR^- derivative. Although the ability to bind Congo red serves as a convenient marker for virulence and for the

TABLE 1. Virulence of *S. flexneri* strains containing wild-type or recombinant plasmids for allantoically inoculated 11-day-old chicken embryos

Strain	50% lethal dose at 24 h
8-2031 CR^+	3.0×10^1
8-2031 CR^-	3.2×10^6
8-2031 CR^-/pTKS2	1.0×10^5

presence of the wild-type plasmid, it is not solely responsible for the virulence attributed to this plasmid.

The cloned CR^+ sequences do not exhibit the same temperature dependence for binding the dye as is seen with the strains carrying the wild-type plasmid, although binding was reduced at 30°C compared to 37°C. The high copy number of the recombinant plasmid may increase the levels of gene products sufficiently to allow Congo red binding even at 30°C. Alternatively, regulatory sequences responsible for the temperature dependence may not be contained on the cloned fragment. Subcloning the fragment into a low-copy-number plasmid and cloning a larger fragment of the 140-kb plasmid to include more of the flanking sequences should indicate why binding occurs at 30°C in strains carrying the recombinant plasmids.

Preliminary studies of the cloned DNA indicate that sequences encoding Congo red binding are associated with the insertion sequence IS*1*. IS*1* mediates DNA rearrangements such as inversions and deletions (1). The presence of this sequence could explain the relatively high frequency of deletions causing loss of Congo red binding and virulence noted by Maurelli et al. (12). Inversions of sequences adjacent to IS*1* could lead to loss of Congo red binding without an apparent change in the size of the plasmid. In addition, the presence of IS*1* could provide a site for recombination between this plasmid and other plasmids containing IS*1* or between the plasmid and the chromosome. Additional studies will focus on the role played by this insertion sequence.

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LITERATURE CITED

1. **Cornelis, G., and H. Saedler.** 1980. Deletions and an inversion induced by a resident IS1 of the lactose transposon Tn951. Mol. Gen. Genet. **178**:367–374.
2. **Dempsey, W. B., and N. S. Willetts.** 1976. Plasmid co-integrates of prophage lambda and R factor R100. J. Bacteriol. **126**:166–176.
3. **Falkow, S., H. Schneider, L. S. Baron, and S. B. Formal.** 1963. Virulence of *Escherichia-Shigella* genetic hybrids for the guinea pig. J. Bacteriol. **86**:1251–1258.
4. **Gemski, P., and S. B. Formal.** 1975. Shigellosis: an invasive infection of the gastrointestinal tract, p. 165–169. *In* D. Schlessinger (ed.), Microbiology—1975. American Society for Microbiology, Washington, D.C.
5. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatability group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
6. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
7. **Lawlor, K. M., and S. M. Payne.** 1984. Aerobactin genes in *Shigella* spp. J. Bacteriol. **160**:266–272.
8. **Mandel, M., and A. Higa.** 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
9. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. U.S.A. **72**:1184–1188.
11. **Maurelli, A. T., B. Blackmon, and R. Curtiss III.** 1984. Temperature-dependent expression of virulence genes in *Shigella* species. Infect. Immun. **43**:195–201.
12. **Maurelli, A. T., B. Blackmon, and R. Curtiss III.** 1984. Loss of pigmentation in *Shigella flexneri* 2a is correlated with loss of virulence and virulence-associated plasmid. Infect. Immun. **43**:397–401.
13. **Nyman, K., K. Nakamura, H. Ohtsubo, and E. Ohtsubo.** 1981. Distribution of the insertion sequence IS1 in gram-negative bacteria. Nature (London) **289**:609–612.
14. **Payne, S. M., and R. A. Finkelstein.** 1977. Detection and differentiation of iron-responsive avirulent mutants on Congo red agar. Infect. Immun. **18**:94–98.
15. **Reed, L. J., and H. Muensch.** 1938. A simple method of estimating 50 percent endpoints. Amer. J. Hyg. **27**:493–497.
16. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
17. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.