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Molecular Alteration of the 140-Megadalton Plasmid Associated with Loss of Virulence and Congo Red Binding Activity in *Shigella flexneri*

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A plasmid of about 140 megadaltons has been associated with the invasiveness of *Shigella flexneri*. Upon subculturing in liquid media of fully virulent isolates of *Shigella flexneri* 2a YSH6000, which contains only a 230-kilobase-pair (kbp) plasmid in addition to 3.3- and 4.2-kbp cryptic plasmids characteristic to all *S. flexneri* strains, loss of invasiveness, loss of Congo red binding activity (Pcr), and complete loss of, or a deletion, or even a single-site IS insertion in the plasmid occurred simultaneously. This was ascribed to the fact that, once a noninvasive Pcr$^-$ cell has emerged, it overgrows the wild type as a consequence of its selective advantage in artificial media. A deletion map of the 230-kbp plasmid was made by analyzing *Sal*I digests of 39 deletion derivatives plus 1 formed by insertion of an IS*1*-like element in independently isolated, noninvasive Pcr$^-$ mutants. Of 39 deletion derivatives, 16 belonged to a single type, and 6 belonged to another, suggesting deletion hot spots. The deletion map was confirmed and extended by analyzing 359 *Sal*I-generated partial digests of the wild-type plasmid cloned into pBR322. Three copies of IS*1*-like elements were found on three different *Sal*I fragments by Southern hybridization. Segments required for the Pcr$^+$ phenotype seemed to occur at several different locations in the plasmid. Each of 28 representative Pcr$^-$ mutants were negative by the Sereny test. Hence, many, or possibly all, Pcr determinants were required for full virulence.

An essential factor in the ability of shigellae to provoke bacillary dysentery is invasion and proliferation in colonic epithelial cells (5), and strains that lack this ability are avirulent. The virulence of shigellae is highly unstable in subculture or storage (7, 10). Recent studies (7, 10, 11, 17) with animals (15) and tissue culture cells in virulence assays have demonstrated the association of large plasmids with the virulence of shigellae. Although the invasiveness of shigellae has been considered a phenotype expressed through cooperative actions of complex genes on the large plasmid (3), genetic analysis of virulence determinants has not yet been properly undertaken because of the large size of the plasmid, the difficulty in isolating it in pure form, and the lack of an easily scorable marker to distinguish invasive and noninvasive derivatives. Recently, Maurelli et al. (7) demonstrated that loss of Congo red binding ability (Pcr) of *Shigella flexneri* 2a consistently accompanied loss of virulence and that the majority of avirulent Pcr$^-$ mutants analyzed had some deletions in the 230-kilobase-pair (kbp) plasmid. We report here a more detailed genetic analysis of this important plasmid.

MATERIALS AND METHODS

Bacterial strains used. *S. flexneri* 2a YSH6000 was kindly supplied by M. Takasaka, Tsukuba Primate Center for Medical Science, National Institute of Health, Japan, and was originally isolated from a primate suffering from shigellosis. *Escherichia coli* K-12 MC1061 (13) was the host for the cloning of *Sal*I partial digest fragments of the 230-kbp plasmid (pMYSH6000) into pBR322.

Media and chemicals. LN (12) and Penassay broths (Difco Laboratories, Detroit, Mich.) and their solidified agar media were used. Congo red binding activity was tested by growing cells at 37°C on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.), to which Congo red (Sigma Chemical Co., St. Louis, Mo.) was added at a concentration of 0.01% (9).

Kinetics of molecular alterations of pMYSH6000 plasmid, loss of invasiveness, and loss of Congo red binding activity during subculturing. Fully virulent, fresh isolates of YSH6000 taken from eyes of guinea pigs suffering from keratoconjunctivitis shigellosa were plated on LN agar plates, and six single colonies thus formed were each inoculated into 2 ml of LN broth and grown at 37°C up to the stationary growth phase (about 5×10^9/ml). Each culture was then diluted 10^7-fold with LN broth and grown again. This subculturing was repeated once a day (see Fig. 2). To confirm the reproducibility of the results, the six independent cultures at the first subculturing were kept frozen at −20°C.

Tests for invasiveness in tissue culture monolayers. Strains of shigellae were assayed for virulence in an in vitro cell culture model similar to that described by Maurelli et al. (7) except for the use of rhesus monkey kidney epithelial (MK) cells designated as LLC-MK_2 (17), kindly supplied by A. Nakamura of the National Institute of Health, Japan.

Sereny tests. Unless otherwise described, Sereny tests (15) were performed with mice as reported elsewhere (7a).

In vivo and in vitro population change of a mixture of Pcr$^-$ and Pcr$^+$ shigellae. Frozen cultures of fully virulent Pcr$^+$ and Pcr$^-$ (noninvasive, Pcr$^-$, but without detectable molecular alteration on pMYSH6000 plasmid) cells derived from YSH6000 were spread on Congo red agar and grown at 37°C for 24 h. The confluent bacterial growth of each strain was suspended in Penassay broth, diluted to an optical density at 600 nm of 0.05 in Penassay broth and then grown at 37°C for

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TABLE 1. Correlation among four different characteristics exhibited by 50 *S. flexneri* 2a derivatives and their plasmids

Expt. no.	Selection of mutants (no.)[a]	Generation time (min)	No. Pcr⁻/ no. tested	No. of large colony mutants on Trypticase soy agar	No. with detectable molecular alteration/ no. tested[b]	No. with negative mouse Sereny test/ no. tested
1	Pcr⁻ (42)	22	42/42	42/42	40/42	16/16
2	Large colony (50)	22	50/50	50/50	43/45	12/12
2	Small colony (50)	25	0/50	0/50	0/12	0/12

[a] Large and small colonies as described in the legend to Fig. 5.
[b] Molecular alterations of pMYSH6000 plasmid derivatives were detected by *Sal*I restriction digestion followed by 0.9% agarose gel electrophoresis.

12 h. Then the cells were collected, suspended in 1 ml of saline to give high-density suspensions of approximately 3×10^{10}/ml, and mixed to give mixtures of Pcr⁺ and Pcr⁻ cells (see Table 2). Mixtures of 30 μl were inoculated into the conjunctival sacs of guinea pigs. After 72 h, cells were recovered from the sacs and immersed in 2 ml of LN broth, which was then spread on Congo red agar to score the ratio of Pcr⁺ to Pcr⁻ cells and separately subjected to in vitro subculturing as described above.

Isolation of plasmid DNA. Large-scale preparation of plasmid DNA was performed as described previously (14). Further purification was made by cesium chloride-ethidium bromide density gradient ultracentrifugation at 34,000 rpm for 40 h at 20°C in a Beckman Instruments, Inc., SW50.1 rotor. Small-scale preparation for detection of plasmid DNA and for restriction enzyme digestion was performed by the rapid alkaline lysis method of Kado and Liu (4) with a slight modification. The modification was only to add 250 μl of distilled water–50 μl of 3 M sodium acetate (pH 4.8)–1 ml of ethanol to 200 μl of the phenol-chloroform-extracted aqueous phase in an attempt to remove sodium dodecyl sulfate as well as possible. Extraction of *Sal*I fragments of pMYSH6000 cloned into pBR322 was performed by the rapid alkaline lysis method of Birnboim and Doly (1).

Restriction endonuclease analysis and agarose gel electrophoresis. Restriction endonuclease was used according to the method described by Maniatis et al. (6). Electrophoresis of restriction endonuclease-cleaved DNA was performed in 0.7% horizontal agarose slab gels in a Tris-acetate buffer system (160 mM Tris base, 80 mM sodium acetate, 8 mM EDTA [pH 8.3]) (12) at 70 mA for 18 h. To detect uncleaved plasmid DNA bands derived from pMYSH6000 or its large derivatives, the concentration of the Tris-acetate buffer was reduced to one-fourth the original.

Cloning of *Sal*I partial digests of pMYSH6000 plasmid into pBR322. pMYSH6000 plasmid DNA (10.8 μg) was treated with 1.5 U of *Sal*I for 30 min at 37°C, and the partial digests thus obtained were ligated with T4 ligase to pBR322, which had been cleaved with *Sal*I and dephosphorylated with calf intestine alkaline phosphatase. Reaction was at 14°C for 12 h. $CaCl_2$-treated competent cells of strain MC1061 were transformed with the ligation products, and ampicillin (Ap) resistance transformants were selected on LN agar with 250 μg of Ap per ml. Ap-resistant transformants were then examined for tetracycline resistance. Upon reconfirmation by digestion with *Sal*I, the majority of Ap-resistant, tetracycline-susceptible transformants were found to contain an insertion of *Sal*I fragments of pMYSH6000.

DNA-DNA hybridization. Hybridization on nitrocellulose filters was performed under stringent conditions according to the Southern blot hybridization method (16) with slight modifications. The probe was a 0.64-kbp *Pvu*II-*Tth*III internal fragment of IS*1* (8) prepared by digesting pBR333 (13)-cloned IS*1*L derived from Tn*9* and nick translated as described by Maniatis et al. (6).

RESULTS

Choice of YSH6000. For the present study, the plasmid profiles of 87 strains of *S. flexneri* collected from different geographical locations were analyzed by agarose gel electrophoresis. Multiple plasmids were found in each virulent isolate. *S. flexneri* 2a YSH6000 showed the simplest plasmid profile, 3.3, 4.2, and 230 kbp, a set of plasmids common to all virulent strains tested, and was used for further genetic analysis of virulence.

Coordinated loss of Congo Red binding activity and invasiveness accompanying molecular alterations of pMYSH6000 plasmid DNA. Changes in plasmid DNAs during subculturing were monitored by agarose gel electrophoresis. Faint bands of deletion derivatives from pMYSH6000 appeared during subculturing in each of the six clones tested. The new band became progressively more abundant on subsequent subculturing, and this was consistently accompanied by decreased amounts of the original 230-kbp plasmid

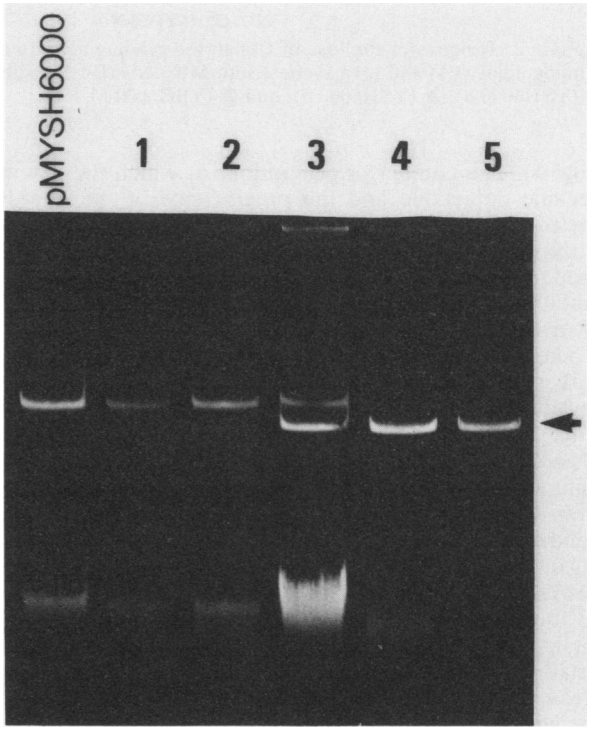

FIG. 1. Molecular transition of pMYSH6000 plasmid DNA in YSH6000 during successive subcultures. Plasmid DNA extracted from samples of subclone YSH6000-1 at each subculture was separated electrophoretically in a 0.7% agarose gel. The numbers 1 to 5 above the gel indicate the number of subculturings, and the leftmost lane shows the intact pMYSH6000 plasmid band as the control.

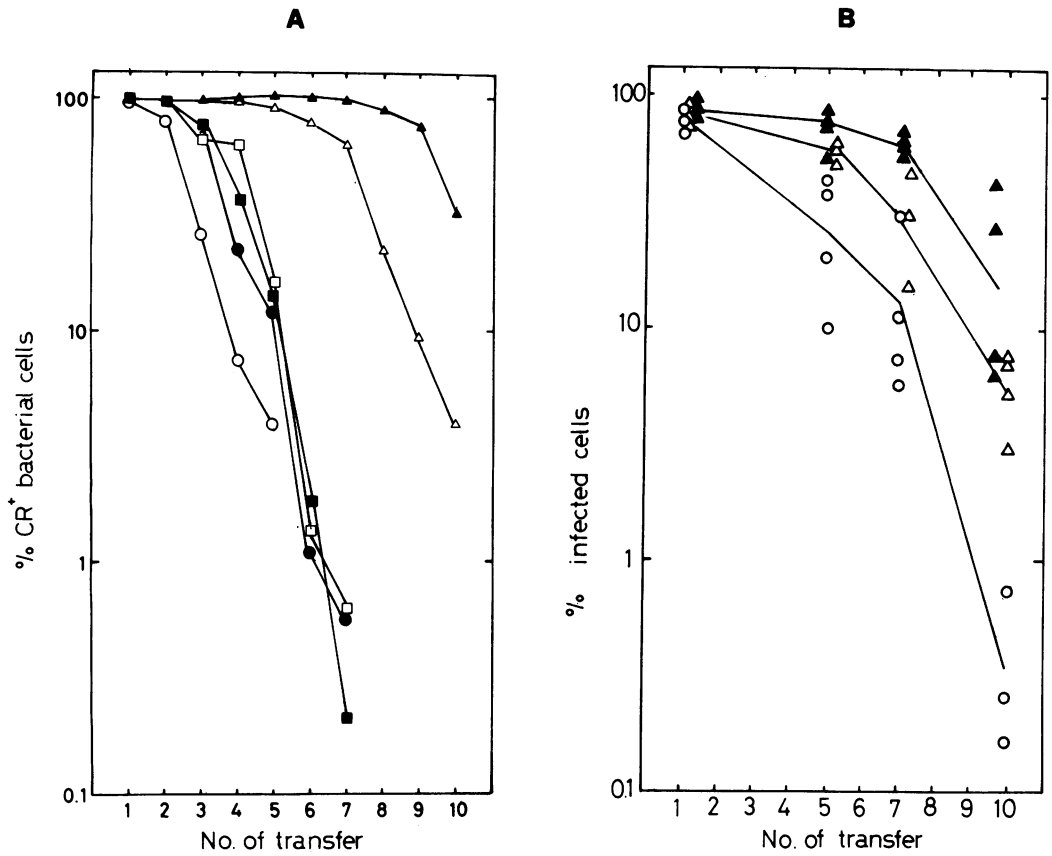

FIG. 2. Kinetics of the loss of Congo red binding activity and invasiveness. YSH6000 at each subculture was examined for Congo red binding ability (A) and invasiveness into MK cells (B). Six subclones are represented by ○ (YSH6000-1), △ (YSH6000-2), □ (YSH6000-3), ● (YSH6000-4), ▲ (YSH6000-5), and ■ (YSH6000-6).

(Fig. 1). The number of generations at which the new band became detectable and the precise size of the new band varied among the six clones and during repetitions with frozen samples of the same clone. Only a single size of product accumulated in any single culture, suggesting that mutation is rare, but that any mutant which happens to arise is strongly selected (Table 1).

The reduction in the size of the plasmid was correlated with reductions in the abilities to bind Congo Red and to invade MK cells, which were also lost with similar kinetics (Fig. 1 and 2). To test whether the observed changes in plasmid size were responsible for the Pcr^- phenotype and failure to invade MK cells, 42 Pcr^- derivatives were isolated from similar successive subcultures originated from 42 independently isolated Pcr^+ subclones of YSH6000, and their plasmid DNAs were studied. *Sal*I digestion of intact pMYSH6000 plasmid DNA from strain YSH6000 generated 23 fragments ranging from 1.5 to 43.1 ± 5.6 kbp (from the intact pMYSH6000 plasmid) in addition to two uncleaved, small plasmid DNAs (Fig. 3). Of 42 Pcr^- plasmid derivatives cleaved with *Sal*I, 40 produced detectable differences from the wild type and could be classified into 13 classes (Fig. 4). The remaining two plasmids did not exhibit an obvious difference in any of the 23 fragments, but this does not exclude the possibility that a smaller point mutation in their plasmids also caused Pcr^- and noninvasiveness (2, 7). Of the 13 classes, 12 were deletions (Fig. 4), and the remaining one was an insertion (see Fig. 6). The most frequent type of deletion (del-17) was found in 16 of 39 deletion derivatives and yielded a 9.1-kbp fusion fragment upon *Sal*I digestion and a 3.6-kbp fragment upon *Pst*I restriction endonuclease digestion (data not shown), suggesting that the endpoints coincide with a deletion hot spot. In an insertion derivative designated as ins-33, the *Sal*I fragment H of 9.9 kbp was replaced by a new band of 10.7 kbp (see Fig. 6, lane O, arrow). A total of 16 Pcr^- derivatives, including at least one from each of the 13 classes, were chosen for the mouse Sereny test; all were negative. The association of Congo red binding ability, invasiveness (even positive Sereny tests), and molecular changes in pMYSH6000 suggested by these observations was tested further by the correlation between colony size and Congo red binding (7). Fifty small and 50 large colonies of independent origin were isolated on Trypticase soy agar with Congo red and tested further. Each of the large colonies was Pcr^-, whereas each of the small colonies was Pcr^+. Of 45 large colonies tested, 43 contained plasmids with reduced molecular sizes, whereas no changes were detected in the plasmids of each of 12 small colonies tested. Each of the 12 large colonies tested had negative mouse Serenty tests, whereas each of the 12 small colonies tested was positive by Sereny test. The generation times in liquid media of the strains forming small and large colonies was 25 and 22 min, respectively. Thus, it is clear that colony size on agar reflects in vitro growth rate and supports the interpretation that, once an avirulent cell has emerged, it overgrows the liquid culture. In contrast, inoculation of a mixture containing some Pcr^+ cells and a great excess of Pcr^- cells into guinea pig eyes resulted in a positive

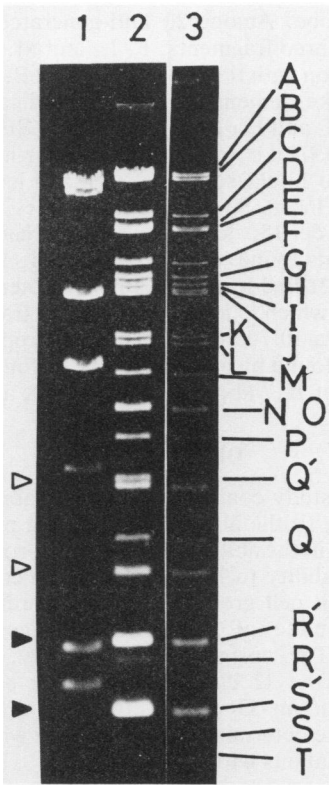

FIG. 3. Agarose gel electrophoresis profiles of *Sal*I endonuclease cleavage fragments of pMYSH6000. A through T indicate *Sal*I restriction fragments of the plasmid, and the three fragments Q', R', and S' frequently comigrated with those bands corresponding to CCC (▲) or OC (△) molecules of the two cryptic plasmids. Lanes 2 and 3 show *Sal*I restriction fragments of pMYSH6000 plasmid (5 and 1.5 μg of DNA, respectively). Lane 1 contains *Hin*dIII-digested λ DNA as the reference.

TABLE 2. In vivo and in vitro population change of a mixture of Pcr⁻ and Pcr⁺ shigellae[a]

Expt no.	% Pcr⁺ cells upon:				
	Infection of guinea pig eyes		Subculturing in artificial media		
	Before	After	1st	2nd	3rd
1	100	100	100	100	ND[b]
2	35	99	97	53	5
3	8	96	68	34	3
4	0	0	0	0	ND

[a] Experimental procedures are described in Materials and Methods.
[b] ND, Not done.

Sereny (virulence) test and overgrowth of the Pcr⁺ cell type (Table 2).

***Sal*I restriction map of pMYSH6000.** A preliminary map of the deletions in pMYSH6000 was inferred from the results obtained in the *Sal*I restriction cleavage analysis of 13 different classes of deletion or insertion derivatives (Fig. 4). To make this more accurate and to extend the map of the entire plasmid, a library of partial *Sal*I cleavage fragments of intact pMYSH6000 plasmid DNA was ligated to the *Sal*I site of pBR322 and used to transform MC1061 to Ap resistance. Plasmid DNAs from 359 Ap-resistant and tetracycline-susceptible transformants were analyzed by *Sal*I digestion. The majority of plasmids contained one or more *Sal*I cleavage fragments characteristic of pMYSH6000 plasmid ranging from 1.5 to 32.6 kbp. No transformant carrying pBR322 with an insertion of the largest 43.1 ± 5.6-kbp *Sal*I fragment A was obtained. Some of the small *Sal*I fragments, which had been difficult to detect earlier owing to the comigration of four bands, to open circular and covalently closed circular molecules of the two cryptic plasmids, were identified among the clones (*Sal*I fragments Q', R', and S'; Fig. 5). Sixteen recombinant plasmids containing two or more adjacent *Sal*I fragments coupled with the assay of deletion

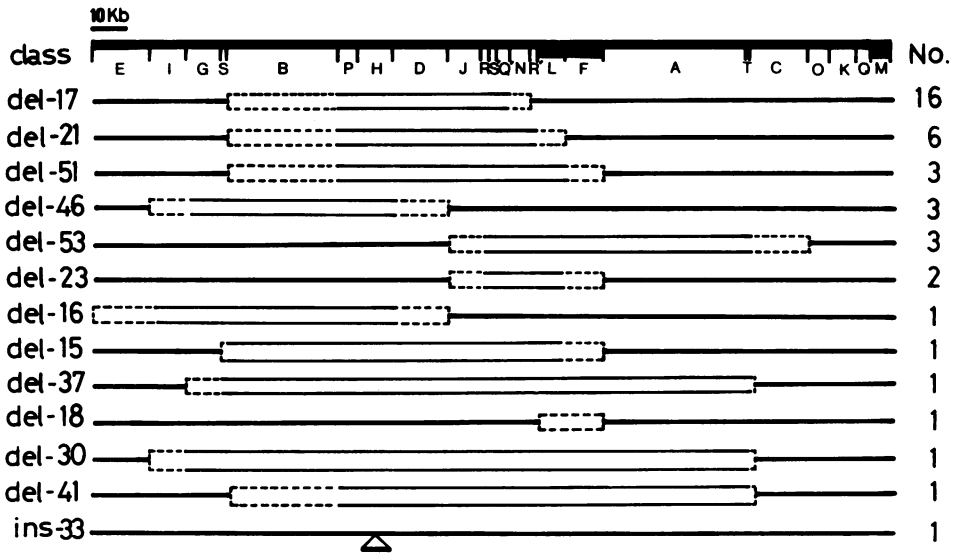

FIG. 4. Deletion map and classification of the deletion derivatives of pMYSH6000. Forty noninvasive Pcr⁻ derivatives of pMYSH6000 were divided into 13 classes according to the *Sal*I restriction patterns shown in Fig. 6A, and the deletion segments or the site of IS insertion are located on the *Sal*I restriction map of pMYSH6000 in Fig. 5. The boxes beneath the top line indicate the *Sal*I fragments containing IS*1*-like elements (Fig. 6).

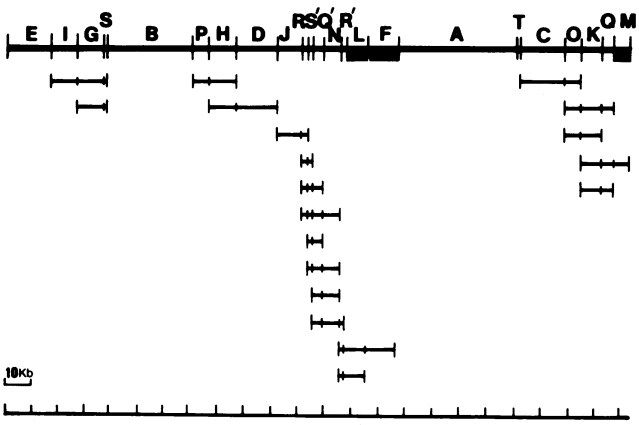

FIG. 5. *Sal*I restriction cleavage map based on a pBR322-cloned library of pMYSH6000 plasmid DNA. The upper horizontal line is the map. Designations A to T correspond to those in Fig. 3. The boxes beneath this line indicate the *Sal*I fragments containing IS*1*-like elements (Fig. 6). The lower horizontal line shows the scale in kilobase pairs. Solid bars between these two horizontal lines indicate two or more contiguous cloned *Sal*I fragments.

derivatives (Fig. 4) permitted the construction of a more complete *Sal*I map (Fig. 5).

IS*1*-like elements found on pMYSH6000 plasmid. A copy of IS*1* was reported to exist in another virulence-associated large plasmid in *S. flexneri* 1b (2). pMYSH6000 plasmid DNA was digested with *Sal*I and hybridized with the ^{32}P-labeled IS*1* probe. Among 23 *Sal*I-generated fragments of pMYSH6000, three fragments, F, L, and M, revealed positive hybridization with the IS*1* probe (Fig. 6B, lane B). Three copies of IS*1*-like elements were further indicated by hybridization to *Pvu*II- and *Eco*RV-digested pMYSH6000 (data not shown). The IS*1*-like segments were further studied by hybridization to 13 classes of deletion and insertion derivatives of pMYSH6000. Seven deletion classes, del-51, del-23, del-53, del-37, del-15, del-30, and del-41 had lost the two IS*1*-like elements found in *Sal*I fragments L and F, whereas del-18 and del-21 had lost one, either fragment F or L (Fig. 6). In ins-33, which lacks 9.9-kbp *Sal*I fragment H and instead carries a 10.7-kbp fragment, four copies of IS*1*-like elements were found by hybridization, the fourth copy in the new fragment of 10.7 kbp (Fig. 6B, lane O, arrow).

DISCUSSION

The present study confirmed that four parameters for the *Shigella* bacilli, (i) the ability to provoke a positive Sereny reaction (Ser), (ii) the ability to invade and proliferate in cells (Inv), (iii) the ability to bind Congo red (Pcr), and (iv) the ability to inhibit cell growth (Igr) are coordinately altered as a consequence of molecular changes in plasmid pMYSH6000. The correlation among Inv, Pcr, and Igr phenotypes in the 12 classes of deletion plasmid and in insertion plasmid ins-33 suggests that they are coded for by the same genetic determinants. The reason why the deletion and insertion mutants with phenotypes Ser$^-$, Inv$^-$, and Pcr$^-$ are selectively isolated is ascribed to selectively advanta-

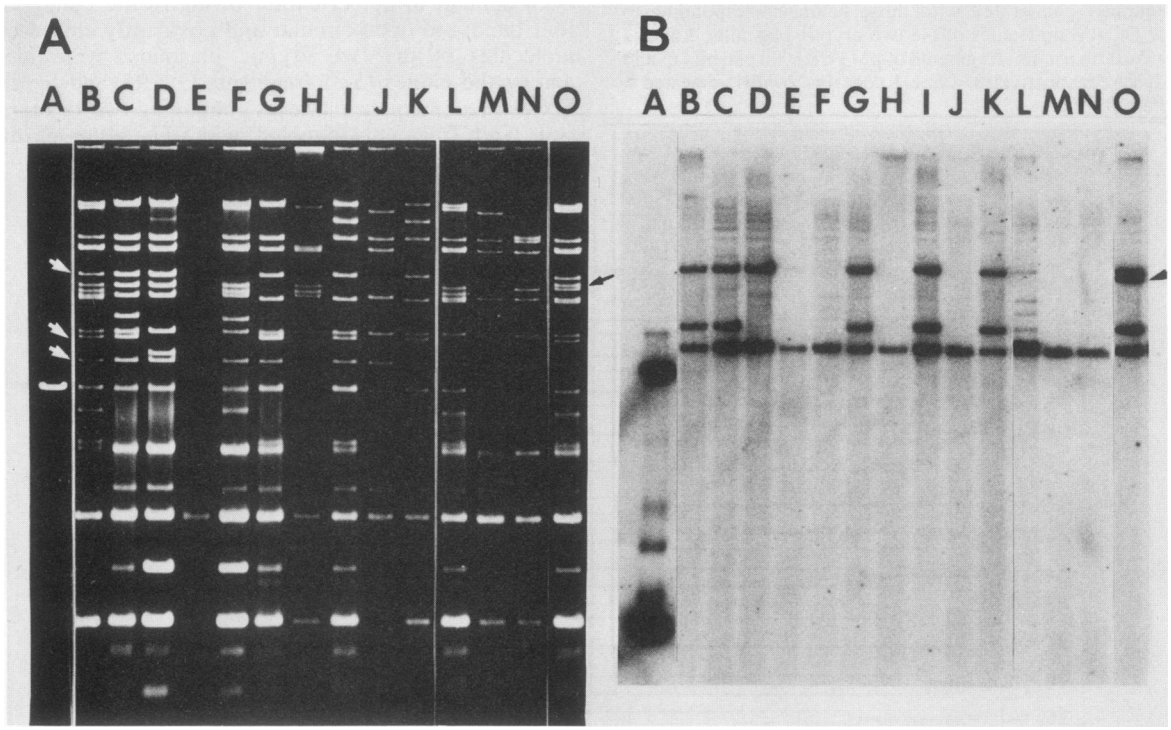

FIG. 6. *Sal*I restriction cleavage patterns of 13 different classes of pMYSH6000 plasmid derivatives and identification of IS*1*-like elements in the plasmid by Southern hybridization. (A) Ethidium bromide-stained 0.9% agarose gel (lanes): A, pBR322::Tn*9* cleaved with *Eco*RI; B, pMYSH6000 cleaved with *Sal*I; C to O, del-17, del-21, del-51, del-46, del-23, del-53, del-16, del-15, del-37, del-18, del-30, del-41, and ins-33, all cleaved with *Sal*I. (B) Autoradiogram of Southern blot hybridization of the gel in A with IS*1*-probe. Open arrows in A, lane A, indicate fragments F, L, and M (from top to bottom), and the closed arrow in A, lane O, indicates an IS*1*-inserted fragment corresponding to the arrow in B, lane O.

geous growth of such mutants in in vitro culture owing to the Igr^- phenotype.

The deletion and insertion mutations isolated in the present study extend over more than half of the entire plasmid. Among them, the smallest deletion (del-18) and insertion (ins-33), both having resulted in the loss of all four phenotypes, are separated about 50 kbp from each other on the map (Fig. 4). A 1.0-kbp DNA sequence derived from pMYSH6000 was cloned in *E. coli* K-12 MC1061 by selecting with the Pcr^+ phenotype. It was mapped and localized in *SalI* fragment F on pMYSH6000 (9a). This cloned segment converted del-18 to full virulence, but not YSH6000 without the 230-kbp plasmid or one with an extensive deletion, del-37. Furthermore, 9.9-kbp *SalI* fragment H cloned into pBR322 gave a positive Sereny test when the recombinant plasmid was introduced into ins-33 but not del-18 (data not shown). These results indicate that the regions affected by the deletion and the insertion play a direct role in Pcr^+ and virulence phenotypes and exclude the possibility that an additional, smaller mutation in another *SalI* fragment is responsible for these phenotypic changes. According to our unpublished data, Tn*5* inserted into different *SalI* fragments, B, P, H, D, and F, has resulted in Ser^-, Inv^-, Pcr^-, and Igr^- phenotypes. Similar Tn*5* insertions into *SalI* fragment G have resulted in Ser^- but Inv^+, Pcr^+, and Igr^+ (manuscript in preparation). We propose that multiple genes required for virulence are scattered throughout the plasmid. Our current efforts are directed toward identifying, mapping, and cloning all the DNA sequence in pMYSH6000 associated with virulence (positive Sereny test) in *S. flexneri*.

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